

Options for the Control of Influenza VI

June 17-23, 2007 • Toronto, Ontario, Canada

Editor
Jacqueline M Katz, PhD

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Options for the Control of Influenza VI

Proceedings of the International Conference on Options for
the Control of Influenza VI held in Toronto, Ontario, Canada
June 17-23, 2007

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Options for the Control of Influenza VI **Conference Proceedings**

In June 2007, Toronto, Ontario, Canada was the site of the *Options for the Control of Influenza VI* conference, an international scientific forum exclusively devoted to influenza. Since its inception in 1985, the *Options* meetings, held every 3 to 4 years, have continued to grow in scope and size. *Options VI* was the largest in the series to date, with 1600 delegates from over 66 countries in attendance. This record attendance reflected the expanded interest in influenza, in part due to the continuing threat to animal and public health posed by the circulation of highly pathogenic avian influenza viruses. The circulation of H5N1 and other avian subtypes have spurred enhanced surveillance efforts, research for improved measures for influenza detection, control and prevention, and extensive pandemic preparedness strategies. The accumulation of this new knowledge on seasonal and pandemic influenza was reflected in the *Options VI* Scientific Program.

The Scientific Program showcased international agricultural and public health challenges in outbreak response and pandemic preparedness, the latest advances in basic or clinical research, and their application for the control and prevention of influenza. The core of the meeting's information was delivered through over 600 abstracts submitted to 14 workshop categories, presented in either oral or poster form, encompassing every aspect of influenza surveillance, epidemiology, and research.

The success of the *Options VI* was due to a culmination of outstanding efforts by Canadian federal and local hosts, the Organizing and Scientific Committees, Session and Workshop Chairs, and MediTech Media Conferencing, and of course the conference participants. Finally, the generous financial contributions of the sponsors made the vision a reality. We also offer special thanks to CDC colleagues for the review of the manuscripts submitted to this Conference Proceedings.

Although it has been over ten years since the first recognized human cases of H5N1 virus infection, the threat of a pandemic due to this or other influenza subtype remains ever present, and new challenges with respect to seasonal influenza control continue to emerge. The need for expanded research on influenza viruses, the impact of the disease, and control and prevention strategies is as urgent as ever before.

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Invited Speakers

What is a Clade? And Why Should I Care?

Helen Branswell

The Canadian Press, Canada

I often get the sense the people we journalists interact with are quite curious about our profession. They occasionally exhibit other reactions as well — irritation and frustration for instance. Sometimes you have every right to be frustrated, annoyed or worse. We sometimes under-prepare or over-hype. Other times we misunderstand things or we make mistakes. In our defence, I don't know a single journalist who doesn't take seriously the responsibility to be accurate. Most are mortified when they make mistakes that find their way into print or on air. But we're human and most of us have to produce our reports very quickly. I know that makes a lot of scientists uneasy. But the reality is the tempo of our working environment is very different than the tempo of science. With the explosion of new media, with people reading headline news on their cell phones and eschewing newspapers for online news sources, there is growing pressure to speed up our output. There is also intense competitive pressure to diversify how we package news. In many news rooms, the distinction between print, radio and TV is blurring. Increasingly, print reporters are required to record interviews for radio and/or capture video footage for use on their newspaper's websites — and write print articles as well. These demands to produce more and produce it faster are being heard in newsrooms all over. And they are having an impact on the calibre and the depth of the journalism we can do. Even without these new duties, keeping abreast of scientific developments is a huge challenge. Studies can be difficult and time consuming to read, and some days there can be a dozen that need at least cursory consideration. While it's not desirable, there are times when there is only time to read a study's abstract. Sometimes complaints don't relate to accuracy, but to the approach or the slant of the journalism. You thought the key message was X, but the reporter seized on Y. You spent 40 precious minutes explaining a development only to find it reduced to a paragraph or two at the bottom of an article. Or you discover that the reporter, in the interest of fulfilling the time honoured tradition of "on the one hand and on the other hand" journalism, has counterbalanced your opinions with those of someone who has never published in your field. Those things will happen occasionally and there is little that can be said to make that less irksome. But perhaps knowing a bit about who we are and how we go about crafting news will help explain how it happens. If you haven't done a lot of interviews in the past, you might expect health, science or medical reporters to have a background in medicine or science. You might think many are physicians. And you almost certainly would be wrong. Many of the medical and science writers you will encounter will have had little or no training in this type of specialized reporting, coming to the beat with no idea of what a p-value is and why a journal would let scientists use the word

"significant" to describe the difference between 12% and 12.9%. Researchers from the University of British Columbia's science journalism program recently surveyed science, health and medicine print reporters in Canada, asking about their experience and training. They found 84% did not have a science degree; 37% said they had no training for the beat at all. A study of health reporters in five Midwestern U.S. states published in the American Journal of Public Health in July 2002 reported that nearly 83% of surveyed health reporters had no training to cover health news. There is a culture in journalism of learning on the job, and that's very much at play here. We are teaching ourselves as we go along. And we're being taught by the scientists and public health officials who take our calls. Ironically, we're the newsroom experts. But we will make up a fraction of the army of journalists who will report on the next pandemic when it occurs or on major developments with pandemic threats in the meantime. That's because when a science story hits the top of the newscasts — as influenza did in the fall of 2005 — journalists from all sorts of subspecialties will attempt to pile on the train. And those reporters are far less likely to know anything about the science of influenza or the nature of public health responses. So you all will get more calls — and at a time when you have no time. How do you handle them? Beyond triage and teleconferences, I don't really know. I hope you have a plan. You see, it is hugely important for you to make the time to inform journalists, so that we can report accurately. Because we are going to report. We have to report. And if you don't make the time, someone else will happily answer our calls. And the public may not be the better for it. It was an eye opener for me how many new "bird flu experts" emerged in September and October of 2005. I was surprised both by the credentials of some of the experts and by some of the quotes. Maybe journalists don't have a monopoly on wanting to get in on a hot story. At the risk of boring you, let me reiterate: If the true experts don't talk to the media, someone else will; Not reporting the story because the true experts don't return our calls is not an option; Writing a story in which we quote no one is not an option. So if we don't get the best expert, we try for No. 2, or the next best person who will pick up the phone. There are other factors that influence the way we work and may contribute to frustration with the way we report on science. A major one relates to the language we use — or do not use. Words like clade. The first time I heard the word clade, it struck me as such an odd word and I recall thinking: That's a word I'd never be able to use in a story. You see, journalists avoid words that are commonplace in science but Martian to non-scientists because we want the public to understand what we are saying — and what you are saying, through us. The accepted wisdom is if news consumers have to turn to a dictionary to look up the words in an article, they will abandon the piece. I agree, up to a point. But I like to think people who consume news do it to learn something new. I like to use the right word, the proper word, to describe something, even if it is a word readers will have to learn through my article. But when journalists introduce words, phrases or concepts that

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are not widely understood, we have to explain them. And we cannot assume everybody who is going to read today's story on vaccine adjuvants is going to have read last month's story on vaccine adjuvants. So we explain and explain again. If we worked in a world of limitless words, that would be no problem. But most reporters don't live in that world. In fact, word limits are one of the primary drivers behind the simplification of news presentation. Consider this: at news agencies like the Associated Press, The Canadian Press and Reuters writers are encouraged to keep stories within a range of between 400 and 800 words. And print reporters are verbose in comparison to TV or radio reporters. That's not a lot of room for findings, analyses and the opinion of outside experts. Now, having explained a bit about us, I'd like to discuss something that makes covering H5N1, and influenza in general, such a challenge for journalists. I apologize if I am speaking out of turn, but the truth is the lexicon of influenza sucks, at least from the point of view of trying to communicate useful and accurate information about it to the public. Reporting on the pandemic threat is already a very complex message, given the complete uncertainty over when one will occur, what strain will cause it and what the attack rate and case fatality rates will be. Then we add in additional layers of confusion because of the way viruses are named. So then we get situations like: "Yes, we think we found an H5N1 virus in goslings on a farm in Prince Edward Island, Canada, but we're almost certain it's not that H5N1..." Or even worse: "The lab in Prince Edward Island got a weak positive for an H5N1 virus in birds, but we can't find any virus so we can't say for sure it was an H5N1. But if it was an H5N1, it wasn't that H5N1." How are people who aren't steeped in the science of influenza supposed to understand that those goslings were not the first sign that "bird flu" had landed in North America? Influenza, not the inaccurately named stomach "flu" but the real, respiratory ailment, is a virus many people are just now coming to learn about. Some are getting to the point where they understand that "bird flu" is not a single entity, that there are multiple types of avian influenza viruses. But it's more complex than that and that's where it really gets sticky for journalists. During Britain's H7N2 outbreak this spring I was talking with Dr. Alejandro Thiermann at the OIE. He raised concerns about the way some reporters were trying to explain the virus and the risk associated with it by comparing it to other H7 viruses. In the course of that conversation, Dr. Thiermann said this: "This H7N2 cannot possibly be compared with any other H7N2 — because there may be nothing in common between the two, other than the fact that they react to that H7 test." How in the world is a journalist meant to explain that to news consumers? People who are interviewed, whether they are politicians or scientists or public health officials, hate it when we journalists don't put their remarks or scientific developments in context. But if it is true this H7N2 may be a completely different virus than that H7N2, what is a journalist to tell people about the virus and the risk, if any, to their health? And of course the complexity doesn't stop there. Then there are lineages, which takes us back to that poor farm on Prince

Edward Island. Because the answer there was if there was an H5N1 virus, it was a North American lineage H5N1, not the Eurasian or just Asian H5N1. Some people complain that description unfairly stigmatizes Asia. But there really is no other way to differentiate between the groupings of viruses. WHO has been trying to figure out what to call the next pandemic, so that another region or country doesn't become permanently associated with a major disease event. And the organization has had an expert group working to clarify the nomenclature, so that there is a better understanding even among scientists about where clade divisions occur. It would be nice if some thought might be given to finding ways to make things clearer for non-scientists too. Influenza is no longer the preserve of scientists. And if you want the public to be able to understand it and put various risks in context, to continue to fund influenza research and pandemic preparedness, you have to be able to lay it out for them in language that makes sense. Language to which they can anchor meaning. It has to be frustrating for those of you working in public health agencies when you have to use precious time responding to unfounded concerns caused by a misunderstanding at the level of journalists or of the public. But if the science doesn't have a vocabulary that allows for clear identification of which viruses we need to be concerned about and which are incidental findings, it's inevitable that these misinterpretations are going to occur. I told you about word limits. With so few words to describe such complex science, it becomes imperative to get the words right.

Evolutionary Shifts in Pandemic Preparedness and Response: The Canadian Experience

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Canadian pandemic preparedness has evolved from a primarily health sector activity into a multi-sectoral collaborative approach. A complementary suite of public health strategies, including measures to reduce community and health care setting transmission, domestic vaccine capacity and antiviral stockpiling, provides a broad based approach to dealing with the next pandemic. One of the current challenges is the ongoing enhancement of domestic pandemic preparedness to keep pace with the rapid evolution in scientific knowledge, technologic advances, and international strategies. Since the emergence and spread of the Asian strain of highly pathogenic avian influenza H5N1, there has been paradigm shifts in international pandemic preparedness strategies including pandemic prevention through prevention and control of avian influenza outbreaks and the containment of an initial focus of novel influenza virus in humans to halt or slow down a pandemic. While there is expectation to attempt such measures, the feasibility of implementation, resource and infrastructure requirements, such as surveillance systems, are considerable. No country can plan in isolation and supporting regional and international preparedness is a key component of enhancing domestic plans. The development of prototype pandemic vaccines and increased availability of antivirals present greater opportunities to enhance preparedness, however, pandemic planners continues to grapple with competing priorities as we strive to optimize our preparedness strategies and capacities. Background: After the emergence of highly pathogenic avian influenza (HPAI) H5N1 with transmission to humans in Hong Kong in 1997, the federal government provided initial funding to facilitate national coordination in pandemic preparedness. A federal, provincial and territorial (F/P/T) Pandemic Influenza Committee (PIC) was formed in 2002 to provide advice to governments and to facilitate the harmonization of planning activities amongst jurisdictions. Outside of Asia, Canada was the country hardest hit by the Severe Acute Respiratory Syndrome (SARS) outbreak in 2003. Although a relatively small outbreak, SARS tested the ability of the Canadian health care system to cope with public health emergencies. The National Advisory Committee on SARS and Public Health established in May 2003 provided recommendations to strengthen public health infrastructure in Canada. In follow-up to the Committee's recommendations, the Government of Canada create the new Public Health Agency of Canada in 2004, led by a Chief Public Health Officer. A federal, provincial and territorial Public Health Network (PHN) was also created in 2005 to facilitate communication, sharing of knowledge, and the development of

public health strategies and policies amongst the jurisdictions. New approaches to pandemic preparedness in the pre- and post-SARS era (Table 1).

Table 1. Pandemic preparedness – before and after SARS.

Then:	Now:
Health Sector (simple, vertical)	Inter-sectoral and interdisciplinary (complex)
Invisible, unscrutinized	Highly visible, highly scrutinized
Limited support	Political support + funding
Slow evolution of science	Rapid evolution of science and technology
Individual country planning	International and regional preparedness

With the re-emergence of HPAI H5N1 epizootic in December 2003, political support for avian and pandemic influenza preparedness in Canada greatly escalated. The Government of Canada's Budget 2007 included \$1 billion for avian and pandemic influenza preparedness and response, and all provinces and territories are fully engaged. Influenza pandemics will impact not only the health of the population but also societal function. In the post-SARS era the approach to pandemic planning in Canada has changed to a whole-of society approach that aims to link governments, civil society and the private sector, and an increasing emphasis on individual and family preparedness. Effective communication and cooperation between the different sectors and disciplines before and during a pandemic will help mitigate major threats to societal function. While governments have to set standards and guidelines and provide a realistic estimate of pandemic risk, the challenges for the private sector and non-government organizations are adapting generic guidelines for their own use and making links with local and regional stakeholders in pandemic planning.

Pandemic Prevention – A New Paradigm. In the past, human pandemics emerged suddenly with little warning and the key goal of the public health response was to reduce the impact of the novel human influenza subtype. With the spread of HPAI H5N1 and ongoing intermittent transmission from birds to humans, the pre-emption of a potential pandemic through prevention measures in the animal population has emerged as a key strategy. Developing more comprehensive strategies that incorporates veterinary surveillance and improvements in veterinary infrastructure to respond to AI outbreaks/epizootics is now considered part of pandemic preparedness planning. Improving the ability of resource poor countries to respond to AI outbreaks is recognised by the international community as one way to prevent potential pandemic deaths in resource rich countries. Although Canada has not experienced the devastating impact of HPAI H5N1, it experienced an avian influenza H7N3 outbreak amongst poultry in British Columbia in Feb 2004, with transmission to two workers involved in depopulation activities. This outbreak highlighted the need to strengthen the linkage and coordination between human and animal health response for avian influenza and other zoonotic diseases. The Human Health Issues Related to Domestic Avian Influenza Outbreaks guideline was published in 2004 to inform

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outbreak investigation, surveillance, use of personal protective equipment, antivirals and seasonal influenza vaccine. This era also gave birth to the concept of early detection and rapid response to contain a novel human virus at source, in order to halt or slow down a pandemic. While there is expectation to attempt such measures, the feasibility of implementation, resource and infrastructure requirements, such as surveillance systems, are considerable.

Progress and Challenges in Canadian Pandemic Preparedness. The Canadian Pandemic Influenza Plan (CPIP) first released in 2004 and further updated in 2006 is primarily aimed at health sector preparedness, while recognising that the health response must integrate into an overall multi-sectoral response. Planning assumptions such as attack rates, clinical illnesses and absenteeism rates do differ from country to country. Although assumptions simply offer reasonable scenarios for planning purposes and there may be justifiable reasons as to why countries choose different parameters, it is still a challenge to try and explain such differences to decision makers and the public. Strengthening our capacity to monitor, detect and promptly report severe respiratory illnesses including novel influenza viruses is a key component of Canada's pandemic preparedness. While weekly seasonal influenza surveillance is in place, including reporting of paediatric hospitalizations through a sentinel hospital network, real-time adult severity indicators such as hospitalization and mortality data are lacking. While Canada does have a web-based tool for electronic dissemination of health alerts, the extent of the dissemination of information from public health to front line health care workers and response to the Alerts remains to be evaluated. Canada has enhanced its laboratory preparedness with the establishment of PCR rapid diagnostics for the identification of H1, H3, H5 and H7 influenza A subtypes in 14 laboratories across the country, in addition to the strengthening of the National Microbiology Laboratory's capacity to perform antiviral resistance testing. Key challenges include the stockpiling of laboratory supplies and reagents and specimen transport to remote regions. There is also a need for all jurisdictions to have a system in place to triage and prioritise laboratory testing of clinical specimens. CPIP guidelines on public health measures includes public education (e.g. on respiratory hygiene and the need to stay home if sick), case and contact management, community based strategies to reduce transmission (such as school closures) and travel and border measures. There is agreement amongst F/P/T jurisdictions that widespread quarantine is not recommended during the pandemic period. There is a recommendation to consider school and day care closures and the restriction of other indoor public gatherings if high-risk settings based on the epidemiology can be identified. The use of masks by individuals with ILI to reduce the spread of virus is identified, as well as the consideration for the use of surgical masks for care providers in the community as per the current infection control guidelines for health care settings. Preventing and controlling the spread of infection in health care settings is key to enhance patient safety and protect health care workers. The strengthening of infection

control measures requires a multifaceted holistic approach that includes, engineering (e.g. ventilation systems), administrative controls and processes, education and training, pharmaceutical measures (vaccines and antivirals), early detection, triage and cohorting, in addition to personal protective measures. Routine practices and large droplet and contact precautions are currently recommended in the CPIP. An addendum was recently published on recommendations regarding aerosol generating procedures, including the use of respirators. Canada established a ten year pandemic vaccine readiness contract in 2001, with a goal to immunise every Canadian as soon as possible after the start of a pandemic. As technology (including reverse genetics and adjuvants) became available over the last decade, a number of countries have stockpiled H5N1 vaccine as an extra insurance against the potential for a H5N1 human pandemic. Canada does not have a stockpile of H5N1 vaccine and is continuing to focus on strategies to increase general preparedness against pandemics of any influenza subtype, however, it will continue to review the science related to H5N1 vaccines to inform further decision making. A 55.7M dose National Antiviral Stockpile (enough treatment courses for approx 17.5% of the population), cost shared by the F/P/T governments, has been established for early treatment of the sick who seek clinical care. In addition, the federal government maintains a separate antiviral stockpile (current target 10M doses of antiviral) to provide a surge capacity to provinces and territories and can potentially be used for early containment strategies. A F/P/T communications network has been established and work is ongoing to further coordinate the international communications response during a pandemic. Public and professional information on seasonal, pandemic and avian influenza have been produced. A series of briefings were conducted to inform the Canadian media. Citizen engagement, including a deliberative dialogue process on antiviral prophylaxis, is being used to help inform key policy decisions.

International Collaboration. No country can prepare for a pandemic on its own and international collaboration is crucial, including regional coordination and support for developing countries. Developed countries have a vested interest in ensuring that resource poor countries have the capacity for early detection and reporting of an emergent novel influenza virus. Canada is the second largest donor in the world supporting avian and pandemic influenza preparedness and response and supporting regional and international preparedness is a key component of enhancing domestic plans. Under the Security and Prosperity Partnership, Canada, Mexico and the United States published the North American Plan for Avian and Pandemic Influenza to enhance regional planning and coordination. There are now multiple international fora to discuss and collaborate on avian and pandemic influenza preparedness include the Global Health Security Initiative (G7 and Mexico), G8, APEC, International Partnership on Avian and Pandemic Influenza. The revised International Health Regulations accepted by the World Health Assembly in May 2005 and enforced June 15th 2007 offers a new proactive framework for dealing with infectious

diseases of international significance. Under the IHR(2005) all cases of human influenza caused by a new subtype must be automatically notified to the WHO. Work is ongoing to examine the implications of the IHR for Canada.

The Way Forward. The Canadian approach for pandemic preparedness must continue to include multiple complementary strategies that can deal with any pandemic virus and form the platform to strengthen our response to other emerging infectious diseases of national and international concern. Preparedness activities and strategies must contribute to the building of a stronger public health system in Canada. While science and technology are progressing at a more rapid rate, we have to continue to balance appropriate interventions against opportunity costs and the feasibility of implementation within the resources of the public health system. In the near future Canada's key preparedness activities will include enhancing our vaccine readiness capacity, ensuring that implementation plans are in place for rapid delivery to those who need treatment and to examine the role of antivirals for prophylaxis. There is an ongoing review on guidelines and policies on infection control measures during a pandemic, including the use of masks. Research partnerships will be forged to address key knowledge gaps and provide a research capacity that can respond during a public health emergency.

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Nuclear Import and Assembly of the Influenza Virus RNA Polymerase Complex

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Influenza A virus RNA polymerase is a heterotrimeric complex composed of three subunits PB1, PB2 and PA. It catalyzes viral RNA transcription and replication in the nucleus of infected cells. Here we review our recent work on nuclear import and assembly of the newly synthesized RNA polymerase complex. We found that PB1 needs the presence of PA for its efficient nuclear accumulation whereas PB2 alone accumulates in the nucleus efficiently. An *in vitro* reconstitution experiment further showed that an active polymerase complex can be reconstituted *in vitro* between a preformed [PB1-PA] dimer and the PB2 monomer. A host nuclear import factor (RanBP5) was also identified which interacts with the PB1 subunit, facilitating the nuclear import of the [PB1-PA] dimer. Therefore, we proposed a partly cytoplasmic and partly nuclear assembly model: PB1 interacts with PA in the cytoplasm and is transported into the nucleus as a dimeric complex. Final assembly of the polymerase complex occurs between [PB1-PA] and individually transported PB2 in the nucleus.

Introduction

The eight segments of influenza virus negative-sense genome are transcribed and replicated in the cell nucleus by a virus-encoded RNA-dependent RNA polymerase complex [1]. Primary viral RNA transcription (vRNA→mRNA) is catalyzed by viral RNA polymerase complex from incoming virions. Secondary viral RNA transcription (vRNA→mRNA) and replication (vRNA↔cRNA), however, are catalyzed by newly synthesized polymerase. The three subunits of the influenza virus RNA polymerase complex are synthesized in the cytoplasm as independent proteins, and need to be transported into the nucleus and assembled into a trimeric complex during this process. The nuclear import of large proteins or complexes requires specific interaction with nuclear import receptors which direct them across the nuclear membrane [2]. This process is highly selective and energy-dependent. There are nuclear import receptor families, e.g. importin α family, importin β family, which, either alone or cooperatively, are responsible for binding specific nuclear localization sequences (NLS) in their cargos and directing them through the nuclear pore complex. In order to understand the detailed mechanisms of nuclear import and assembly of the influenza virus RNA polymerase complex, we performed intracellular localization [3], *in vitro* reconstitution [4], and protein co-purification studies [5] with individually expressed single polymerase subunits, and co-expressed dimeric complexes in human embryonic kidney cells (293T).

The data from these studies has led us propose a model for the influenza virus RNA polymerase assembly [5].

In vivo localization studies of influenza virus RNA polymerase subunits.

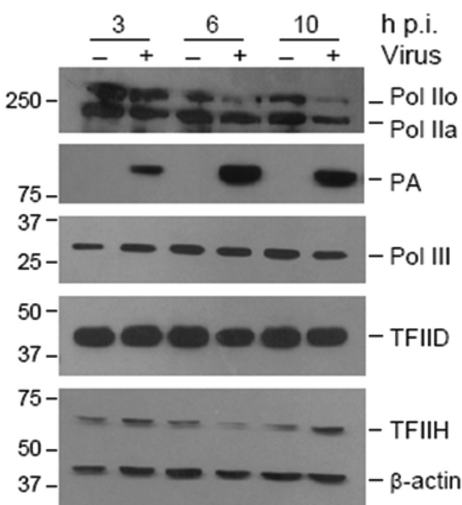
In order to study the cellular localization and the assembly of the polymerase complex, green fluorescent protein (GFP)-tagged polymerase subunits derived from influenza A/WSN/33 virus were generated [3]. Then the localization of the individually expressed GFP-tagged polymerase subunits and co-expressed [PB1-PA] and [PB1-PB2] dimers were examined by fluorescence microscopy [3]. The results of these experiments showed that individually expressed PB1 and PA subunits were distributed in both the cytoplasm and the nucleus, while the PB2 subunit accumulated in the nucleus. Surprisingly, coexpression of PA with PB1-GFP led to efficient accumulation of PB1-GFP in the nucleus, whereas coexpression of PB2 with PB1-GFP did not alter PB1-GFP localization. In addition, coexpression of PB1 with PA-GFP also resulted in PA-GFP nuclear accumulation. Therefore, these data support the hypothesis that PB1 and PA are imported into the nucleus as a heterodimeric complex; in contrast, PB2 is imported as a monomer [3].

In vitro assembly of a functional influenza trimeric polymerase complex.

In order to understand the process of viral RNA polymerase assembly, an *in vitro* reconstitution approach was used [4]. As shown in Figure 1A, partially purified individually expressed PB1, PB2 and PA monomers and co-expressed [PB1-PA], [PB1-PB2] dimers and the 3P trimeric complex using a TAP (tandem affinity purification) tagged subunit were prepared. The reconstitution of an active RNA polymerase complex was tested by mixing these monomers, or dimers with monomers in different combinations in solution. The polymerase activities were assayed by ApG and globin mRNA primed transcription which represent cap-independent and cap-dependent transcription, respectively. Short synthetic RNA oligonucleotides corresponding to the 5' end (15 nt) and 3' end (14 nt) of the wild-type influenza A virus vRNA promoter were used in these assays [4]. Figure 1B showed that, in both ApG-primed and globin mRNA-primed transcription assays, significant transcription was observed when monomeric PB2 was assembled with coexpressed [PB1-PAtap] dimer (lanes 8 and 13). No activity was observed when monomeric PA was assembled with [PB1-PB2tap] dimer (lanes 7 and 14). Furthermore, no transcription activity was detected with any monomers or the two dimeric complexes [PB1-PAtap] and [PB1-PB2tap] (lanes 1-5, 9-12), which confirmed that all three subunits were required for significant transcriptase activity. An assembly of the polymerase by mixing three individually expressed subunits showed no activity (data not shown). Interestingly, these results were entirely consistent with our cellular localization studies which indicated that PB1 and PA were transported into the nucleus as a heterodimeric complex. Thus, we proposed a model in which the trimeric polymerase

complex could be assembled sequentially in the nucleus from a dimeric [PB1-PA] complex and individually transported PB2 [3].

Figure 1. PB1-PA dimers can assemble with PB2 in vitro to produce functional polymerase. (A) Purification of TAP-tagged RNA polymerase subunits from 293T cells transfected with expression plasmids for various combinations of the three polymerase subunits (PB1, PB2 and PA) analyzed by silver staining of 8% SDS-PAGE gel. Sizes of protein standards in kDa are shown on the left. Arrows indicate the position of unknown protein X co-purified with polymerase subunits. The open circles indicate the position of copurified Hsp90. (B) Lanes 1-8: in vitro ApG-primed transcription with all possible combinations of monomers, dimers and 3P complex. Lanes 9-15: in vitro globin mRNA-primed transcription with all possible combinations of monomers, dimers and 3P. The transcription products (TP) are indicated on the right. This figure is adapted from reference 4.



Role of RanBP5 in nuclear import and assembly of the viral RNA polymerase complex.

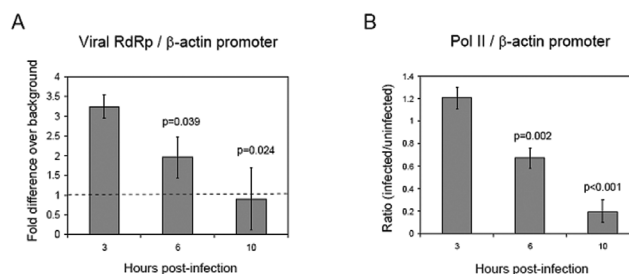
As shown in Fig. 1A, band X, migrating at about 120 kDa, was found to be co-purified with PB1tap and [PB1-PAtap] dimer, but not with PB2tap, PAtap, [PB1-PB2tap] dimer. To characterize this band, standard in-gel trypsin digestion followed by liquid chromatography (LC) and MS/MS analysis (LC-MS/MS) were performed. The LC-MS/MS analysis identified band X as Ran binding protein 5 (RanBP5), also known as karyopherin $\beta 3$, importin $\beta 3$ or importin 5. A western blot using a rabbit polyclonal anti-RanBP5 antibody confirmed the identity of RanBP5 [5]. RanBP5 is a member of the importin β family of transport receptors. Authentic nuclear import complexes formed by these nuclear import receptors and their import substrates are typically characterized by their sensitivity to RanGTP. Indeed, in vitro treatment of partially purified [PB1-PA] with RanGTP resulted in RanBP5 release from the [PB1-PA] dimer [5]. We also found that the release of RanBP5 upon incubation with RanGTP facilitated the assembly of PB2 with the [PB1-PA] dimer as indicated by the increased amount of PB2 assembled with the [PB1-PA] dimer, and a corresponding increase of in vitro transcription activity [5]. Therefore, we concluded that association of RanBP5 with [PB1-PA] in the cytoplasm is

required for nuclear import of the dimer, and dissociation of RanBP5 from [PB1-PA] facilitates the assembly between [PB1-PA] and PB2 in the nucleus. The biological significance of the observed interaction between RanBP5 and PB1 was examined by siRNA-mediated knock-down of RanBP5. We observed an inhibitory effect of RanBP5 knock-down resulting in delayed levels of viral RNA at early time points post-infection [5]. This delayed accumulation of viral RNA levels was consistent with the observation of reduced accumulation of [PB1-PA] dimer in the nucleus in RanBP5 knock down cells, as examined by immunofluorescence [5]. These results further supported our assembly model and confirmed that RanBP5 plays a role in the viral life cycle.

Discussion

We used various in vivo and in vitro approaches to study nuclear import and assembly of the influenza virus RNA polymerase. The data from these studies give rise to the following model (Figure 2): PB1 and PA form a dimer in the cytoplasm where a host import factor, RanBP5, specifically interacts with the PB1 subunit, forming a nuclear import complex with [PB1-PA]. After the [PB1-PA] dimer has been transported through the nuclear pore into the nucleus, the high RanGTP concentration in the nucleus triggers release of RanBP5 from the [PB1-PA] dimer, which then facilitates the assembly of [PB1-PA] with individually transported PB2 in the nucleus.

Figure 2. Model for the transport and assembly of the influenza virus RNA polymerase complex (see text for details). This figure is from reference 5 published in *Journal of Virology*.



We still do not know which host import factor is responsible for PB2 nuclear import. Interestingly, Tarendeau et al. (2007) have reported a co-crystal structure of the C-terminal domain of PB2 with importin $\alpha 5$ (karyopherin $\alpha 1$), suggesting that importin $\alpha 5$ could be a potential host import receptor involved in PB2 nuclear transport [6]. However, direct biological evidence is still needed to confirm this. Although our in vivo and in vitro data support our assembly model, we cannot exclude other assembly pathways for newly synthesized viral RNA polymerase that may operate in infected cells. Recently, Naito et al. (2007) reported that Hsp90 is involved in the assembly and nuclear transport of viral RNA polymerase subunits which led them to propose an alternative polymerase assembly pathway, in addition to the

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pathway proposed here [7]. However, it is likely that the model proposed here represents a major assembly pathway.

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Intracellular Trafficking of Influenza A Virus RNAs

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Influenza A virus replicates and transcribes its RNA genome in the nucleus of infected cells but enters and leaves the cell via the plasma membrane. This necessitates a complex series of intracellular trafficking events involving bidirectional movement of multiple species of viral proteins and RNAs across the nuclear membrane. Trafficking of viral RNAs has mostly been studied by cell fractionation methods or by immunofluorescent analysis, using antibody-staining of proteins likely to be associated with the RNA as a proxy. We have recently developed fluorescent *in situ* hybridization (FISH) protocols that allow direct visualization of the intracellular localisation of viral RNA. This methodology has revealed unexpected complexities in the nuclear export of both viral mRNA and vRNA.

Introduction

The genome of influenza A virus consists of eight segments of negative strand RNA, each separately encapsidated into ribonucleoproteins (RNPs) by the viral RNA dependent RNA polymerase and multiple copies of the single-stranded RNA binding protein, NP [1]. Viral RNA synthesis occurs in the nucleus of infected cells [2] and this has been attributed to a dependence on the cellular transcription machinery. It provides ready access to the capped mRNA substrates necessary for priming viral mRNA synthesis as well as the cellular splicing machinery necessary for processing of two viral pre-mRNAs [1]. However, use of this cellular compartment requires the trafficking of multiple viral macromolecules both into and out of the nuclear pores. On infection, RNPs deposited in the cytoplasm after fusion of viral and cellular membranes must enter the nucleus. Newly synthesised viral mRNAs must then exit to the cytoplasm for translation, after which, some of the new viral proteins enter the nucleus to support the process of genome replication. Later in the viral lifecycle, replicated RNPs are exported to the cytoplasm where they are packaged into new viral particles at the apical plasma membrane. These viral trafficking events are active, energy dependent and regulated processes that depend on multiple interactions between viral and cellular components, not all of which have been satisfactorily defined [3]. In particular our understanding of the trafficking mechanisms of the viral RNA species is incomplete. Little is known about the nuclear export pathway(s) used by viral mRNAs, and while several protein factors have been identified as playing a role in the trafficking of the virus genome [3], the control mechanisms are not fully understood.

One technical difficulty faced by these studies is in following the intracellular localisation of viral RNA. Most published experiments have used either cell fractionation methods or (for vRNA), immunofluorescence directed against the viral nucleoprotein. Cell fractionation is bedevilled by problems of artifactual cross contamination between fractions and historically has proven difficult with influenza virus infected cells [2]. Immunofluorescent detection of NP suffers from the problem that the protein exhibits a complex intracellular trafficking profile in the absence of the virus genome [4-6] and that not all NP in infected cells is necessarily RNP associated. However, we have recently developed fluorescent *in situ* hybridization (FISH) protocols that allow direct visualization of the intracellular localisation of viral RNA [7]. This methodology has revealed unexpected complexities in the nuclear export of both viral mRNA and vRNA.

Materials and Methods

FISH probes were generated by *in vitro* transcription of the appropriate plasmid after linearization by restriction enzyme digestion (pCDNA-PB2; Xba I, pCDNA-NP; Kpn I, pCDNA-M1, Hind III) in the presence of digoxigenin-UTP (Roche) or Cy3-UTP (Perkin Elmer) as previously described [7]. MDCK and 293T cells were cultured and infected with influenza A/PR/8/34 (PR8; Cambridge strain) as described [7]. After infection, cells were processed for FISH staining and imaged as previously described [7].

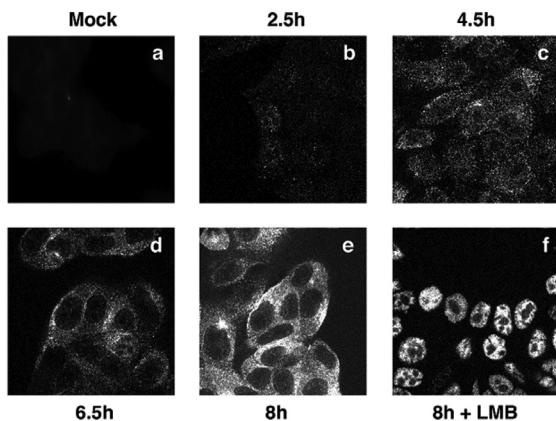
Results and Discussion

Many studies examining vRNA trafficking have used immunofluorescent detection of NP as a marker of RNP subcellular localisation [8-15, 6]. In infected cells, NP is found almost entirely within the nucleus until around 4 h post infection (p.i.). Thereafter it increasingly accumulates within the cytoplasm such that later in infection, cytoplasmic NP staining is markedly more intense than nuclear [8, 12, 13]. The protein also exhibits specific localisation patterns within both compartments. Early in infection nuclear NP localises throughout the organelle in a granular pattern. However, midway through the virus lifecycle, it accumulates at the nuclear periphery, internal to the nuclear lamina [6]. This pattern is also seen late in infection after treatment with the drug leptomycin B (LMB) which blocks RNP nuclear export by inactivating the cellular exportin, CRM1 [12, 13]. Cytoplasmic NP often localises diffusely in granules, but it also specifically concentrates under the apical plasma membrane, the site of virus budding [15, 16]. All of these intracellular localisation patterns are also displayed by non-RNP-associated NP, expressed in the absence of other viral proteins or genomic RNA [15, 6]. This raises the possibility that in infected cells, NP may not be a perfect marker for vRNA localisation. We have recently developed a protocol for FISH staining of specific viral mRNAs that allows visualisation of their intracellular localisation [7]. This technique also detects the negative sense virus genome and as an example, Fig. 1 shows a time-course of infected cells stained for segment 1 vRNA.

Options for the Control of Influenza VI

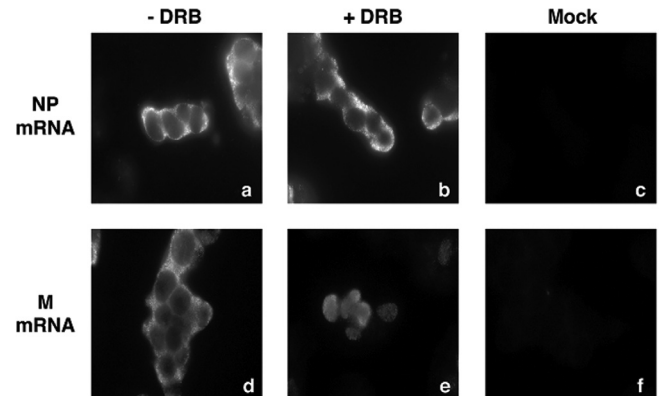
Uninfected cells displayed only background levels of staining (Fig. 1 a), whereas in infected cells at 2.5 h p.i., faint staining that was most intense in the nucleus was detected (Fig.1 b). This most likely reflects vRNA derived from the infecting virus particles. At 4.5 h p.i. the overall levels of segment 1 staining were increased and although both nuclear and cytoplasmic vRNA was visible, the cytoplasmic staining was more intense (Fig. 1 c). At later time points (6.5 and 8 h p.i.) vRNA-specific staining was markedly more intense than at the early time points and was also overwhelmingly cytoplasmic with some apparent concentration at the plasma membrane (Fig. 1 d, e). However, when cells were treated with LMB, the vRNA showed almost total nuclear retention (Fig. 1 f), consistent with a block to RNP nuclear export [12, 13]. Consideration of these staining patterns with similar published experiments examining NP localisation [12, 13, 15, 6] suggests a rather imperfect correlation between vRNA and total NP staining. In particular, NP staining is predominantly nuclear at 4.5 h p.i. while vRNA is not. In addition, at 4.5 h p.i. in normal cells, and in LMB-treated cells, NP apparently concentrates at the inner nuclear periphery and this was not seen for vRNA. Thus direct staining of vRNA is likely to prove a better method for following the intracellular trafficking of the influenza virus genome; experiments are currently underway to further examine this possibility. In addition, the hypothesis that the concentration of NP at the inner nuclear periphery represents RNPs awaiting nuclear export [13, 6] may not be correct.

Figure 1. Intracellular localisation of the virus genome during a time-course of infection. MDCK cells were infected or mock infected and stained for segment 1 vRNA at the indicated time points. One set of cells was treated with 11 Nm LMB from 90 min. post infection.



FISH detection of viral mRNA is also proving invaluable. We have recently used this approach to confirm that the differential sensitivity of 'early' gene NP and 'late' gene M1 and HA expression to a variety of drugs that inhibit RNA polymerase II activity operates at the level of mRNA nuclear export [17, 7]. Whereas ordinarily the majority of positive sense segment 5 and 7 transcripts were detected in the cytoplasm of infected

Figure 2. Effect of DRB on nuclear export of influenza virus mRNAs. Infected or mock infected 293T cells were treated (+) or left untreated (-) with 150 μ M DRB from 90 min. p.i. and stained at 6.5 H p.i. for the indicated viral mRNAs.



cells (Fig. 2 a, d), in cells treated with the inhibitor of pro-cessive mRNA transcription 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB; [18]), M1 but not NP mRNA was retained in the nucleus (Fig. 2 b, e). Furthermore, removal of DRB reversed this block unless a variety of chemically and mechanistically distinct RNA polymerase II inhibitors were added instead [7]. We conclude that influenza A virus replication requires cellular transcription activity not just to provide capped mRNA substrates but also to facilitate nuclear export of selected viral mRNAs. This is consistent with recent data regarding the nuclear export of microinjected cellular mRNAs [19]. Work is now ongoing in our laboratory to identify the cellular pathway(s) used for nuclear export of influenza virus mRNAs.

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Veterinary Aspects of Avian Influenza Infections: Challenges and Opportunities

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Notifiable Avian influenza (NAI) is an OIE listed disease that has become of great significance for both animal and human health. The increased relevance of avian influenza (AI) in the fields of animal and human health, has highlighted the lack of scientific information on several aspects of the disease, which has hampered the adequate management of some of the recent crises. Millions of animals have died, and there is growing concern over the loss of human lives and over the management of the pandemic potential. Until 1999, Highly Pathogenic Avian Influenza (HPAI) was considered a rare disease of animals and only 18 primary outbreaks had been reported since 1959. Around the turn of the millennium, a series of HPAI outbreaks occurred with different characteristics to those seen previously. The Italian H7N1 1999-2000 and Dutch H7N7 2003 epidemics caused outbreaks of unprecedented magnitude which were only a prelude to the ongoing H5N1 crisis. The latter appears to represent possibly the greatest threat yet to animal health, with very serious implications for public health, that the veterinary community has ever been called to face [1]. HPAI caused by subtype H5N1 virus has become widespread in vast areas including Asia, the Middle East, Europe and Africa. This widespread dissemination of the virus has greatly increased its impact, affecting the health of wild animals, domestic animals and humans. Currently, human health is affected both in terms of the reduction of food security and by the cases of the infection of humans, with the potential that these may lead to the emergence of a new pandemic virus. Viruses causing HPAI originate from H5 or H7 precursor viruses of low pathogenicity harboured in wild birds, primarily *Anseriformes*. The introduction of these progenitors into domestic poultry has resulted, on several occasions in the mutation of the low pathogenicity virus to a highly pathogenic mutant containing multiple basic amino acids at the cleavage site of the haemagglutinin molecule. This mutation enables the virus to spread systemically in the infected bird, replicating in vital organs and bringing about the death of the bird [3]. Historically, HPAI had usually been a self-limiting disease as most birds affected died as a result of infection. The virus that infected primarily chickens and turkeys, was in most cases apathogenic for waterfowl and did not infect wild birds. The latter were only very rarely infected and usually only found as dead birds on or near poultry farms with HPAI, and thus played a minor role in the epidemiology of this infection. Since its appearance in 1996, the Asian lineage HPAI H5N1 progenitor virus and its descendants have spread to several interconnected compartments of the animal husbandry systems in Asia, which has resulted in amplification of infection and establishment of an endemic state in the domestic avian reservoir. This has

in turn resulted in a spill-over of infection to wild birds. So far, relatively few species have been shown to be infected, but the epidemiological consequences of this unique situation are impossible to predict. What is clear is that the HPAI H5N1 virus actively circulates in birds reared for agricultural purposes in Asia, the Middle East and Africa and could be present in the Eurasian wild bird population. Additionally, the persistence of a HPAI H5N1 virus in poultry compartments that are farmed traditionally with low biosecurity standards and in areas with a high human population density has generated a unique set of opportunities for the virus. There have been numerous reports of infections of humans and some domesticated mammals, especially felids. There is evidence that the viruses involved in such infections have developed mutations that may result in a greater capability for causing clinical disease resulting in death of the host. Humans confirmed to be infected with the virus have shown a >50% fatality rate [2]. The extent of wild bird involvement in the spread and perpetuation of HPAI H5N1 virus has been the subject of much debate. However, the virulence of the virus for most bird species and the reports that more recent isolates have acquired the capacity to cause severe disease and death in domestic and wild ducks suggests that the maintenance in wild birds will be fairly limited unless spill-over from the domestic sector continues. Therefore, the crucial issue in resolving this situation is to limit the circulation of the virus in the poultry reservoir, as this represents a never-ending source of virus. Although specific tools are available, the infrastructure and economic conditions of most of the affected areas are insufficient to react to the emergency. At the rural level, even basic hygienic measures are rarely respected in animal husbandry and farmers have no concept of disease control measures. The social behaviour of the rural human population includes habits which facilitate the spread of infection, within the same village and, through trade, to other villages. International interventions must therefore be focused primarily on education programmes and on veterinary support to farmers. Control programmes have been developed on a country by country basis, and vary from very limited interventions to strict stamping out policies. Some countries have applied blanket vaccination campaigns with or without veterinary support; very few of these have employed monitoring of vaccinated birds with a view to stamping out those vaccinated birds found to be infected. What appears to be lacking in certain countries, regardless of the chosen method of control, is a follow up on the results of the interventions, a prerequisite to improving management efforts worldwide [4]. The scientific veterinary community has the duty and responsibility to address the complex issue of AI ecology and epidemiology in the countries it affects. The complex epidemiology of this infection requires tailored interventions in collecting and analysing the data available from affected areas and identifying the links with husbandry methods and social practices that are typical of each environment. The generation of similar data from different parts of the world, integrated with molecular epidemiology studies represents the baseline to understand the mechanisms

by which the virus appears, disappears and reappears – which in turn is essential to the development of adequate control strategies. The medical, veterinary and agricultural scientific communities are challenged with a virus that is moving in a tri-dimensional fashion, modifying itself as it adapts to different species and reassorting with other influenza viruses of avian and potentially mammalian origin, as it infects new species. A significant collaborative and financial effort in a transparent scientific environment are required to generate data and ideas contributing to the eradication effort. Until the extensive circulation of the virus is limited in the poultry reservoir, avian influenza will continue to remain an issue for food security and a global threat for animal and human health. The veterinary community should take ownership of this responsibility as it has the knowledge and understanding to offer sustainable solution for the management of this infection.

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Influenza A/H5N1 Vaccine Development: Building on What We Have Learned

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Introduction

The emergence of human infections caused by novel avian viruses in recent years has raised concerns regarding their pandemic potential [1]. The ongoing epizootic caused by highly pathogenic (HP) influenza A/H5N1 has resulted in several hundred human fatalities, as well as the death of millions of diverse avian species—both wild and domestic. The continued spread of influenza A/H5N1 infections has stimulated intense control efforts, including the development of candidate vaccines. This update will summarize the current status of A/H5N1 vaccine development.

Recent Progress in Influenza A/H5N1 Vaccine Development

Much progress has been made in the 10-year period since the appearance of HP influenza A/H5N1 infections. Expanded global surveillance of influenza has improved our ability to track the epidemic and to identify the emergence of antigenic variants. Reverse genetics (rg) techniques permit removal of the polybasic amino acid sequence on the attachment protein (the hemagglutinin, or HA) allowing for growth in eggs [2]. Laboratory methods for assay of immune responses following infection or immunization have been developed to overcome the insensitivity of standard serological methods: avian receptors differ from human receptors [3], and horse RBCs are more sensitive than turkey or chick RBCs for detection of HAI responses to A/H5N1 viruses [4, 5]. Enhanced international cooperation has facilitated identification and transport of potential vaccine seed strains. Reagents and libraries of avian viruses for vaccine standardization and development are being developed. Expansion of manufacturing capacity and transfer of technology to developing countries will facilitate a more timely response when a pandemic develops. Finally, new approaches to immunization are being explored. These efforts are proceeding on a global level: clinical trials of candidate vaccines are underway in Australia, Belgium, China, the Czech Republic, France, Germany, Hungary, Japan, Switzerland, The Netherlands, and the United States [6].

How Are Pandemic Vaccines to Be Evaluated?

Benchmarks for assessment of responses after immunization with interpandemic vaccines provide useful reference points for assessment of responses after immunization with novel influenza strains [7, 8]. Recommended antibody response profiles following immunization with pandemic vaccine candidates have been published by the European and American regulatory agencies [Committee for Human Medicinal Products (CHMP) and the US Food and Drug Administration (FDA), respectively] [9, 10] (Table 1).

Table 1.

RECOMMENDED SERUM HAI ANTIBODY RESPONSE PROFILE

Pandemic Influenza Vaccines (See references 9 & 10)

European Guidelines	US FDA Guidelines
<65 / ≥65 years	<65 / ≥65 years
$\frac{GMT_{post}}{GMT_{pre}} > 2.5 / > 2$	Post titer ≥40 in >70% / 60%*
Post titer ≥40 in >70% / 60%	SCR >40 / 30%*
SCR >40 / 30%**	$\frac{GMT_{licensed}}{GMT_{new}} \leq 1.5^{***}$
<i>Neut Ab also should be measured</i>	$\frac{SCR_{licensed}}{SCR_{new}} \leq 10\%^{***}$

Abbreviations: GMT=geometric mean titer; post= after immunization; pre=before immunization SCR= seroconversion rate; GMTlicensed= GMT after immunization with licensed vaccine; GMTnew=GMT after immunization with a new vaccine

*Lower bound of 2-sided 95% CI

**SCR defined as ≥4-fold rise or increase from <10pre to ≥40post

***Upper bound of 2-sided 95% CI

These guidelines will require validation following identification of correlates of protection against avian influenza. Although immunization goals have been proposed, clinical trial results are difficult to compare. Vaccine standardization and formulation are variable. Lack of assay standardization was documented by Stephenson et al [11]. Variability from one lab to another is a function of the type of assay; the substrates and reagents used; and assay protocols. International standards for assay of antibodies against influenza A/H5N1 are being prepared; their use will facilitate comparison between trials and candidate vaccines (Wood J; [http:// www.who.int/ vaccine_ research/ diseases /influenza/ meeting_150207/ en/index2.html](http://www.who.int/vaccine_research/diseases/influenza/meeting_150207/en/index2.html)).

Summary of Clinical Trials

Non-Adjuvanted Subunit Vaccines Emergence of A/H5N1 in 1997 triggered initial vaccine development efforts [12]. Because the vaccine strain (A/Hong Kong/97, or A/HK) was lethal to chick embryos, the HA gene was cloned into a baculovirus vector, and then expressed in insect cells. Treanor et al described the safety and immunogenicity of a recombinant H5 HA (rHA) vaccine among healthy adults given two intramuscular (IM) doses containing 25, 45, or 90µg of rHA/dose [13]. Dose-related increases in antibody responses were observed, but only ~50% given the 90µg dosage developed a neutralizing (Neut) antibody response. The reemergence of A/H5N1 viruses in 2003 stimulated intensified development efforts [14]. Initial candidates were prepared using licensed approaches following HA modification and plasmid rescue of a virus expressing the HA and NA from A/Vietnam/1203/04 (A/VN) and the internal genes from A/PR/8/34 [2]. Treanor et al described the safety and immunogenicity of an inactivated subvirion (SV) vaccine prepared from rg A/VN/1203/04 among healthy adults given 2 IM doses containing 7.5, 15, 45 or 90µg HA/dose [15]. Vaccines were well tolerated. Dose-related increases in antibody responses were demonstrated. As observed previously, two-

90µg doses of HA were necessary to stimulate responses in ~50% of the subjects. Forty-six percent of elderly subjects given two-90µg doses achieved an HAI titer of ≥ 40 , similar to results seen in younger adults [J.Treanor et al, abstract, Options for the Control of Influenza VI, Toronto, Ontario, Canada, June 2007]; and 38% of 91 healthy 2-9 year olds given two 45µg doses developed ≥ 4 -fold rises in HAI titer (Campbell J; http://www.who.int/vaccine_research/diseases/influenza/meeting_150207/en/index2.html).

Adjuvanted Vaccines. The requirement for high dosages of vaccine is a matter of concern, because vaccine availability will be limited during the early phases of a pandemic. Therefore, dosage-sparing approaches are being evaluated. Adjuvants are substances that enhance the immunogenicity of a vaccine. Several adjuvants are licensed for use in human vaccines in the US and/or Europe, including mineral salts (aluminum hydroxide, or AIOH; aluminum phosphate, or AIPO; etc), an oil-in-water emulsion (MF59), and AIPO plus monophosphoryl lipid A, a microbial adjuvant. Others types of adjuvants are being evaluated, including other microbial (cholera toxin, CpG, flagellin), particulate (viroosomes, liposomes), synthetic (polyphosphazene, copolymers), genetic (plasmids expressing costimulatory molecules) and cytokine (IL-2, IL-12, GM-CSF) adjuvants. The mechanism(s) of action include depot effect, antigen protection, cytokine release, and induction of maturation and activation of antigen-presenting cells. Several studies have assessed the ability of adjuvants to reduce the amount of H5 antigen required. Bresson et al reported the safety and immunogenicity of a SV rg A/VN/1194/04 vaccine among healthy adults given 2 IM doses containing 7.5, 15 or 30µg HA/dose formulated with or without AIOH just before administration [16]. Serum HAI and Neut antibody responses were lower in groups given 7.5 or 15µg with AIOH compared with nonadjuvanted groups. A modest but statistically insignificant beneficial effect of AIOH was seen only at the 30 µg level. Keitel et al assessed the safety and immunogenicity of preformulated AIOH-adjuvanted and non-adjuvanted SV rg A/VN/1203/04 vaccines in 600 healthy adults given 2 IM doses containing 3.75, 7.5, 15 or 45µg of HA/dose. Dose-related increases in antibody responses were observed, but a clinically significant benefit of AIOH was not observed: at the 45µg dosage with or without AIOH, 33% and 25%, respectively, developed ≥ 4 -fold increases in HAI antibody titer [W. Keitel et al, abstract, Options for the Control of Influenza VI, Toronto, Ontario, Canada, June 2007]. Brady et al reported similar results among elderly subjects: 33% and 34% given two 45µg doses of vaccine with or without AIOH, respectively, had significant rises in titer [R. Brady et al, abstract, Options for the Control of Influenza VI, Toronto, Ontario, Canada, June 2007]. Influenza vaccines formulated with MF59 are licensed for use in Europe [17]. Nicholson et al described the safety and immunogenicity of a purified surface antigen (PSA) vaccine derived from a non-pathogenic A/Duck/Singapore (H5N3) [18] among healthy adults randomized to receive two IM doses of MF59-ajuvanted or non-adjuvanted vaccine containing 7.5, 15, or 30µg HA/dose.

Vaccines were well tolerated. Non-adjuvanted vaccines were poorly immunogenic. Addition of MF59 significantly augmented responses: HAI and Neut responses were observed in 6 and 8 of 10 subjects, respectively, who were given the 7.5µg dosage. A 3rd dose of vaccine given 16 months later significantly boosted responses compared to those achieved after the 2nd dose [19]. Similar results were reported by Atmar et al using a vaccine prepared from A/Hong Kong/97 (H9N2): >75% of subjects given 3.75µg responded [20]. The safety and immunogenicity of AIOH-, MF59-, or non-adjuvanted SV rg A/VN vaccines among healthy adults were reported by Bernstein et al [D. Bernstein et al, abstract 6293.15, Pediatric Societies Annual Meeting, Toronto, Ontario, Canada, May 2007]. Inclusion of AIOH did not enhance immunogenicity at any dosage level. In contrast, 63% of subjects given two-15µg doses of MF59-adjuvanted vaccine developed ≥ 4 -fold increases in HAI titer. The adjuvant effect of a proprietary adjuvant system (AS; GlaxoSmithKline Biologicals) on immune responses after immunization of healthy adults with inactivated H5N1 vaccine containing 3.8, 7.5, 15, or 30µg of A/H5N1 HA/dose resulted in an EU license application for a pre-pandemic vaccine early in 2007 [http://www.fda.gov/ohrms/dockets/ac/07/slides/2007-4282OPH1_1_files/frame.htm]: over 80% of subjects given two-3.8µg doses responded compared with <50% of subjects given the highest dosage level of nonadjuvanted vaccine.

Whole Virus Vaccines. Another dosage-sparing approach is the use of whole virus (WV) vaccines, which were shown to enhance immune responses among unprimed persons in previous trials of pandemic vaccine candidates [21]. However, WV vaccines elicited a higher frequency of adverse events, including fever in young children [22]. The safety and immunogenicity of an egg-grown, AIOH-adjuvanted WV vaccine prepared using A/VN/1194/04 among healthy adults given two IM doses containing 0, 1.25, 2.5, 5 or 10µg of HA/dose were reported by Lin et al [23]. Most of the subjects given the 10µg dosage developed an antibody response; however, the effect of the adjuvant is unknown because non-adjuvanted preparations were not evaluated. Tashiro and colleagues reported the safety and immunogenicity of egg-grown WV rg A/vaccines among healthy 20-39 year old males [http://www.who.int/vaccine_research/diseases/influenza/meeting_150207/en/index2.html]. Seroconversion rates (Neut Ab) after two-15µg doses were 84% and 96% after subcutaneous (SQ) and IM vaccination, respectively; and 72% and 71% after two 5µg doses. Kistner et al described the safety and immunogenicity of a Vero cell culture-grown WV vaccine prepared using wt A/VN/1203/04 virus among healthy adults given two IM doses containing 7.5 or 15µg of HA/dose with or without AIOH, or two doses containing 3.75 or 30µg of HA/dose with AIOH [http://www.who.int/vaccine_research/diseases/influenza/meeting_150207/en/index2.html]. Seventy-six percent of subjects given the 7.5µg dosage level achieved a Neut titer of ≥ 20 vs. A/VN/1203/04 and A/HK/156/97; 45% of these also had Neut antibody titers ≥ 20 vs. A/Indonesia/05/05. Interestingly, non-adjuvanted formulations were more immunogenic than adjuvanted formulations. An

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NIH-sponsored trial of a cell culture-grown WV A/H5N1 vaccine with or without AIOH is in progress.

Intradermal (ID) Immunization. Administration of lower than usual dosages of influenza vaccine by the ID route has been shown to elicit similar responses when compared with IM injection in previous trials [24, 25]. Our group reported the results of a pilot evaluation of a SV A/VN/1203/04 vaccine among healthy adults who were given 2 ID or IM doses of vaccine 1 month apart (S Patel et al, abstract presented at the VIII International Symposium on Respiratory Viral Infections, Kohala Coast, HI, March 2006). Response frequencies following 3 or 9µg given ID and 15µg IM were low and similar. Administration of a 3rd dose modestly enhanced responses, particularly among subjects given 45µg IM (S Patel et al, abstract LB-4, 44th Infectious Diseases Society of America (IDSA) meeting, Toronto, Ontario, October 2006). ID administration of vaccine was associated with a higher rate of injection site redness, swelling, and transient hyperpigmentation when compared with IM administration. A phase II trial evaluating a 30µg dosage levels by the IM or ID route is underway to test the hypothesis that the ID route is superior to the IM route when the same dosage level is given by both routes. Results of several other unpublished clinical trials have been reported [26]: an AIPO-adjuvanted PSA vaccine elicited Neut antibody responses in 41% of subjects given two-30µg doses; and one 6µg dose of an AIPO-adjuvanted WV vaccine was reported to elicit HAI antibody responses in 68% of subjects in Hungary.

Live Attenuated Influenza Vaccines. Another approach being explored is the use of live attenuated influenza vaccines (LAIV) [27, 28]. Karron et al reported the results of a phase I clinical trial of a LAIV produced by reassortment of the master donor strain influenza A/Ann Arbor/6/60 (H2N2) with rg A/VN (H5N1). Vaccine was well tolerated, but replication was highly restricted. Only 11% of subjects had an antibody response after two-10^{6.7} TCID₅₀ doses. Rudenko reported that most subjects given 2 doses of a LAIV [A/Leningrad (H2N2) master donor strain X A/duck Potsdam/86/92 (H5N2)] containing 10^{6.5} EID₅₀/dose shed virus (55% and 70% after dose 1 and 2, respectively); 47% had a ≥4fold rise in HAI antibody (http://www.who.int/vaccine_research/diseases/influenza/meeting_150207/en/index2.html).

Antigenic Drift. Influenza A/H5N1 viruses continue to undergo antigenic drift: several clades and subclades have been identified [29, 30]. Information regarding the ability of vaccines from one clade of virus to stimulate antibody against other clades of virus is critically important. Stephenson et al tested sera collected after immunization of subjects with 3 doses of MF59 or non-adjuvanted A/duck/Singapore/97 (H5N3) vaccine against a panel of A/H5N1 variants [31]. Immune responses against drifted viruses were greater with adjuvanted vaccine, but were lower than those against homologous or antigenically similar strains. In another study, administration of a dose of A/VN vaccine resulted in significant responses to the new virus vaccine among subjects previously primed with A/HK rHA, suggesting that priming with a variant could be a useful

strategy (J Treanor et al, abstract LB-4, 44th IDSA meeting, Toronto, Ontario, October 2006). Animal studies suggest that immunization with drifted variants can confer significant protection against challenge despite low levels of antibody to the challenge strain, underscoring the need for further work on correlates of protection [32-35].

Lessons Learned. Results of recent trials suggest non-adjuvanted SV and PSA vaccines appear to be poorly immunogenic. Vaccines formulated with MF59 or other novel adjuvants and/or WV preparations are promising candidates. Although adjuvants may enhance immunogenicity, their safety and tolerability profiles remain a significant consideration. Modest enhancement of immunogenicity has been reported in some trials using aluminum-containing adjuvants, but dosage-dependence and differences in formulations require additional study. Next steps should include expeditious evaluation of promising adjuvants (MF59; AS) and formulations (WV) with drifted viruses in all age groups; dose-ranging of promising adjuvants; and further investigation of the infectivity of LAIV candidates. Other approaches being explored for development of pandemic vaccines include use of novel adjuvants [36], DNA constructs [37], and vaccines based on conserved, broadly cross-reactive epitopes [38]. Finally, passive immunization may play a role in the treatment of established infections [39-41]. The overarching priority of current and future vaccine development efforts is to develop a broad programmatic approach that emphasizes international collaboration and harmonization of assay procedures. This will facilitate surveillance of emerging viruses, strain selection, and trial comparisons; help to better define correlates of protection and requirements for effective immunization; and ensure that all nations will benefit from vaccine development activities. The goal is to develop a range of vaccines/approaches that could be used in the setting of an evolving pandemic. It is unlikely that any single product would be indicated for the entire population in all countries. Age, underlying medical conditions, cost, delivery methods, cold chain requirements and other factors need to be taken into consideration. Relative advantages and disadvantages to the various approaches are outlined in Table 2.

Table 2. Potential advantages and disadvantages of immunization with various types of vaccines/formulations.

TYPE OF VACCINE	POTENTIAL ADVANTAGES	POTENTIAL DISADVANTAGES
SUBUNIT	Safety; broad indications (age to 6months, underlying diseases, pregnancy); licensed processes	Requirement for 2 high(er) dosages
SUBUNIT WITH ADJUVANT	Antigen-sparing	Potential safety concerns; increased reactogenicity; increased cost
WHOLE VIRUS	Potential antigen-sparing; current/past licensed processes	Reactogenicity in young children
LIVE ATTENUATED	Potential antigen-sparing; broader immune responses; needle-free; possible need for only 1 dose; possible early protection	Pandemic only; safety issues (age; health/ immune status); low infectivity

In conclusion, wherever the road takes us from this point forward, future development of promising candidates should take advantage of the lessons we have learned.

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Humidity and Temperature Affect Influenza Virus Transmission: Studies in the Guinea Pig Model

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We have recently shown that the guinea pig is highly susceptible to infection with human influenza viruses and transmits these viruses by both aerosol and contact routes. Thus, the guinea pig represents an ideal small mammal in which to examine influenza virus transmissibility. To gain insight into the role of weather conditions in the seasonality of influenza, we used the guinea pig as a tool to investigate the effects of relative humidity (RH) and temperature on transmission. Although the seasonal epidemiology of influenza is well characterized, the underlying reasons for predominant winter-time spread in temperate climates are not clear. By performing twenty replicate transmission experiments under controlled conditions of RH and temperature, we have shown that aerosol spread is dependent upon both of these parameters: cold and dry conditions favor influenza virus transmission. We found that infected guinea pigs housed at 5°C shed virus at peak titers for approximately two days longer than animals housed at 20°C, an observation which likely accounts for the improved transmission seen at 5°C. The observed relationship between transmission via aerosols and RH is similar to the reported relationship between the stability of influenza virus in aerosols and RH, implying that the effects of humidity act largely at the level of the survival of virus particles in the environment. Our data provide direct, experimental evidence to support the role of climatic conditions in the epidemiology of influenza. Furthermore, our findings are likely to be applicable to efforts to control epidemic influenza: the spread of influenza virus in healthcare and community settings could potentially be curtailed through internal environmental control.

Introduction

The efficiency with which influenza viruses spread from host to host is clearly of great importance to their potential to cause an epidemic or a pandemic. Nevertheless, there is a paucity of research into the viral, host and environmental factors affecting influenza virus transmission, partly due to the lack of a convenient animal model to study this phenomenon. Although the mouse is a well-established and convenient laboratory animal, mice are relatively resistant to infection with human influenza viruses, and mouse-adapted influenza viruses transmit inefficiently [1]. Influenza in the ferret model more closely mimics that seen in humans [2], and human influenza viruses are transmitted from ferret to ferret [3]; thus ferrets are a valuable tool in influenza research. However,

several practical considerations [4] make ferrets less suitable for use in an academic setting than rodents. Most importantly, their size and cost often preclude the implementation of large-scale studies. For these reasons, we recently characterized the guinea pig as a model host for the study of influenza virus [5] and have exploited this model to investigate the effects of humidity and temperature on transmission [6]. Surveillance data dating back several decades shows that human influenza recurs with a highly predictable, seasonal, pattern in temperate climates. Both in the northern and the southern hemispheres peak influenza activity is tightly associated with the winter months [7]. Nevertheless, the mechanisms underlying the seasonal epidemiology of influenza remain unclear. The periodic incidence of various infectious diseases has attracted much interest over the years and theories aimed at explaining seasonality therefore abound. Hypotheses for influenza include variation in immune competence mediated by seasonal host factors such as melatonin [8] and vitamin D [9]; seasonal changes in human behavior, such as air travel [10], indoor crowding during cold or rainy weather, and school attendance; and environmental factors including temperature [11], relative humidity (RH) and the direction of air movement in the upper atmosphere [12]. Of particular interest to the studies reviewed herein are the common perception that cold weather brings the “flu”, and the more scientifically supported idea that relative RH plays a role in influenza seasonality through its effects on viral stability in an aerosol. Several articles published in the 1960’s and 1970’s reported an impact of RH on the viability of influenza virions suspended in aerosol particles [13-17]. The most recent of these [16] shows that the airborne virus is most stable at low RH of 20-40%, least stable at intermediate RH of approximately 50%, and more stable at high RH of 60-80%. As a result of these data it has long been thought that, through an effect on aerosol transmission, RH could be the seasonal force underlying the dynamics of influenza. This hypothesis had not been tested, however, until very recently [6]. Using an environmental chamber to achieve controlled conditions, we assessed the effects of RH and temperature on the aerosol transmission of influenza virus between guinea pigs.

The guinea pig as a model for influenza virus transmission. We have shown that the human influenza virus isolate A/Panama/2007/99 (Pan/99; H3N2) grows to high titers in the upper respiratory tract (10^7 PFU/ml of nasal wash) and to moderate titers in the lower respiratory tract (10^5 PFU/g of lung) of guinea pigs [5]. The 50% infectious dose (ID_{50}) of Pan/99 virus in guinea pigs was 5 PFU, indicating that guinea pigs were also highly susceptible to infection. Furthermore, we found that Pan/99 virus transmits from infected to exposed guinea pigs, both under conditions where the two animals are in direct contact and when the animals are housed in separate but adjacent cages [5]. More recently, we have shown that two other human influenza A virus isolates (A/Texas/36/91 [H1N1] and A/Beijing/46/92 [H3N2]) and an influenza B isolate (B/Shanghai/361/02) transmit between guinea pigs, while the avian influenza virus A/duck/Ukraine/1/63 (H3N8) does not

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(unpublished observations). Thus, overall, the results obtained to date in guinea pigs accurately model the growth and transmission of influenza viruses in humans.

Influenza virus transmission is dependent on RH. To test the effect of RH on influenza virus spread, ten replicate transmission experiments were performed in which the only variable that changed was the ambient RH. Each experiment involved eight guinea pigs, four of which were infected intranasally with 1000 PFU of Pan/99 virus. At 24 h post-inoculation all eight animals were placed in individual transmission cages (that is, cages which allow air flow through a wire mesh top and one wire mesh side, but do not allow contact between guinea pigs) and housed in the environmental chamber. The four naïve animals were thereby exposed to the four infected animals under controlled conditions of RH and temperature for a total of seven days. During this time, and up to 12 d post-inoculation, nasal washings were collected from all eight guinea pigs every two days. The infection status of each guinea pig was then monitored by performing plaque assays on the nasal washings. Duplicate experiments were carried out at 20°C and the following relative humidities: 20%, 35%, 50%, 65%, and 80%. The data, summarized in Table 1, indicated that the efficiency of transmission did indeed vary with ambient RH. Spread to exposed animals was most efficient at low RH of 20 or 35%, less efficient at 50% RH, more efficient again at 65% RH, and completely blocked at 80% RH. With the exception of the lack of transmission at 80% RH, our data on influenza virus transmission parallel those on influenza virus stability reported by Schaffer et al. [16]. The effects of RH on transmission are therefore likely to be mediated largely by effects on the virus particle. We propose that, at 80% RH, an independent effect of humidity comes into play. Namely, at high relative humidities the formation of droplet nuclei from larger respiratory droplets occurs less efficiently. As small respiratory droplets either fail to lose water through evaporation or take on water from the air, they settle more rapidly, thereby reducing their potential to contribute to transmission.

Table 1. The efficiency of influenza virus transmission varies with relative humidity.

	Transmission Efficiency (%)*	
	Experiment 1	Experiment 2
20%RH 20°C	100	75
35%RH 20°C	100	100
50%RH 20°C	25	25
65%RH 20°C	75	75
80%RH 20°C	0	0

*Transmission efficiency is defined as the percentage of exposed, naïve, guinea pigs that became infected during the course of the experiment

Influenza virus transmission is dependent on temperature. The impact of both low (5°C) and high (30°C) temperature on transmission were also examined. Thus, transmission experiments were performed exactly as described above, but rather than 20°C, the ambient temperature in the environmental chamber was set to either 5°C or 30°C. Again in duplicate experiments, a range of RH conditions was tested at 5°C (35%, 50%, 65%, and 80%), and a low RH of 35% was tested at 30°C. The results are summarized in Table 2. While transmission was completely blocked at 30°C and 35% RH, the incidence of infection of exposed guinea pigs was increased at 5°C relative to 20°C. In particular, at 50% and 80% RH, the transmission rate was significantly higher at 5°C than at 20°C ($p < 0.05$). The increase in transmission efficiency at lower temperature is likely due to the increase in viral shedding observed at 5°C. As reported in [6], guinea pigs housed at 5°C shed virus at high titers ($\geq 10^6$ PFU/ml) for an average of 41 h longer than guinea pigs housed at 20°C. The exact mechanism leading to increased viral shedding is less clear. The induction of innate immune mediators in nasal turbinates in response to viral infection was found to be similar between animals housed at both temperatures [6], arguing against the hypothesis that immunosuppression of animals kept at 5°C plays a role. Instead, a more direct effect on physical barriers to infection such as mucociliary clearance may lead to increased viral growth at 5°C. Alternatively, it is possible that lower temperature increases virus stability in the upper respiratory tract and thereby leads to increased (and prolonged) shedding of viable progeny virions.

Table 2. The efficiency of influenza virus transmission varies with temperature and RH.

	Transmission Efficiency (%)*	
	Experiment 1	Experiment 2
35%RH 5°C	100	100
50%RH 5°C	100	75
65%RH 5°C	50	50
80%RH 5°C	50	50
35%RH 30°C	0	0

*Transmission efficiency is defined as the percentage of exposed, naïve, guinea pigs that became infected during the course of the experiment

Conclusions

We have exploited our recently described guinea pig model of influenza virus transmission to test the effects of RH and temperature on the spread of this important human pathogen. Our data show that the efficiency of influenza virus transmission among guinea pigs is dependent on both RH and temperature, implicating these climatic factors in the epidemiology of influenza. Although other factors may

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contribute, our data suggest that humidity and temperature could drive the seasonal periodicity of human influenza through effects on aerosol transmission. It is furthermore apparent that the limitation of influenza virus spread in indoor settings could potentially be achieved through modification of air temperature and humidity.

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Age as an Independent Risk Factor for Deaths From Pandemic Influenza

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The great 'Spanish Influenza' pandemic of 1918 was unique in the 20th century both in its virulence, causing greater than 40 million deaths worldwide and in the age groups that fell victim to the disease and died. Unlike seasonal influenza, both then and now, where it is the old, young and infirm who are its usual victims, in 1918, there were an unprecedented number of deaths in the age range 20 to 44 years. Many of the individuals in this age range, who died, did so within 24 hours of falling ill, with well described symptoms and signs of primary viral pneumonia. Many theories have been put forward for both the scale and age distribution seen for deaths in 1918, ranging from social unrest, malnutrition and lack of social distancing in the army camps along the Western Front in Europe. This study will explore the role of age as an independent risk factor for deaths due to new emergent pandemic influenza virus. Results will be presented on the subsequent pandemic of Hong Kong influenza 1968 in England and Wales and the impact of this across the age spectrum. These findings will be discussed in the context of pandemic planning and the defining of 'at risk' groups for antiviral drugs and vaccine.

Introduction

At the end of the 'Great War' in Western Europe where societies and civilisation itself, had been brought to its knees, the 'Spanish Influenza' pandemic spread rapidly through the already war ravaged lands and decimated the lives of men and women who should have been in their prime. Alfred Crosby has reminded us, in his seminal work 'America's Last Great Plague', that this epidemic was not confined to the 'Old World's' war torn lands but caused an equal, if not more, catastrophic impact on the 'New World' of North America and beyond [1]. The more we study this period in history, the more influenza seems to have impacted on the world's population. Potentially some 40 million people may have succumbed to the 'second wave' of the 1918 pandemic, deaths occurring in far flung lands well away from the death, disease and social upheaval caused by war. One major characteristic of the 1918 visitation by influenza was the unusual age profile of its victims. Cases of acute respiratory distress and rapid death occurred more frequently in young adults than one would expect from experience gained with seasonal influenza. Frost [2], writing in 1919 reported that while the highest case fatality rates were seen in the under 1 age group and the elderly between the ages of 70 and 74 years (5.2% and 5.3% respectively), across the age spectrum the profile of deaths varied markedly from seasonal influenza [2]. Young children between the ages of 5 and 9 years and middle aged adults had the lowest rates (0.5% and 0.9%) while in the age range 25 to

29 years a higher than expected rate of 3.1% was observed. This higher than expected rate persisted at around 2% for those aged less than 45 years, with nearly half the total death toll occurring between the ages of 20 and 40 years [3]. The extreme elderly, older than 75 years, by comparison to previous seasonal epidemics, seemed to fare more favourably. Many theories have been put forward as to why the pandemic of 1918 had this 'W' shaped curve in relation to mortality, not seen in other epidemics, which will be explored later. Simonsen et al., [3], used national vital statistics for the United States for the period 1965 to 1995 to generate a mathematical model from which they could estimate the excess mortality from influenza, by age group, year on year. They showed that when the new H3N2 (Hong Kong) virus emerged during the winter of 1968 to 69 in North America, persons less than 65 years accounted for 45% of the estimated excess mortality, this proportion fell to 10% after ten years as the virus became endemic. The present study explored, using national mortality statistics for England and Wales in the United Kingdom, UK, whether during the pandemic emergence of the Hong Kong virus, any age related differences in response occurred such as those reported previously [2, 3].

Methods

Monthly mortality data was obtained from the Office of National Statistics, ONS, in the United Kingdom for the period 1965 to 2004. Data were obtained on all cause mortality, deaths registered as Influenza and the more general label of Influenza and Pneumonia. This study is based on the deaths registered as Influenza which occurred during the months when Influenza A virus was known to be circulating and causing significant epidemics. Winters when no influenza epidemics occurred were excluded. Each year of the study ran from the summer period to the next summer in order to capture the period of peak winter seasonal activity and avoid confusion that would arise should the study include calendar years. Data from the period January 1966 to April 1976 were used for the analysis. This time period included the last two seasons of significant Influenza deaths that occurred due to H2N2, Asian Influenza, and all influenza epidemic years from 1968 to 1976 which were caused by the emergent H3N2, Hong Kong, virus. Analysis of the data was in ten year age bands from 5 years to 84 years data on the <1 year olds, the 1-4, and 85 years and older being presented separately. Age-band specific population estimates for each year in the study were obtained from ONS and used in the analysis to calculate age band specific mortality rates. Age-band specific mortality rates per 100,000 population were then calculated for each influenza epidemic period as well as the average age-band specific mortality for the decade as a whole. The ratio between an epidemic year's age-band specific mortality and the average age-band specific mortality for the whole period was calculated in order to analyse the differential impact of influenza, each season, across the age bands. Weaknesses in any ecological study of influenza mortality are multiple. While mortality, as used in this study, represents a 'hard' end-point, a number of questions about validity and reliability remain.

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Many deaths from influenza would have been excluded from this study as the specific cause of death, Influenza, was used rather than the more general, Influenza and Pneumonia. In addition, many deaths exacerbated by influenza illness, such as those recorded as Heart Disease, would not be accounted for. Finally, many deaths recorded as being attributed to influenza, were not substantiated before death certification by laboratory methods. Fluctuations in recorded deaths from influenza can be due to the dynamic interplay between attack rate and virulence, with epidemics of low attack rates with a highly virulent virus producing as many, if not more, deaths, than one which sees the opposite i.e. high attack rate with a low pathogenic virus. In comparison to 1918, the H3N2, Hong Kong, virus that emerged in 1968 had low pathogenicity with moderate attack rates. The present study, by averaging age-band specific mortality rates across all seasons used in the study and excluding those years with little or no influenza, is likely to underestimate the age specific impact of the pandemic emergent strain. The inclusion of deaths only registered as influenza, again is likely to underplay the collateral impact of influenza on other death registration categories. The methodology of comparing the seasonal age-specific mortality to an expected average was used to try to address the attack rate/virulence problem discussed earlier.

Results

During the study period from autumn 1965 to spring 1976 influenza epidemics occurred in each of the 11 winter seasons except for the winters of 1966-67 and 1970-71. During the spring of 1968 the world saw the transition from Influenza H2N2, Asian, virus being the sole strain of Influenza A epidemics from 1957-68, to H3N2 'Hong Kong' virus for the rest of the study period. In the United Kingdom and Western Europe, while very mild epidemics occurred due to the emergent strain in the 1968-69 season, the following winter the true impact of the disease was felt, Table 1.

Table 1. Ratio of the age-specific mortality per 100,000 of population during the influenza seasons from 1966 to 1976 compared with the average age-specific mortality per 100,000 of population for this period.*

Age group	Jan-Apr 1966	Dec 67-Mar 1968	Jan-Apr 1969	Dec 69-Feb 1970	Dec 71-Feb 1972	Dec 72-Feb 1973	Mar-Apr 1974	Jan-Apr 1975	Jan-Apr 1976	Average Mortality Rate
All ages	0.93 (0.25)	1.31 (0.83)	0.34 (2.26)	2.73 (18.36)	0.46 (2.06)	1.05 (7.05)	0.17 (1.17)	0.32 (2.03)	1.70 (11.45)	(6.72)
<1	1.72 (0.89)	1.36 (2.07)	0.73 (1.10)	2.61 (3.99)	0.43 (0.66)	0.46 (0.71)	0.10 (0.15)	0.32 (0.48)	1.22 (1.85)	(1.52)
1-4	2.00 (0.46)	0.87 (0.20)	0.30 (0.57)	2.48 (0.57)	0.48 (0.11)	0.95 (0.22)	0.13 (0.03)	0.13 (0.03)	1.85 (0.38)	(0.23)
5-14	2.08 (0.25)	0.82 (0.11)	1.08 (0.13)	1.58 (0.19)	0.33 (0.04)	1.17 (0.14)	0.42 (0.05)	0.08 (0.01)	1.58 (0.19)	(0.12)
15-24	0.97 (0.30)	1.00 (0.31)	0.90 (0.28)	2.82 (0.78)	0.42 (0.13)	1.39 (0.43)	0.19 (0.06)	0.19 (0.06)	1.92 (0.47)	(0.31)
25-34	1.07 (0.44)	0.73 (0.20)	0.80 (0.23)	2.85 (1.21)	0.46 (0.19)	0.90 (0.37)	0.12 (0.05)	0.31 (0.13)	1.73 (0.71)	(0.41)
35-44	0.90 (0.82)	0.57 (0.52)	0.88 (0.58)	3.88 (3.62)	0.51 (0.46)	0.93 (0.85)	0.11 (0.10)	0.22 (0.21)	1.07 (0.97)	(0.91)
45-54	0.79 (1.80)	0.89 (1.97)	0.63 (1.45)	3.89 (6.15)	0.80 (1.37)	1.13 (2.39)	0.16 (0.37)	0.26 (0.80)	0.75 (1.72)	(2.29)
55-64	0.87 (1.59)	0.82 (1.54)	0.42 (2.06)	4.10 (26.17)	0.95 (0.89)	0.96 (0.94)	0.10 (0.18)	0.22 (1.41)	0.95 (0.57)	(6.40)
65-74	0.80 (17.51)	1.03 (21.56)	1.38 (7.96)	3.99 (83.86)	0.82 (10.79)	0.88 (18.48)	0.12 (2.83)	0.22 (4.80)	0.89 (85.80)	(28.31)
75-79	1.08 (48.19)	1.91 (65.87)	0.34 (15.30)	2.80 (114.77)	0.48 (11.91)	1.22 (54.07)	0.17 (7.85)	0.31 (13.81)	1.48 (85.99)	(48.33)
80-84	0.81 (85.84)	1.29 (125.62)	0.17 (17.68)	1.16 (122.40)	0.27 (28.85)	0.88 (82.37)	0.13 (13.65)	0.26 (26.91)	1.57 (165.20)	(76.48)
85+	1.11 (188.85)	1.96 (335.93)	0.22 (28.31)	1.30 (228.30)	0.32 (64.07)	0.97 (164.95)	0.22 (37.83)	0.38 (69.72)	2.32 (427.72)	(169.76)

*Mortality rate per 100,000 of the population of England and Wales is shown in brackets ()

It can also be seen, again in Table 1, that England and Wales experienced a severe epidemic in the last season of the reign of the H2N2 virus, with the result, it has been proposed, that the cross immunity generated by the N2 antigen, common to both

'Asian' and 'Hong Kong' viruses, protected Western Europe from the first wave of the new virus [4]. Specific comparisons of the mortality during the 1969-70 season, across age-bands, shows that, mortality was 2.73 times the seasonal average for the study period (including 1969-70), however, those less than aged 25 years did better than this overall, <1 year was 2.61 times, 1 to 4 age group was 2.48 times, 5 to 14 age group was 1.58 times and the 15 to 24 age group was 2.52 times. Those between the ages of 25 and 75 years fared worse than the overall average, with those between 35 and 74 years being 4 times more likely to die than in an average winter season. Those aged over 75 years being preferentially spared, a finding, on face value, to be counter intuitive knowing the nature of normal seasonal influenza mortality. This profile of age-specific mortality with the elderly fairing better than expected, while those aged 25 years and older doing worse persists for the first few seasons of the new H3N2 virus, until the 1974 to 75 season when the normal pattern of the young and old being affected returns.

Discussion

Any discussion of pandemic influenza is always coloured by reference to the spectre that was 1918 'Spanish Influenza' and the profound impact it had in terms of morbidity and mortality in the young and most productive years of life. Frost [2] has demonstrated, by the W shaped pattern of deaths seen, that this particular pandemic involved relative sparing of the elderly and young children between the ages of 5 and 9 years, while young adults were profoundly affected. The present study has demonstrated that in 1968, in England and Wales, during a much milder pandemic emergence of a new virus, H3N2, there existed some variation across the age spectrum with relative sparing in those over 75 years. Simonsen et al, demonstrated an increase in relative mortality in the less than 65 age group during the 1968 pandemic in the United States, with a trend towards a more 'normal' seasonal distribution over subsequent years [2]. This study builds on this work and demonstrates a relative increase in susceptibility of death in younger age groups during the early phase of a pandemic emergence. Further support for my findings, as a real phenomena, can be drawn from the fact that the results demonstrate that less people, relatively, based on the impact of the pandemic in England and Wales, died over the age of 75 years than one would expect from an epidemic of similar magnitude from a seasonal virus. Masarel [5] has demonstrated antibodies from stored blood samples drawn before 1968, against the H3N2 virus in individuals over the age of 70 years, the highest levels being in those over 75 years, suggesting prior exposure, perhaps in the 1890's and hence protection against the virus when it re-emerged in 1968, an hypothesis supported by the findings of this study [5]. The profile of the high age-specific mortality affecting young adults and the relative sparing of the elderly and children, between 5 and 15 years of age, fits the profile of deaths in 1918 with a much more virulent virus. The elderly being spared by immunity gained from prior exposure and the 5 to 15 year olds are as an age-group immuno-tolerant before they develop fully adult,

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immune systems. The first contact, robust immune response, of young adults, resulting in high levels of cytokine, could explain the increase death rates to both the 1918 virus and new emergent viruses such as 'Hong Kong' influenza in 1968. During the middle years of life, a period of declining thymic function, the cell mediated host response is diminished and individuals die, more often, from secondary invaders than primary viral disease. The high prevalence of chronic diseases of the Heart and Lungs in the middle aged of the UK in the 1960's and 70's may explain why this age group fared less well, comparatively to other age groups, in 1968 than comparatively in 1918. With an increasing global population and an average age in the developing world of 30 years or less, the implications of a global pandemic could result in a disproportionate burden, in terms of morbidity and mortality, being felt by young adults with key roles in civil society. Global pandemic planning, involving the use of antiviral therapies and vaccines, needs to take into account the differential host/pathogen response, felt across the seven ages of man, if it is to minimise the impact of the last great plague of antiquity on mankind.

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Workshop Topic #1

**Disease Surveillance
and Impact**

**Oral
Presentations**

Intra-Familial Transmission of Influenza A and Influenza B

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Introduction

Active vaccination programs, early diagnosis, and early treatment are essential in controlling influenza outbreaks. It is becoming increasingly important to effectively block the spread of these viruses. In our previous presentation [1] – “Option V in Okinawa,” we showed that the influenza virus persists for three days after fever has abated in patients treated with anti-influenza drugs. We further showed that this persistence of the influenza virus in patients contributes to intra-familial infection over a long period of time. We studied 1,636 flu cases in the last five influenza seasons, all confirmed by a positive result by rapid diagnostic test kit. How the first infected member of a family spread the virus to other members of the family was observed. Herein we report the results of our study of the infection rate, route, and period, with a focus on the role of children in how the virus is transmitted.

Method

Our study was of influenza cases over five flu seasons from 2001/2002 to 2005/2006. We define influenza patients as those with a body temperature no lower than 37.8 degrees Celsius; cough, sore throat, and other upper respiratory symptoms; and general feelings of illness such as headache and myalgia. People with these symptoms had influenza confirmed by the rapid test kits Capilia Flu A,B,[®] (Nippon Becton Dickinson, Japan) or Capilia Flu A+B[®] (Alfresa Pharma Corporation, Japan). Intra-familial infection was observed for two weeks after the first patient in the family developed symptoms.

Results

The incidence of influenza in one community. Attack rate from the 2001/2002 to 2005/2006 flu seasons. The scale of the outbreaks differed from one season to another, but the annual incidence of influenza A remained largely unchanged, at 4 to 6%, over the period. Influenza B occurred every other year, with an average incidence of 2.7% over the five seasons.

Attack rate and recurrent attack rate by age group over the five flu seasons. Overall, 30.7% of all groups became infected with either influenza A or B. Of those, 19.8% were infected more than once. The infection rate among children aged 4 to 6 years old was 59.7%. Of them, 33.3%, or 19.9% of the total, were infected more than once. The infection rate among children aged 7 to 12 was 55.1% and the percent who caught influenza more than once among this age group was 28.8%, which is high for children.

Infection rate, route and period in Intra-familial infection. The

Number of patients and families observed by influenza season. 1,636 flu patients from 1,255 families were tracked over the last 5 seasons. Of these families, 381, or 30.4%, had multiple infections. 983 people from 754 families were infected with influenza A. Of them, 229 families had multiple infections. 626 people from 477 families were infected with influenza B. Of those families, 149 had multiple infections. The influenza type of 26 patients from the 2001/2002 season are unknown because the use of rapid test kits other than Capilia prevented us from distinguishing between the A virus and B virus. The precise data for one patient from the 2003/2004 season is also unknown because the patient tested positive for both Capilia A and B.

Incidence rate of intra-familial infection and the families' infection rate in intra-familial infection. Intra-familial infection by influenza A and B was determined for families comprised of two to eight members. The number of four member families with influenza A was 360, or about half of the 756 families we monitored. The next largest in number was three-member families, at 224, followed by five-member families at 112. Families of up to five members accounted for 95.5% of the total. Influenza B affected 484 families ranging from two members to seven. As in the case of influenza A, families with four members accounted for about half of the total, or 235 families, followed by 123 three-member families and 70 five-member families. Secondary infection with influenza A did not occur in two-member families. It occurred in 43 three-member families, this is 19.2% of the inter-familial infection; 22.5% was in four-member families and 25.0% in five-member families. As the figures indicate, the larger the family, the more likely it was to suffer secondary infection. In all, 164 families, or 21.7%, suffered secondary infections. Secondary infections of influenza B occurred in 7.9% of two-member families, 13% of three-member families and 22.6% of four-member families. As the figures indicate, the larger the family, the more likely it was to suffer secondary infection. In all, 103 families, or 21.3% of the total, had secondary infections. This trend is similar to that for influenza A. Next, we observed the number of family members with influenza A and B exposed to influenza viruses by index cases in respective families and the number of family members infected by the index case (or the family infection rates). Among families that suffered secondary infections of influenza A, about 10% suffered further spread of the disease. The number of secondary infections rose with larger families but the rate of infection stayed the same. The percentage of people with influenza B who suffered secondary infection was especially high in five-member families, at 14.3%, but the overall secondary infection rate of the B virus was 9.8%, a level similar to that for influenza A (Table 1).

Incidence rate of inter-familial infection and the family infection rate in inter-familial infection by index case age. Table 1 shows familial infection of influenza based on an index case in each family. Index cases included grandparents, fathers, mothers, and children. Child index cases were further classified into five age groups: zero to three years, four to six, seven to nine, ten to twelve, and 13 and older. With influenza A, among children under age four who were infected first, 28.3% infected other

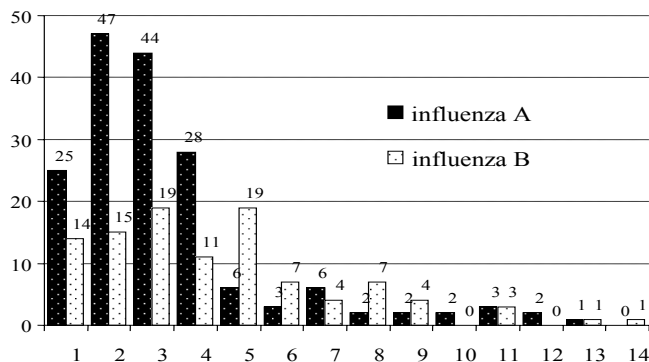
Table 1. Incidence rate of intra-familial infection and the families' infection rate in intra-familial infection.

	Influenza A				Influenza B			
	No. of families	No. of families with secondary infection (%)	No. of observed family members	No. of infected members (%)	No. of families	No. of families with secondary infection (%)	No. of observed family members	No. of infected members (%)
No. of family members								
Two	26	0 (0.0)	26	0 (0.0)	38	3 (7.9)	38	3 (7.9)
Three	224	43 (19.2)	448	47 (10.5)	123	16 (13.0)	246	18 (7.3)
Four	360	81 (22.5)	1080	109 (10.1)	235	53 (22.6)	705	65 (9.2)
Five	112	28 (25.0)	448	37 (8.3)	70	26 (37.1)	280	40 (14.3)
Six	25	8 (32.0)	125	13 (10.4)	14	3 (21.4)	70	5 (7.1)
Seven	7	3 (42.9)	42	3 (7.1)	4	2 (50.0)	24	2 (8.3)
Eight	2	1 (50.0)	14	1 (7.1)	0	- (-)	-	- (-)
Index case age								
0-3 yrs old	113	32 (28.3)	301	41 (13.6)	47	11 (23.4)	124	14 (11.3)
4-6 yrs old	123	39 (31.7)	368	49 (13.3)	105	29 (27.6)	312	36 (11.5)
7-9 yrs old	83	20 (24.1)	256	24 (9.4)	74	17 (23.0)	230	24 (10.4)
10-12 yrs old	99	20 (20.2)	302	31 (10.3)	38	9 (23.7)	121	13 (10.7)
13 yrs old more	146	21 (14.4)	434	25 (5.8)	73	8 (11.0)	215	11 (5.1)
Mothers	107	15 (14.0)	284	18 (6.3)	81	19 (23.5)	194	22 (11.3)
Fathers	82	16 (19.5)	226	21 (9.3)	61	9 (14.8)	151	13 (8.6)
Grandparents	3	1 (33.3)	12	1 (8.3)	5	1 (20.0)	16	1 (6.3)
Total	756	164 (21.7)	2183	210 (9.6)	484	103 (21.3)	1363	134 (9.8)

family members. For children four to six years old, the figure was 31.7%. Children under seven were highly likely to pass on their infections to family members. The overall rate of secondary infection was 21.7%. Among index cases, 9.6% passed the virus to family members. When index cases were children under four, the infection rate was 13.6%. For those aged four to six, the rate was 13.3%. For familial infection of influenza B based on an index case in each family, the 484 index cases included 337 children, or 69.6% of all index cases. The overall incidence rate of inter-familial infection was 21.3% – a level similar to that for influenza A. Among children under 12 who were infected first, 23.0 to 27.6% infected other family members. In the case of mothers who were index cases, the secondary infection rate was also high, at 23.5%. The overall family infection rate was 9.8% – a level similar to that for influenza A. But unlike influenza A, there were no significant differences in the secondary infection rates for the age groups under the age of 12, and the rates of influenza B infection from mothers was also high, suggesting that relatively older members of families are also likely to spread the virus.

Time distribution of secondary infection. The onset of secondary infection was observed for two weeks after an index case showed symptoms. The chart shows the daily number of secondary patients who developed symptoms (Figure 1).

Figure 1. Time distribution of secondary infection.



Tertiary infections are not included in the analysis. The number of people developing influenza A symptoms the day after the onset of the index case was 25; on the second day, 47; on the third day, 44; and on the fourth day, 28. The average interval between onset of the index case and a secondary infection was 2.57 days. The number of people developing influenza B symptoms the day after the onset of the index case was 14; on the second day, 15; on the third day, 19; on the fourth day, 11; and on the fifth day, 19. The average interval between the index case and a secondary infection was 3.54 days – nearly 24 hours longer than for influenza A.

The route of intra-familial transmission of influenza. As has been noted, 164 patient pairs who spread or contracted influenza A and 103 pairs with influenza B were observed. On analysis of the route of intra-familial transmission of influenza A, fathers were shown to account for 9.8% of the index cases, mothers 9.1%, children zero to three years 19.5%, four to six 23.8%, seven to nine 12.2%, ten to twelve 12.2%, and 13 and older 12.8%. Children up to age six made up a significant percentage of the index cases in families. Children of any age group passed the virus to either the mother or younger siblings. The results showed many secondary infections in mothers (43.3%) and children under four (17.7%). With influenza B as well as influenza A, babies (10.7%) and infants (28.2%) were the major source of intra-familial infection, transmitting the virus to their mothers (25.2%) and siblings under four years old (27.2%). Interestingly, more cases of infection among children under four were observed with influenza B than with influenza A. Intra-familial infection routes of the influenza virus are divided into two groups based on the persons who spread the virus and based on the persons who contracted the virus. Of the 348 infected children, 206, or 59.2%, contracted the virus from outside their families and spread the virus to their families. Of the 132 infected mothers, 98, or 74.2%, contracted the virus from a family member.

Discussion

The incidence of influenza in one community during a five-year period was 4.3% for influenza A and 2.7 % for influenza B. However, when the virus spread in families, the incidence rose to 9.6% for influenza A and to 9.8% for influenza B. The incidence rate of intra-familial infection was 21.7% for influenza A and 21.3% for influenza B. The family infection rates were two or three times higher than the general infection rate. Secondary infection in families was particularly high when the index cases were children under age seven. The infection rate in such cases was higher than 11%, suggesting that the virus survives longer in small children than in adults. In our previous examination, we observed the number of patients with detectable viruses among children under 16 compared to people 16 years or older, including parents. In people 16 years or older, the virus was completely gone 5.5 days after onset, whereas for those under 16 it took more than 6.5 days. The figures substantiated that children are major contributors to the spread of influenza. The average interval between the onset of an index case and

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secondary infection was 2.57 days for influenza A and 3.54 days for influenza B. The B virus has been shown in other studies to survive longer than the A virus.[1] The result is substantiated by the fact that the influenza A virus of our study survived in 50 % of the patients for 3.5 days after onset and the influenza B virus survived for 4.5 days.

Conclusions

The secondary infection rate in households is significantly high: 9.6% for the A virus and 9.8% for B. The key to containing influenza outbreaks, therefore, is preventing children from both contracting and spreading the virus. The results of this study also may be useful for determining who should be given priority in the administration of anti-influenza drugs when supplies are limited.

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Proceedings Topic #2

**Virus Structure/Function
and Receptor Binding**

**Oral
Presentations**

Structural Comparison of the Nucleoprotein From Influenza A Virus With Other NSV Nucleoproteins

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Crystal structures of the N proteins from borna disease virus (BDV) [1], two rhabdoviruses (vesicular stomatitis virus (VSV) [2] and rabies virus (RABV) [3]) and influenza A virus (FLUAV) [4], have been reported. The structures of the rhabdovirus N proteins were determined with RNA bound in a cavity. The cavity is located between two separated domains that accommodate the RNA with both hydrophobic and charged/polar interactions. Extended N-termini and a loop in the C-terminal domain reach over neighboring molecules to form an extended protein network along the RNA. The BDV and FLUAV N proteins were determined as a tetramer and a trimer, respectively, in the absence of RNA. The collection of N protein structures from three negative strand RNA viruses makes it possible to identify conserved structural motifs in the nucleoprotein from different virus families. We found that the RNA binding region of the N protein contains an N-terminal domain and a C-terminal domain with similar topology in all three virus families. In the RNA binding cavity, a central α -helix surrounded by four α -helices in the N-terminal domain continues to a central α -helix surrounded by two α -helices in the C-terminal domain. By superimposing the rhabdovirus N protein structure with that of BDV and FLUAV, this structural motif was also present in the other two structures. This suggests that the (5H+3H) structure may be a common motif in the nucleoprotein of negative strand RNA viruses.

Superposition of Individual Domains

The BDV N protein consists of 370 amino acids, the N protein from VSV and RABV consists of 422 and 450 amino acids, respectively, and the FLUAV N (commonly known as NP) protein consists of 489 amino acids. There is no detectable homology at the amino acid sequence level among these N proteins. When the flexible alignment of the VSV and BDV N protein structures was calculated, 280 residues were aligned with five twists and an RMSD of 4.26 Å (P-value of 8.20e-3) by use of the FATCAT program [5]. The superposition of the RABV and the BDV N proteins resulted in similar statistics with five twists and an RMSD of 4.60 Å (P-value of 1.73e-2). Since the two N structures from the two rhabdoviruses are nearly identical, only the VSV N protein was used as the representative rhabdovirus N protein for subsequent analyses. The topology of the protein fold is essentially the same between the VSV and BDV N structures each of which is composed of two domains. A similarity in protein topology is an indication of the evolution lineage and functional similarities. The central core of the N protein structure contains 7 aligned helices in the N-terminal

domain and 5 aligned helices in the C-terminal domain. This may suggest that the N-terminal domain and the C-terminal domain are more conserved as individual domains. They may change their relative orientations in the RNA binding region if the N protein needs to encapsidate the RNA in a slightly different mode, such as binding a more or less number of nucleotides per N protein molecule. A negatively charged surface groove was identified in the FLUAV N structure, but the RNA binding region was not clearly mapped in the previous report⁴. We found that the region comprised of residues 21-271 of the FLUAV N protein is structurally similar to the RNA binding region of the VSV N protein, but the two domains within this region are positioned differently in the FLUAV N structure. This shows that the two domains in the FLUAV N structure have a large change in their relative orientations compared to those in the VSV N structure. As a result, each domain in the two proteins could only be superimposed separately (Figure 1). The C-terminal domain again is structurally more conserved between VSV and FLUAV N proteins with secondary structure elements arranged by a similar topology. When residues 219-341 of the VSV N protein were superimposed with residues 213-271 of the FLUAV N protein, the RMSD was 3.60 Å (P-value of 3.11e-2) for 46 aligned residues, about half of the aligned residues between the VSV and BDV N proteins. When the C-terminal domain of the FLUAV N protein was superimposed with that of the BDV N protein, 53 aligned residues were found with an RMSD of 4.23 Å (P-value of 5.14e-3). The N-terminal domain of the FLUAV N structure could not be superimposed with that of the VSV or BDV N structure if all the residues (21-202) were included. If only the core residues 56-147 from the N-terminal domain were included in the superposition, 40 aligned residues were found between the N-terminal domain of the FLUAV and VSV N proteins, with an RMSD of 3.23 Å (P-value of 1.40e-1), and 45 aligned residues were found between the N-terminal domain of the FLUAV and BDV N proteins, with an RMSD of 3.09 Å (P-value of 1.33e-2), respectively.

RNA Binding Cavity

The two domains of the FLUAV N structure corresponding to the RNA binding region of the VSV N protein have very different positions compared to the other structures. For this reason, residues that may be similar to the RNA binding residues in the VSV N cavity could not be definitely identified. If the C-terminal domain of the FLUAV N protein is aligned with that of the VSV N protein, the N-terminal domain and the additional domain at the C-terminal end of the FLUAV N protein would close the cavity that is present in the rhabdovirus N protein. The structural comparisons discussed above have shown that the two domains are separately conserved structural domains and may assume various orientations relative to each other. It is not unacceptable that the orientations of the two domains in the FLUAV N protein in an RNA-free conformation may be changed in an RNA-bound conformation. To explore that possibility, an open conformation was simulated by aligning each domain individually, i.e. the N-terminal domain and the

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C-terminal domain of the FLUAV N protein were aligned with those of the VSV N protein. Next, the additional domain at the C-terminal end is manually positioned to match the extreme C-terminal end of the VSV N protein. This maneuver requires only rotations (twists) of two clearly defined structural domains in the FLUAV N protein. The final simulated open conformation of the FLUAV N protein (Figure 2) is essentially derived from the N conformation that is observed in the VSV N-RNA complex.

Discussion

Comparisons of the N protein structures from three virus families showed that the RNA binding region in each N protein has a similar structure containing two domains. The overall structure of the rhabdovirus N protein can be superimposed with that of the BDV N protein by flexible alignment, whereas the FLUAV N protein could only be superimposed with the other N proteins as separate N-terminal and C-terminal domains. However, it appears that the fold of the individual domains are conserved in the N proteins to a degree similar to that of the β -barrel fold in the capsid proteins of spherical viruses. There are at least five helices in the N-terminal domain and three helices in the C-terminal domain that are common among the N structures of the three virus families. This motif, which we have named the (5H+3H) motif, may be a common motif responsible for encapsidating RNA by the N protein of negative strand RNA viruses. The helices $\alpha 8$ and $\alpha 9$ named as in the VSV N protein are at the center of the motif and connect the two domains in the motif. However, the spatial geometry of the helices in the (5H+3H) motif is variable when the structures were compared. One possible explanation for this observation is that the structures of the BDV and FLUAV N proteins were determined without RNA bound, whereas those of the VSV and RABV N proteins were determined with a random RNA molecule bound in the RNA binding cavity. The orientation of the helices in the BDV or FLUAV N protein might change when the N protein binds RNA. This question could be answered when the structure of the BDV or FLUAV N protein is determined in the presence of bound RNA. An alternative explanation could be that there are intrinsic differences in the three dimensional structure of the N proteins, a likely result of evolution despite commonality of the structure and function of the N proteins among negative strand RNA viruses. The structural alignments of the N proteins from three negative strand RNA virus families have significant predictive values in recognizing the RNA binding site and the side-by-side interactions of the BDV N protein, which was not revealed when the BDV N structure was determined alone in the absence of bound RNA. The chemical properties of the homologous cavity in the BDV N protein and the pattern of intermolecular interactions are consistent with its functions to assemble the viral RNP. It also suggested a possible conformation of the FLUAV N protein which may be more suitable for RNA binding than the conformation observed in the recent crystal structure.

Figure 1. Topology drawings for the C-terminal domain (top panel) and the N-terminal domain of the N proteins. Large circles represent α -helices and triangles represent β -strands. Small circles represent 3_{10} helices. Color codes are from blue to red orange, corresponding to the sequence distance to the N-terminus similar. Lines above the circles represent connections on top of the helices, whereas lines below the circles represent the connections at bottom of the helices. The secondary structure elements are labeled the same as in the reported crystal structures. The dotted gray colored circle in the FLUAV C-terminal domain implies a possible disordered α -helix and the gray circle implies a mismatch of a loop with an α -helix.

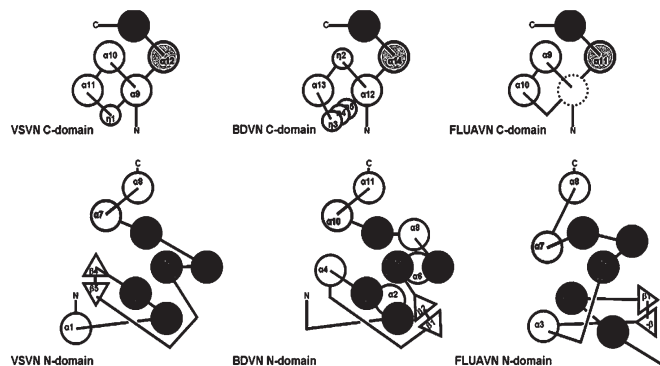
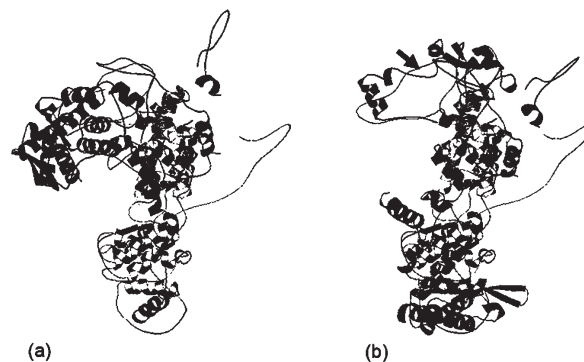


Figure 2. Cartoon drawings for the superposition of the FLUAV N protein with that of the VSV N protein (gray). (a) Superposition of the FLUAV N protein as in the reported crystal structure. Only the C-terminal domain of the putative RNA binding region of the FLUAV N protein was included in the calculation for the structural alignment. The N-terminal domain of the putative RNA binding region in the FLUAV N protein was colored green, the C-terminal domain, yellow, and the addition domain at the C-terminal end of the FLUAV N protein, blue. (b) Superposition of a hypothetical structure of the FLUAV N protein with the structure of the VSV N protein. The orientation of the C-terminal domain is the same in (a) and (b). The N-terminal domain (green) was aligned with the N-terminal domain of the VSV N protein. The additional domain at the C-terminal end (blue) was positioned by twisting the torsion angles of the peptide chain including residues 295-297 (indicated by the red arrow) to match the loop at the end of the VSV C-terminal domain.



Acknowledgements

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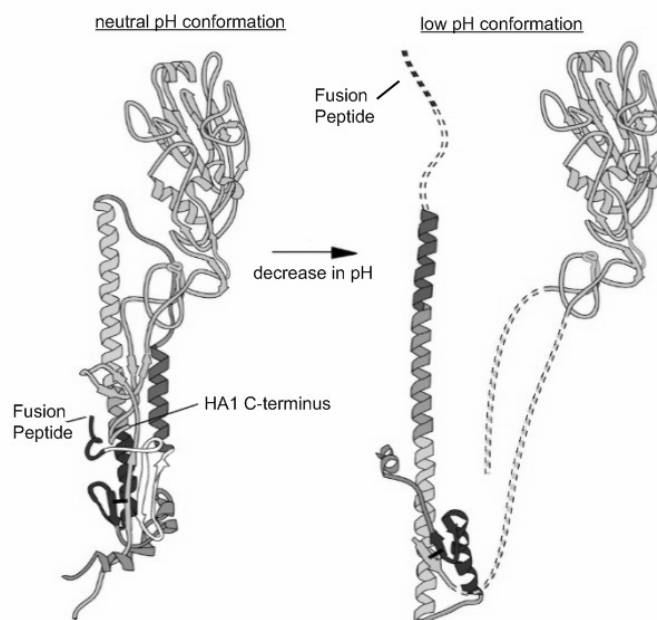
Subtype-Specific Differences at Residues Near the HA Fusion Peptide and Their Potential Roles in Membrane Fusion

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Influenza virus infection begins when the viral hemagglutinin (HA) protein binds to sialic acid-containing glycans on the surface of host cells. Following receptor binding, the virus is internalized by receptor-mediated endocytosis and the acidification of endosomes triggers the HA to undergo extensive structural rearrangements. These conformational changes convert the HA from a metastable conformation into a highly thermostable form of the protein and this transition is required for fusion of the viral and host membranes and release of the viral genome into the host cell cytoplasm. The structure of the neutral pH HA and the conformation that it assumes following acidification have been well characterized (1-3). However, the molecular mechanisms by which protonation of HA trigger the structural rearrangements are lacking, as are details regarding any possible structural intermediates. A comparison of the structures of HAs among different viral subtypes, and the mechanisms by which acid-induced conformational changes are initiated for these, could lead to a greater understanding of membrane fusion and to the design and improvement of drugs that inhibit this process. There are 16 different subtypes of HA which are classified by their antigenic properties and designated as H1-H16 (4). Of these, only three (H1, H2, and H3) have circulated extensively in humans, although others have been isolated from humans and have the possibility of emerging as threats to humans in the future (5, 6). Among HAs, those of H3 subtype viruses have been characterized most extensively, with the HA of A/Aichi/2/68 virus providing the prototype. It is expressed on viral surfaces as a homotrimer. Each monomer of the mature protein is synthesized as a 550 amino acid precursor, HA0, that is proteolytically cleaved into two disulfide-linked subunits, HA1 and HA2. The N-terminus of HA2 that is generated is a highly conserved hydrophobic domain referred to as the fusion peptide. Following cleavage, the fusion peptides of each monomer insert into a cavity in the trimer interior, and the HA molecule is transformed into a metastable protein that can be subsequently triggered to induce membrane fusion upon acidification (7, 8).

Figure 1.

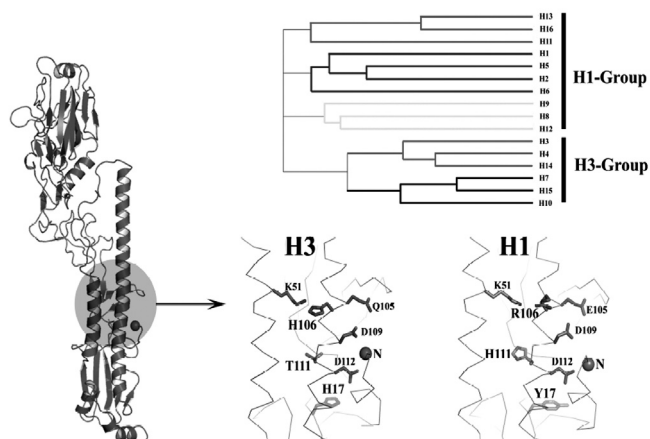


The structural changes that lead to fusion are depicted in Figure 1 and include the following: 1) The membrane distal head domains detrimmerize (light blue). The receptor binding domains and many antigenic regions retain their native structure but are tethered to the stalk domain by residues (light blue, hatched lines) that are disordered in the crystal structure (1). 2) The fusion peptide is extruded from the trimer interior. 3) The extended chain region (orange) that links the long (yellow) and short (red) helices of HA forms a helix and extends the central coiled coil in the N-terminal direction. This functions to direct the fusion peptide toward the target membrane. 4) A peptide segment (green) in the long helix of neutral pH HA near the fusion peptide undergoes a helix-to-loop transition and reorients residues C-terminal to it by 180 degrees. This converts the molecule into a rod-like structure that brings the fusion peptide and the membrane anchor domain into close proximity with one another, and presumably leads to fusion. It is not known whether these changes occur sequentially, and if they do, in what order they take place. The mechanism by which acidification triggers conformational changes is not known, but mutations that lead to an increase in fusion pH can occur at various regions throughout the trimer (9-13). These are known to locate at interfaces between monomers, between HA1 and HA2 subunits within monomers, and between domains that relocate during fusion. The positions of the mutations suggest that acidification leads to localized changes in the structure which destabilize the HA and allow for conformational changes to occur more readily. It is interesting that such changes often occur in and around the region of fusion peptide in neutral pH HA, and that studies with antibodies and double mutants

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suggest that upon acidification, changes in this region may precede or dictate the events elsewhere in the molecule (11, 14, 15). X-ray crystal structures have now been determined for the neutral pH HAs of several influenza A subtype viruses, and a comparison of these may provide insights with regard to acid-induced conformational changes and membrane fusion (3, 16-18). For example, the membrane distal head domains that detrimerize during fusion are situated differently relative to the stalk domains, depending on HA subtype. These can differ in “height” by as much as 4 angstroms relative to the stalk domain, and in rotational symmetry along the three-fold axis by as much as 31 degrees among subtypes (18). The main reason for this involves differences in the structure of the polypeptide chain that links the long and short helices of neutral pH HA. This is the peptide segment that becomes helical during fusion to extend the coiled coil, and in the native HA it makes numerous contacts with the HA1 head domains. Structural comparisons among subtype HAs and relevance for fusion are also intriguing for the region of the neutral pH HA in the vicinity of the fusion peptide, and we will focus some discussion on this region. When precursor HA is cleaved into HA1 and HA2, only six residues at the C-terminus of HA1 and 12 residues at the N-terminus of HA2 are relocated. Thus, the conformational changes that accompany cleavage alter the accessibility to solvent for of only a limited number of ionizable residues in the entire trimer, and these are in the fusion peptide region. Figure 2 shows a phylogenetic tree of the HA subtypes based on sequence and structural analyses, and indicates that they can be segregated into clades. These can be separated into two groups, designated here as the H1 group and the H3 group, based in part on structural features in the fusion peptide region. The H1 group includes H1-, H9-, and H13-like viruses, and the H3 group includes the H3 and H7 clades. The lower panels of figure 2 depict the fusion peptide region for the prototype H1 and H3 subtype HAs representing each group. A comparison of all HAs show that some of the ionizable residues that are buried by the fusion peptide after cleavage are completely conserved, while others vary strictly along group-specific lines. In all HAs, HA2 residues K51, D109, and D112 are completely conserved, whereas the residues at positions HA1 17, and HA2 106, and 111 are group-specific. Among H3 group HAs, such as the Aichi HA analyzed in this study, HA1 17 and HA2 106 are always histidine, and HA2 111 is threonine. In the H1 group HAs, HA1 17 is tyrosine, HA2 111 is histidine, and HA2 106 is either a basic lysine or arginine residue (4, 19-21).

Figure 2. The lower panels of figure 2 depict the fusion peptide region for the prototype H1 and H3 subtype HAs representing each group.



The uncleaved HA0 precursor is relatively unresponsive to reduction in pH compared to cleaved HA. It does not undergo irreversible conformational changes and it cannot mediate membrane fusion. Thus, it seems plausible that cleavage-induced changes in the chemical environment of ionizable residues near the fusion peptide may be relevant for protonation events that provide the initial triggers for the conformational changes that lead to fusion. Several of the ionizable residues noted above are known to be significant for HA stability based on observations of mutants which differ with respect to fusion pH, and we are currently examining these residues in both H1 group and H3 group HAs more comprehensively. The observation that the kinetics of membrane fusion and HA inactivation can differ among subtypes might relate to differences in the fusion peptide region that are group or subtype specific, and such differences could play a role in the ecology of influenza viruses. However, the group-specific differences among subtypes in the region of the fusion peptide may also have significance with regard to antiviral compounds designed to inhibit membrane fusion function. Several compounds have been developed to bind in this region and have been shown to inhibit, or in some cases facilitate HA conformational changes (22-27). The observation that these compounds often select for changes in the fusion peptide region support the hypothesis that they bind locally, and interestingly, some of the drugs that have been characterized are subtype specific. These results suggest that a greater understanding of the roles that particular residues play in the initiation of fusion, and comparative structural analyses among HAs of different subtypes, may aid in the development of new generations of anti-influenza drugs that are designed to inhibit membrane fusion function.

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Neuraminidases of H9N2 Influenza Viruses Isolated From Different Hosts Display Various Substrate Specificity

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Using the fluorescent neuraminidase (NA) assay developed by us earlier [Anal. Biochem., 34 (2005) 190] we have evaluated the specificity of seven influenza H9N2 virus NAs towards seven BODIPY-labeled sialyloligosaccharides: 3`SiaLac, 3`SiaLacNAc, SiaLe^C, SiaLe^A, SiaLe^X, 6`SiaLac, and 6`SiaLacNAc. Patterns of substrate specificity for duck, goose, turkey, chicken, swine and human viruses were different, but their specificity profiles formed several distinct groups. Results obtained suggest that virus replication in different species requires NAs, displaying different oligosaccharide specificities.

Introduction

NA hydrolyzes the α -ketosidic linkage between sialic acid and an adjacent sugar residue. Data on the oligosaccharide specificity of influenza virus NAs are restricted to a few examples. The NAs of N2 influenza viruses isolated from different hosts displayed different ability to discriminate 3`SiaLac and 6`SiaLac [1-4]. It was recently reported that the NAs of human influenza viruses discriminate the inner part of oligosaccharide chains [5]. Earlier, we described the fluorescence method for determining NA activity [6], based on the use of BODIPY-labeled sialo-oligosaccharides as NA substrates and following separation of labeled neutral product from negatively charged labeled substrate on anion-exchange microcartridges. NA specificity for the sialosides was calculated as the slope of the starting linear region of the V_0 against S_0 curve; that is, as the V_0/S_0 value at $S_0 \ll K_M$ [7, 8]. The comparison of V_0/S_0 values for all substrates of the same virus at the same conditions gave substrate specificity profile of the NA. In this work we focused on substrate specificity of N2 neuraminidases of H9N2 influenza viruses isolated from different organisms, namely duck, goose, turkey, chicken, swine and human.

Materials and Methods

4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid succinimidyl ester (BODIPY FLSE) was purchased from Molecular Probes (Netherlands). All other chemicals were purchased from Fluka (Switzerland). All chemicals were of analytical grade. DEAE-Toyopearl 650S was from Tosoh (Japan). The 10- μ l pipette tips with filter and 8-strip thin-wall 0.2 ml PCR tubes with caps were from Porex (USA). Black polystyrene 96-well microtiter plates were purchased from Nunc (Denmark).

The 96-well MultiScreen-DE plates with DEAE anion-exchange paper bottom were from Millipore (USA).

Influenza virus strains. Viruses (A/duck/Primorie/3/82, A/swine/Hongkong/9/98 and) were obtained from the virus repository of the Ivanovsky Institute of Virology (Moscow, Russia) and viruses (A/goose/Minnesota/80, A/turkey/Minnesota/38391-6/95, A/turkey/Wisconsin/01/96 and A/chicken/New Jersey/12220/97, A/Honkong/1073/97) were obtained from Influenza Division, Centers for Disease Control and Prevention (Atlanta, USA). All viruses were propagated in 10-day-old embryonated chicken eggs. Purification of all viruses was done as described previously [9]. Virus concentration was determined in a hemagglutination reaction with human erythrocytes (group 0, Rh⁻).

Assay of NA activity. To select the appropriate virus concentration for the NA specificity assay, a 2- μ l aliquot of purified influenza virus at various dilutions was placed in a microtube containing 0.5 nmol of 3`SiaLac-BODIPY in 3 μ l of 0.1 M Na-acetate buffer (pH 5.0) with 10 mM CaCl₂. The microtube was tightly closed and gently shaken at 37°C for 20 min. To stop the reaction, the tube was heated at 70°C for 10 min and the mixture was diluted with 45 μ l of distilled water. The solution obtained was analyzed by one of the separation procedures microcartridge or plate assay [6, 10]. Analysis of each reaction mixture was performed in three replicates.

Substrate specificity evaluation. NA. Five to six dilutions of each BODIPY-labelled substrate were prepared in 0.1 M Na-acetate buffer, pH 5.0, with 10 mM CaCl₂. Twelve μ l of each solution was placed in a striped microtube and 8 μ l of virus suspension per tube was added simultaneously, using a multichannel pipette. The tubes were tightly closed, shaken gently, and incubated at 37°C. Every 5-10 min 5- μ l aliquots were taken from the strips and transferred into the tubes, containing 45 μ l of water. The DRMs were heated at 70°C for 10 min and analyzed as described in [6, 10]. Virus NA specificity for each sialoside was calculated as the slope of the starting linear region of the V_0 against of S_0 curve; that is, as the value of V_0/S_0 at $S_0 \ll K_M$.

Results

In this study, we used seven substrates (Table 1), differing by: 1) the type of linkage between the Neu5Ac and Gal residues, 2) the presence of an N-acetamide group at position 2 of the Gal residue, 3) core type (1-3 or 1-4), and 4) the presence of fucose at the GlcNAc residue.

Table 1. Structure of BODIPY-labeled oligosaccharides.

Oligosaccharide	Abbreviation
Neu5Aca2-3Gal β 1-4Glc	3`SiaLac
Neu5Aca2-3Gal β 1-4GlcNAc	3`SiaLacNAc
Neu5Aca2-3Gal β 1-3GlcNAc	SiaLe ^C
Neu5Aca2-3Gal β 1-3(Fuca1-4)GlcNAc	SiaLe ^A
Neu5Aca2-3Gal β 1-4(Fuca1-3)GlcNAc	SiaLe ^X
Neu5Aca2-6Gal β 1-4Glc	6`SiaLac
Neu5Aca2-6Gal β 1-4GlcNAc	6`SiaLacNAc

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All NAs studied preferentially hydrolyzed substrates with α 2-3 bound neuraminic acid (i.e. 3' SiaLac, 3' SiaLacNAc, SiaLe^c, SiaLe^A and SiaLe^X). The duck, goose and swine virus NAs (Table 2) did not discriminate the structures of inner core of oligosaccharides, namely presence of N-acetamide group and α 1-3/ α 1-4 bond. The other viruses studied discriminated within linear α 2-3 substrates. The A/turkey/Minnesota/38391-6/1995 (Table 2) and A/Honkong/1073/2003 (Table 2) NAs hydrolyzed with the same efficacy 3' SiaLac and 3' SiaLacNAc, but discriminated 1-3 vs. 1-4 cores. The A/turkey/Wisconsin/01/1996 NA hydrolysed 3' SiaLac 1.3 times better than all other substrates, while A/chicken/New Jersey/12220/97 discriminated core structures as well. More differences between these viruses were observed when fucosylated substrates were used. All the NAs displayed decreased activity for tetrasaccharide SiaLe^A. A/turkey/Wisconsin/01/1996 virus NA hydrolyzed this fucosylated substrate 13.5 times lower than fucose free analog, SiaLe^c. For the duck NA this ratio was of two, while for all others – about five. As for 3' SiaLacNAc-fucosylated derivative, i.e. SiaLe^X, only the chicken virus NA hydrolyzed it slower than 3' SiaLacNAc (2.4 times), while the duck, swine and human viruses digested these sialosides with the same efficacy. Drastic difference between viruses studied are found for 2-3/2-6 activity ratio. It was about 50 for the duck, about 25 - for goose and turkey, 11 - for chicken and human viruses, and about 6 for swine isolates.

Table 2. Substrate specificity of influenza virus NAs.

	3' SiaLac	3' SiaLacNAc	SiaLe ^c	SiaLe ^A	SiaLe ^X	6' SiaLac	6' SiaLacNAc
A/duck/Primorie/3/82	8,00	7,80	8,00	4,00	8,22	0,12	0,15
A/goose/Minnesota/80	17,30	18,00	16,50	1,95	n/d**	0,85	0,90
A/turkey/Minnesota/38391-6/95	14,50	13,30	9,00	1,85	n/d**	0,55	0,55
A/turkey/Wisconsin/01/96	12,50	9,50	9,50	0,70	n/d	0,37	0,37
A/chicken/New Jersey/12220/97	2,70	3,30	1,90	0,35	1,40	0,18	0,25
A/swine/Hongkong/9/98	2,00	1,90	2,00	0,43	1,61	0,37	0,32
A/Honkong/1073/97*	2,08	2,03	1,39	0,36	2,10	0,20	0,20

The data are normalized to one hemagglutinating unit. *For A/Honkong.1073.97 virus data was normalized to three hemagglutinating units. The data represent an average of at least three ** Analysis was not performed

Discussion

The NA substrate specificity was evaluated using seven sialooligosaccharides. Specificity profiles of all viruses studied were unique and no absolutely identical profiles were found. Nevertheless the NAs may be divided into groups on the basis of hydrolytic activity towards α 2-3/ α 2-6 sialosides. First group is formed by duck viruses. High hydrolytic activity towards SiaLe^A and, on the contrary, very low towards α 2-6 sialosides are the main features of these viruses. Duck viruses of H1N1 subtype, studied by us earlier, [10] also fall into this group. The goose and turkey virus NAs fall into the second group. Low activity towards α 2-6 sialosides and SiaLe^A is their main feature. Besides, NAs of turkey viruses distinguish the inner structure of oligosaccharides. The amino acid sequences of all these NAs are rather similar, but differ greatly from all others. The chicken, swine and human virus NAs form the third group. For the third group hydrolysis efficacy towards SiaLe^A is identical to that for

the α 2-6 sialosides. Low level of the α 2-3/ α 2-6 ratio (about 10) is the main feature of the third group NAs. This data, compared with previously obtained [11] on substrate specificity of H1N1 viruses, isolated from different hosts gives us a few hints on NA evolution during its establishment in a new host. For example the α 2-3/ α 2-6 ratio for human viruses, circulating in human population for a long time is usually less than 5, and about 10 for those studied in this work. Conversely, this ratio for swine H1N1 viruses is about 20, but for NA of H9N2 virus it is only 5. So, we propose that the human H9N2 virus has penetrated in human population recently, while the swine virus originated from the human host. Sequences of the third group NAs known to have high homology and differ from that for the duck, turkey and goose viruses. Our data also coincides with [3] where role of 258 and 275 amino acids in neuraminidase specificity towards 6' SiaLac was shown. In brief, Lys258 and Ile275 increase NA activity towards α 2-6 oligosaccharides. 258Lys is found in chicken, swine and A/goose/Minnesota/80 viruses, all of them have reduced α 2-3/ α 2-6 ratio in comparison with duck viruses, but this ratio for goose virus remains at the level characteristic for the group. Chicken and human viruses have 275Ile and both have low α 2-3/ α 2-6 ratio. Interestingly, chicken virus has both amino acids responsible for high activity towards 6' sialosides, but its α 2-3/ α 2-6 ratio is not the lowest of the NAs studied. Our data is also in good agreement with [11] in the activity towards 3 vs. 6 sialosides for all viruses but part of poultry ones. This shows that some regulatory mechanisms still stay unrevealed. The substrate specificity profiles reflect NA origin and seem to give a clue to its evolution. With the use of a new fluorescent neuraminidase assay it is now possible to track the NA substrate specificity evolution in case of virus penetration to a new host and virus adaptation to it.

Acknowledgements

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Expression of Avian Influenza Virus Receptors and H5N1 Virus Infection in Human Respiratory Tract

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Receptor specificity restricts influenza virus cross species transmission, with SA α 2,6 Gal and SA α 2,3 Gal sialic acids recognized by human and avian influenza viruses, respectively. This study investigated the distribution of these two species of sialic acids in the human respiratory tract. The SA α 2,3 Gal species was infrequently detected in the upper respiratory tract, but prevalent in the lower part, while the SA α 2,6 Gal species is more common in the upper respiratory tract. Though alveolus cells are more susceptible than trachea and bronchus epithelial cells to avian influenza H5N1 virus infection in the *ex vivo* experiment, H5N1 virus was found to infect the upper respiratory tract epithelial cells of a human case. It was also found that H5N1 virus infection occurs in the epithelial cells of the respiratory tract which do not express detectable SA α 2,3Gal. These observations may be important to investigate further if the currently-observed limited human to human transmission by H5N1 virus is associated with the differential expression of SA α 2,3 Gal in human upper respiratory tract among individuals.

Introduction

The binding of influenza virus to host cells is mediated via the viral surface protein haemagglutinin, which recognizes cell surface glycoproteins containing terminal sialic acid residues. Haemagglutinin proteins from human and avian influenza viruses differ in their ability to recognize different receptor structures of sialyloligosaccharide molecules on host cell membrane [1, 2]. Host tropism of influenza viruses is restricted by receptor specificity. Human influenza viruses preferentially bind to the terminal sialic acid with an α 2,6 linkage to the underlying galactose (SA α 2,6Gal), whereas avian and equine viruses prefer an α 2,3 linkage to the underlying galactose (SA α 2,3Gal) [2,3], which predominate in their respective target species. In recent years, however, avian influenza virus H5N1 subtype, has repeatedly crossed host barrier and infected humans. Over 300 human cases have been confirmed in various affected countries by the World Health Organization since 2003 when H5N1 virus human infection reemerged in Hong Kong [5]. Genetic analysis has demonstrated that

the H5N1 viruses causing human infections up to now are still of the avian type [6, 7, 8]. The molecular mechanism underlying such cross species transmission is not clear though it is generally believed that the availability (or presence) of α 2,3 linkage terminal sialic acid receptor in human airway may attribute to the susceptibility to avian influenza virus infection. H5N1 virus only showed inefficient human to human transmission up to this stage [9]. It is also suspected that some individuals with high exposure to H5N1 virus do not become infected due to an inherent reduced susceptibility to this virus. Two recent studies demonstrated SA α 2,3Gal expression and influenza virus binding in the bronchioles and alveoli of the human respiratory tract, indicating that avian influenza viruses can replicate relatively efficiently in the lower respiratory tract, providing a possible explanation for inefficient human-to-human transmission of avian influenza viruses [10, 11]. In another study, H5N1 virus was found to be able to infect epithelial cells of the human upper respiratory tract in an *ex vivo* infection model [12]. However, the prevalence and the extent of expression of SA α 2,3Gal and SA α 2,6Gal in the human respiratory tract have still not been fully investigated. In this report we studied the distribution and prevalence of SA α 2,3Gal and SA α 2,6Gal on the airway epithelia of different anatomical areas of the human respiratory tract. Our results demonstrated that SA α 2,6Gal predominates in the upper respiratory tract, while SA α 2,3Gal is patchily expressed in the upper respiratory tract of a portion of individuals but more prevalent in the lower part of the respiratory tract. Examination of *ex vivo* and *in vivo* H5N1 virus-infected human tissues indicated H5N1 virus infection of upper respiratory tract may not completely dependent upon avian influenza virus receptor.

Materials and Methods

Tissue samples. 144 paraffin-embedded human respiratory tissue sections from 88 patients were obtained from the pathological archives in several local hospitals in Guangxi and Guangdong provinces, China. The age of the patients ranged from antenatal to 76 years old. Different anatomical parts of respiratory tract were included based on the availability of tissues, and were then histologically classified as trachea, bronchus, bronchiole and alveolus. Paraffin-embedded respiratory tissue sections from a H5N1 human infection case [4] in Hong Kong in 2003 were also included in this study.

Lectin histochemical staining of human airway tissues. Expression of SA α 2,6Gal and SA α 2,3Gal was examined using biotinylated lectins: *Sambucus nigra* lectin (SNA, EY Laboratories, Inc., California, USA), and *Maackia amurensis* lectin (MAA-I & MAA-II, Vector Laboratories, California, USA). Deparaffinized and rehydrated tissue sections were blocked consecutively for 15 minutes with avidin D and biotin solutions (Vector Laboratories) to reduce nonspecific binding of endogenous biotin and avidin. These sections were then incubated with biotin-labeled MAA-II or SNA at a concentration of 10 μ g/ml or 1 μ g/ml, respectively, for 60 minutes at room temperature, and then with streptavidin/peroxidase complex reagent (Vector

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Laboratories) for 30 minutes followed by color development using 3,3'-diaminobenzidine (DAB, Vector Laboratories).

Ex Vivo infection of human respiratory tissue. For the *ex vivo* infection study, one H5N1 isolate from human infection, A/HK/212/03 [4] and two avian H5N1 influenza viruses, DK/GX4444/05 and DK/GX3546/05 [7,8], were passaged once in 10-day embryonated chicken eggs and stored at -80°C until use. Eight cases of surgically-removed human respiratory tract tissues from lung cancer or non-cancer patients were obtained from local hospitals in Guangdong province in accordance to the local Institutional Ethical Review Board's approved protocols. The tissues were cut into small pieces of about 0.2cm^3 and infected with 10^3 TCID₅₀ of each H5N1 virus in a 500 μl inoculum. The virus was removed after a one hour absorption at $37^{\circ}\text{C}/5\% \text{CO}_2$, and tissues were further incubated at $37^{\circ}\text{C}/5\% \text{CO}_2$ for 24 hour in 3ml of serum-free F12K Nutrient Mixture (GIBCO, New York, USA) supplemented with 25mM HEPES buffer solution and 1% Antibiotic-Antimycotic (GIBCO). The infected tissues were then fixed in 10% neutral formalin for 24 hours before being histologically sectioned for immunohistochemical detection of influenza nucleoprotein (NP).

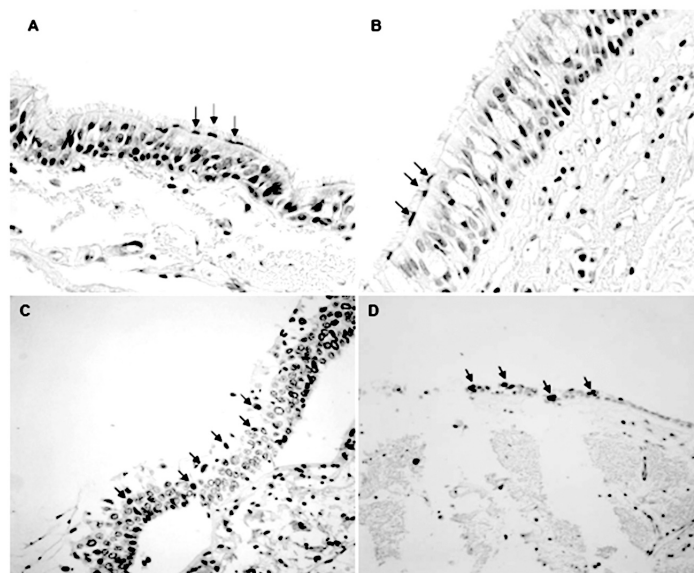
Immunohistochemical staining of tissues. For detection of influenza A nucleoprotein (NP) in human tissues, sections were blocked with 1% bovine serum albumin/PBS, stained with a anti-influenza nucleoprotein monoclonal antibody (17H4 clone) raised with H5N1 strain CK/Yu22/02 [8] at 1:5000 dilution at 4°C for overnight and then incubated with goat anti-mouse IgG, H & L chain specific biotin conjugate (Calbiochem) at 1:2000 dilution for 30 minutes at room temperature. Tissue blocks were then incubated with streptavidin/ peroxidase complex reagent (Vector Laboratories) for 30 minutes at room temperature. Color development and images capture were carried out as described above.

Results and Discussion

Distribution of influenza virus receptors in human respiratory tract. 144 respiratory tissue sections including 10 tracheas, 40 bronchi, 46 bronchioles and 48 alveolar tissues were studied by lectin histochemistry methods. SA α 2,6Gal and SA α 2,3Gal recognized by SNA or MAA-II respectively on the apical epithelial surface were counted as positive in this study, while relative intensity of staining achieved with the lectins was not used as a direct quantitation measure of sialic acid distribution, because the relative affinities of the lectins, and the degree of biotinylation may differ among different preparations. SA α 2,3Gal was only detected in a small portion of the upper respiratory tract tissues, with 20% in trachea and 42.5% in bronchus sections showing positive staining. As shown in Figure 1A and B, only sporadic SA α 2,3Gal positive cells were observed in each positive sample. In contrast, SA α 2,3Gal was more regularly observed in the lower respiratory tract epithelial cells, with 68.7% of the bronchiolar and 79% of alveolar tissues showing positive staining in all cases examined (data not shown). In contrast, expression of human influenza

virus receptor, SA α 2,6Gal, was mainly detected in the upper respiratory tract and to a lesser degree in the alveolar epithelium, with 100% in trachea, 97.5% in bronchus, 80.4% in bronchioles but only 43.8% in alveolar tissues (data not shown). These results, consistent with previous studies, suggested that lower respiratory tract may be more susceptible to avian H5N1 influenza virus infection for its relatively higher expression of SA α 2,3Gal. However, the observation of SA α 2,3Gal expression in some epithelial cells of tissues from the upper respiratory tract argues one possibility that the currently-observed limited human to human transmission by H5N1 virus may be associated with the differential expression of SA α 2,3 Gal in human upper respiratory tract among individuals.

Figure 1. Detection of SA α 2,3 Gal expression in human respiratory tract tissues, trachea (A) and bronchus (B), by immunostaining with lectin probes MAA-II; Detection of influenza viral antigen (nucleoprotein) in trachea (C) from one H5N1 virus infected patient and in *ex vivo* infected bronchus tissue (D) with avian H5N1 virus, DK/GX3546/05, with immunohistochemical staining. Viral nucleoprotein positive cells were shown in brown color.

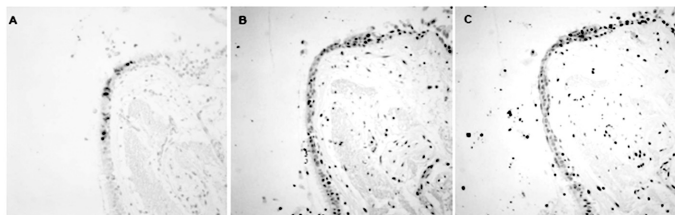


Avian H5N1 virus infection in human respiratory tract tissues. Previous reports suggested that alveolus cells in the lung represent the major site for avian H5N1 virus replication in human infection [13]. Infection of avian H5N1 influenza virus in human upper respiratory tract is less defined. We examined tissues of upper respiratory tract obtained from a postmortem H5N1 human case in Hong Kong in 2003 [4]. Immunohistochemistry staining of influenza NP protein with a monoclonal antibody raised against H5N1 strain Ck/Yu22/2002 (clone 17H4) showed that influenza viral antigen was present in tracheal (Figure 1C), bronchial and also in alveolar epithelial cells (data not shown). To further explore the possibility of H5N1 virus infection in the upper respiratory tract, we then performed H5N1 virus infection in *ex vivo* cultured human respiratory tissues removed from the respiratory tract of patients suffering non-infectious diseases. The H5N1 human

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strain (A/HK212/03) we tested was isolated from a fatal human infection case [4]. A previous receptor binding study found that this strain can bind to both avian and human receptors [14]. These *ex vivo* infection tests further demonstrated that this virus infected both bronchial and alveolar epithelial cells with similar efficiency. In this study, we also tested two avian H5N1 isolates, DK/GX4444/05 and DK/GX3546/05 (data not shown). Genetic analysis of the haemagglutinin gene of these two viruses indicated they are typical avian type [7, 8]. We could also demonstrate that bronchial and alveolar epithelial cells were both infected by these viruses (figure 1D). This evidence supports the notion that upper respiratory tract is also susceptible to avian H5N1 virus infection. It remains to be determined if the presence of SA α 2,3Gal in the upper respiratory tract would predispose towards susceptibility to H5N1 virus infection. It is noteworthy that, when sequential tissue sections from *ex vivo* H5N1 infected bronchus tissues were stained for both virus replication and the expression of influenza receptors, in the area where H5N1 virus infection was observed, staining with the lectin MAA-II failed to detect SA α 2,3Gal species (Figure 2 A, B and C). However, a strong positive staining of SNA and a patchy signal of MAA-I lectin staining were observed in the same region. Previous reports on lectin affinity studies had showed that MAA-I also reacts with other species of sialic acids [15], and the inner fragment of the carbohydrate chain in sialic acid-containing oligosaccharides may also be involved in influenza virus entry into the target cells [16, 17]. However, the exact mechanism of how H5N1 avian influenza virus infection epithelial cells which do not express SA α 2,3Gal remains to be investigated.

Figure 2. H5N1 virus infects bronchus cells without expressing SA α 2,3Gal. Human bronchus tissue infected with DK/GX/4444/05 was stained for viral antigen (NP) expression, consecutive sections of bronchus tissue were stained with monoclonal antibody specific for viral antigen (NP) (A), with lectin MAA_II specific for avian influenza virus receptor SA α 2,3Gal β 1-3GalNAc (B), and with MAA for both SA α 2,3Gal β 1-3GalNAc and SA α 2,3Gal β 1-4GalNAc expression (C), respectively as described in the Materials and Methods.



It is believed that alteration of receptor specificity is essential for the emergence of pandemic viruses from their avian progenitors. Whether this change is now happening to H5N1 is under intensive investigation. This virus may well be becoming less restricted to SA α 2,3Gal sialic acid receptor specificity. If this virus is adapting to become more like a human influenza virus by increasing binding affinity for the SA α 2,6Gal sialic acid receptors which are more widely expressed throughout the human respiratory tract, an imminent pandemic seems likely.

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Proceedings Topic #3

Outbreak and Pre-Pandemic Response

Oral Presentations

Public Health Response to an Outbreak of Highly Pathogenic Avian Influenza (H5N1) on a Poultry Farm in Suffolk, United Kingdom

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Introduction

On the 3rd of February 2007, following a higher-than-normal incidence of deaths in turkeys on a farm in Holton, Suffolk, tests performed by the Veterinary Laboratories Agency (VLA) confirmed the presence of highly pathogenic avian influenza (HPAI) H5N1 of Asian lineage (Clade II). Confirmation of HPAI led the State Veterinary Service (SVS) to order a 3km Protection Zone and a 10km Surveillance Zone around the premises. Later, a further Restricted Zone of 30 km was declared. These zones related to the movements of birds, not humans. The infected premises contained about 160,000 turkeys housed in 22 units. Disease was only observed in one of the units. The turkeys affected were 55 days old, which, given the incubation period of avian influenza in birds, implied that they were exposed to the disease whilst on the unit. Bio security at the site was judged to be of a good standard. Apart from the sheds there were also food processing factories at the farm. The whole complex had been built upon a former airfield. An estimated 1,400 workers were employed mainly at the various factories on the farm. Upon confirmation of disease, the decision was made by the Department of Environment and Rural affairs (DEFRA) to cull the turkeys in all 22 units. On-site facilities and personnel were used but extra catchers and vets were called in to help. The cull was completed on Monday, 5 February. A local Incident Management Team (IMT) consisting of the Health Protection Agency (HPA) and its local public health partners was formed immediately, to address the human health implications, supported by expert advice from the HPA Centre for Infections. The main tasks of the team were to manage the human health aspects of this incident and arrange for the supply of oseltamivir prophylaxis and provide seasonal influenza vaccination for those who had been in close contact with the infected birds and for workers, i.e. cullers, catchers, relevant transport staff and staff at the rendering plant. The IMT also helped communicate with family practitioners, hospitals and the public about the outbreak. Background: Avian influenza H5N1 had been reported in 2006 in Europe, mainly in wild birds, including in a swan in Scotland. At the time of the current incident, the only other reported H5N1 outbreak in a poultry farm in 2007 had been in the last week of January on a goose farm in the eastern Csongrád region of Hungary. In addition, there had been no reports of wild birds in Europe

with H5N1 virus so far in 2007, despite increased surveillance and testing of wild birds.

Public Health Response to the Outbreak

During the initial stages of the outbreak, the number of at-risk, exposed persons was reported to be 50. This was then found to be grossly underestimated as the total number of people who eventually received oseltamivir prophylaxis was 482. Within 12 hours after the IMT was set up, 115 persons had received oseltamivir prophylaxis. Seasonal influenza vaccination was also offered, with an uptake of 68%. The extremely vital co-operation between the local Health Protection Unit (HPU) and its local public health partners from the National Health Service, UK, enabled rapid identification of a local community hospital nearby for use of dispensing prophylaxis. Data collection for the local database, designed by the local HPU, which recorded details of all persons exposed needing prophylaxis was also carried out at that site. The hospital was only 3 miles from the site of the outbreak and was a very convenient place to distribute the medicine. Ready pre-stocked oseltamivir prophylaxis was easily accessible at for use during this outbreak. The dispensing teams, which mainly comprised of 1 doctor in Public Health and 1 nurse per team, worked with great flexibility in the first hours of the incident, and succeeding in dispensing oseltamivir near the site for workers who could not leave site to attend the nearby community hospital. In addition to this, the setting up of the on-call rota and IMT was achieved quickly, often on a voluntary basis. The main interventions carried out included the dispensing of oseltamivir, providing seasonal influenza vaccination and providing advice on the clinical management of symptomatic, exposed persons.

Results

Out of 482 at-risk persons, 36(8%) were female and 446(92%) were male. The majority of the persons were aged 20-49 years. 59% worked for the farm; 78.2% of all persons who received oseltamivir were given pre-exposure prophylaxis. The IMT was guided by HPA algorithm J3 - Management of Personnel Involved in the Response to an Occurrence of Confirmed Highly Pathogenic Avian Influenza (H5N1 only) in Poultry in the United Kingdom, presenting with febrile respiratory illness), [1] (Appendix 1) for the surveillance of symptoms of persons on prophylaxis. In addition to this, the IMT modified algorithm J3 which heightened the surveillance by using the flowcharts to investigate all persons on oseltamivir complaining of any symptoms, not just fever and respiratory symptoms. During the outbreak, 17 at-risk persons on oseltamivir complained of various symptoms and appropriate risk assessment was carried out by the IMT. Out of 17, only 4 people were tested for H5N1 using RT-PCR – all negative. However, one person of the 4 persons tested was positive for seasonal influenza H3N2. Persons not tested according to the J3 algorithm were all followed-up actively for 48 hours in order to detect any deterioration of reported symptoms. None reported further deterioration.

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Figure 1. All reported symptoms during the outbreak.

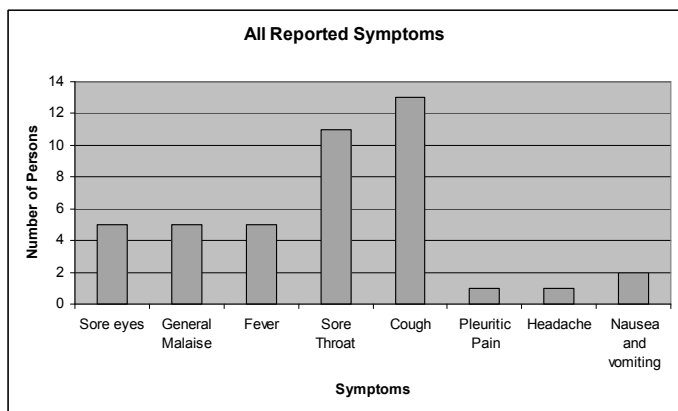


Figure 1 shows the various symptoms reported by a total of 28 persons, to the IMT during the outbreak. Out of these 28 persons, only 17 were at-risk, exposed persons as described above, the other 11 persons were non-exposed persons during the outbreak. Some persons had more than 1 symptom; hence the number of symptoms is greater than the number of individuals reporting symptoms. In addition to this, the IMT also carried out active surveillance for 7 days on 11 at-risk personnel who were working on the farm before the diagnosis of HPAI was made. None of these 11 persons were hospitalized, and all had received post-exposure prophylaxis.

Discussion

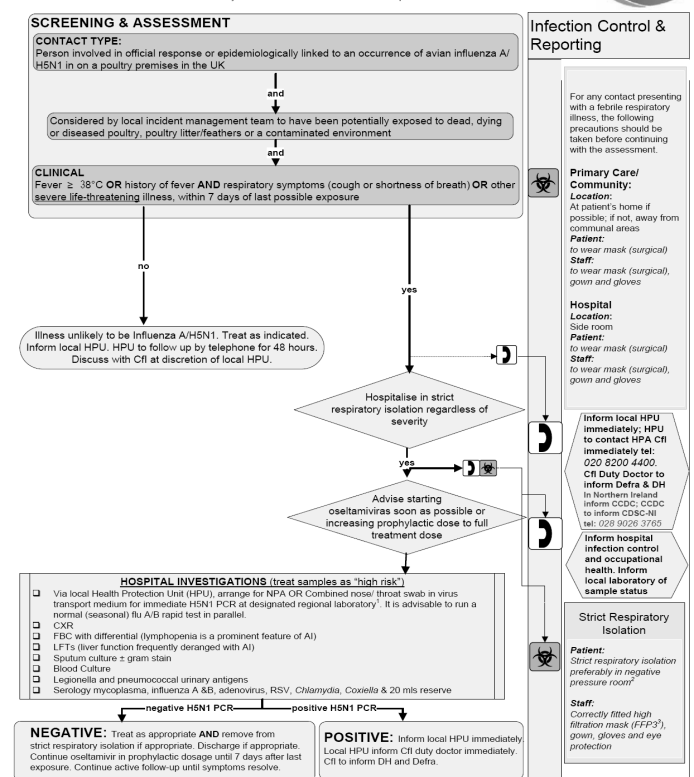
Some lessons identified during this outbreak are listed as follows: Roles and responsibilities always benefit from clarification in any multi-agency incident; Mechanisms for prescribing and distributing oseltamivir to patients need to be well planned and exercised; Case definitions of symptomatic persons can be problematic unless they are well defined; Thorough understanding of the layout of the infected premises is essential for a balanced risk assessment; Meetings management and task allocation must be slick; Recovery time and de-briefing both essential for the successful management of the outbreak. The public health response to this outbreak involved the largest ever mass provision of oseltamivir prophylaxis in the United Kingdom. This was a successful operation but was very resource intensive, especially during the first few days of the incident. However, we believe that the experience gained from this will help us better prepare ourselves for future outbreaks.

Reference

1. Health Protection Agency, UK. Avian Influenza Guidance and Algorithms, reviewed on 6th July 2007. Available at: http://www.hpa.org.uk/infections/topics_az/influenza/avian/documents/J3poultrymanagement080207.pdf Accessed July 13, 2007.

Appendix 1

J3 WHO PANDEMIC ALERT PHASE 3: Draft algorithm for the management of personnel involved in the response to an occurrence of confirmed highly pathogenic avian influenza (H5N1 only) in poultry in the UK, presenting with febrile respiratory illness
08 February 2007. Please check HPA website for updates.



1 Information on local influenza A (H5) testing laboratories is available from: http://www.hpa.org.uk/infections/topics_az/influenza/avian/labs.htm
2 If negative pressure facilities are unavailable an isolation room can be set up in accordance with WHO guidance. Transferring patients for this reason alone is not advised www.who.int/csr/disease/avian_infectioncontrol/en
3 FFP3 standard masks, see HSE guidelines: <http://www.hse.gov.uk/biosafety/diseases/avianflu.htm>. For full avian influenza guideline see http://www.hpa.org.uk/infections/topics_az/influenza/avian/default.htm
4 Standard adult treatment dose=75mg bd unless expert advice is to increase dose.

Successful Control of Institutional Outbreaks of Influenza 2003/2004 Using Anti-virals Tamiflu in Taiwan: Can this Experience Be Applied to H5N1 Control?

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The common clinical manifestations shared by severe respiratory virus infection (SARS) and influenza constitute a big challenge for the successful control of SARS during the winter flu period 2003/2004 in Taiwan. In the belief that the far majority of flu-like illness (ILI) will actually represent influenza in the flu season and to avoid confusion and panic of SARS suspect in the post-SARS period, the Center for Disease Control of Taiwan implemented a national policy of ILI control measure which includes public health measures and the stockpile of antivirals-Tamiflu for the control of flu-like illness (ILI) in the 2003/2004 winter period, particularly for the control of institutional outbreak of ILI illness. The control policy includes the real-time reporting of ILI clustering in the institutions such as nursing home, school, military, and jail. Upon receiving the reporting, a task force team was immediately dispatched to investigate the outbreak. After sampling the throat swab specimens, anti-virals were given to the patients and close contacts. The transmission and clinical outcome were then followed up in the coming weeks. A total of 24 institutional ILI outbreaks were reported in the 2003/2004 winter period. Among which, 23.4 % of the cases (609 samples taken, 143 positive) were laboratory-proved to be caused by influenza by the laboratory by a combination of ELISA kit for influenza, PCR for influenza and SARS-CoV, and viral culture. None of them were caused by SARS-CoV. All the ILI outbreaks were successfully controlled by this measure and no more transmission was reported following the application of anti-virals. In parallel to this control measure, the ILI incidence of ILI cases reported by sentinel physician survey reduced remarkably to 30 cases in 2003/2004 as compared to the 80 cases at the periods before SARS. Laboratory survey of 30 viral isolates collected between 2004-2007 revealed no drug resistance to oseltamivir. Therefore, the control policy of ILI illness by combining public health measures and stockpile of antivirals Tamiflu proves to be effective and successful measures to control the institutional outbreaks of influenza. This strategy can be considered to be applied during the H5N1 pandemic flu.

Background and Control Measures

The outbreak of severe acute respiratory syndrome (SARS) in 2003 caused a catastrophic victim of 76 life, extensive and profound social panic, and a direct economic loss of more than one billion dollars in Taiwan. The potential resurgence of SARS, especially in the winter season of 2003/2004, therefore

constitutes a big challenge to Taiwan government, because of the overwhelming numbers of influenza as compared to SARS epidemic in May 2003. For the successful control of SARS and influenza, the Taiwan government strengthens the public health measures such as expanding flu vaccination program, enhancing the scientific capability and surveillance, and controlling the clustering outbreak of febrile patients in the institutions such as nursery, schools, military, and prisons. The most important policy decision is to stockpile anti-virals oseltamivir Tamiflu for the control of influenza-like illness (ILI) in a total of 2.3 millions dosages on November 2003. On receiving a report of clustering outbreak of ILI illness in the institutions, a task force was dispatched to investigate and laboratory tests including rapid ELISA test, PCR, and viral culture were examined. After sampling, the patients and their close contacts were immediately given the anti-virals. Table 1 summarized the total numbers of institutional outbreaks of ILI during the post-SARS period.

Table 1. Institutional outbreak of ILI and results of laboratory tests.

Influenza lab results	Types of Institutions			
	Nur sing homes, health-care facilities	Schools	Military bases, jails	Others
No of ILI outbreaks	5	9	7	3
No tested for influenza *	4	9	6	3
No positive	4 (100%)	7 (77%)	5 (83%)	2 (67%)
No persons w ILI symptoms	98	301	237	30
No tested for influenza *	137	278	181	13
No positive	38 (27.7%)	66 (23.7%)	34 (18.8%)	5 (38.5%)

* combination of rapid tests, viral culture and RT-PCR

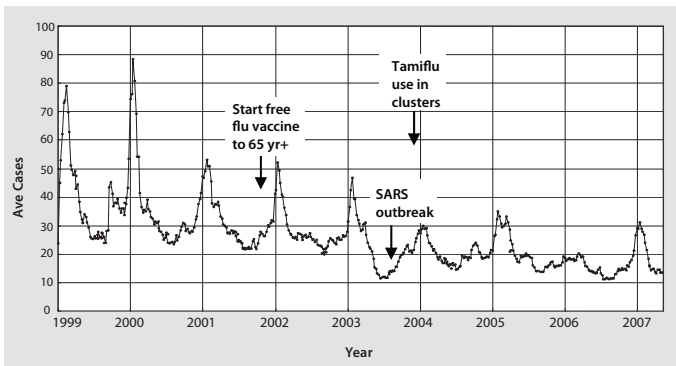
Results and Discussion

Among the 24 institutional outbreak of ILI reported during this period, 22 were confirmed to be caused by seasonal influenza A H3N2. A total of 609 throat specimens were sampled for tests for SARS CoV and influenza and 143 (23.4%) of them were positive for influenza. None of them were tested positive for SARS CoV. These data indicated that the combination of laboratory tests were sufficient to confirm the ILI diagnosis in institutional outbreak of ILI if the cases number in one outbreak exceeded 5 or more. Anti-virals were prescribed to the patients and close contacts in 20 institutions. Surprisingly, all the institutions showed no more transmission of ILI and no new cases documented after the 3rd day. However, the transmission continued in 2 schools, the outbreaks of which were initially interpreted as *Streptococcus pneumoniae* infection and antibiotics were prescribed until the 3rd wave of transmission occurred and the laboratory tests revealed influenza infection. Therefore, the transmission chain of influenza infection could be successfully controlled by anti-virals at its early phase of infection. Besides, the sentinel physician survey (Figure 1) revealed a dramatic

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decrease of ILI cases after 2003 as compared to the data before 2002, further supporting that the combination of public health measures, vaccination, and anti-virals have profound influence on the transmission of influenza. The experience we obtained from the post-SARS control measures therefore should be valuable for the preparedness of coming influenza pandemic. Additional reading: Ho MS and Su IJ: Preparing to prevent severe acute respiratory syndrome and other respiratory infections. *Lancet Infectious Dis.* 2004;4:684-689.

Figure 1. Decrease of ILI cases in sentinel physician survey after vaccination program and anti-viral usage.



Rapid Assessment of an Avian Influenza Outbreak – Juba, Southern Sudan, 2006

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The global spread of Avian Influenza (AI) H5N1 virus among poultry and humans has raised concern that the virus will cause the next pandemic. In South Sudan, poor public and animal health infrastructure make detection of AI outbreaks in birds and humans especially difficult. In Juba, Southern Sudan, H5N1 virus was identified in backyard chickens in September, 2006. To assess for human cases of AI and additional AI cases in poultry in Juba, we conducted a house-to-house survey. Methods: From September 20-25, 2006, five teams of animal and human health workers interviewed households in Juba using a questionnaire to assess whether people had sick or dead chickens in their home and whether people were sick themselves. Households were identified by convenience sampling. We defined a suspected human case of AI as anyone who had fever and cough, shortness of breath or difficulty breathing within the prior three weeks along with a history of contact with sick or dead birds. The Ministry of Health was informed if any people reported symptoms consistent with AI, and veterinary officers were informed if sick or dead poultry were found. GPS, Geographical positioning system coordinates were recorded for households reporting sick humans or sick or dead chickens. Results: A total of 1,613 households were surveyed. 80.6% (1300/1613) of the households reported having either chickens or ducks. In all, 14% (226/1613) of households reported having chickens that died within the previous two weeks. One person met the case definition for suspected AI and tested negative for AI. Conclusions: Our rapid survey did not identify any human AI cases in Juba. In countries with poor surveillance where new AI outbreaks are reported, rapid assessments through door-to-door household surveys can be effective tools in identifying suspected human cases and evaluating the extent of AI outbreaks in poultry

Introduction

The global burden of influenza epidemics is believed to be 3.5 million cases of severe illness and 300,000-500,000 deaths. The risk of serious illness and death is highest among persons aged > 65 years, children aged < 2 years, and persons who have medical conditions that place them at increased risk of developing complications from influenza [1]. In contrast to epidemics, pandemics are rare events that occur every 10 to 50 years. They have been documented since the 16th century [2], and in the last 400 years, at least 31 pandemics have been recorded [3]. But, global spread of Avian Influenza H5N1 virus

among poultry and humans has raised concern that the virus may cause the next pandemic if a novel subtype emerges to which human have no immunity; infection with the virus causes human illness and virus transmission sustained between humans [4]. In Africa; nine countries reported H5N1 in poultry and in humans; Egypt, Nigeria, Burkina Faso, Cote D'Ivoire, Cameroon, Niger, Djibouti, Sudan, Ghana [6]. Sudan is located in NE Africa, with area of 2,505,810 sq km, slightly more than a quarter of US with the population of 39,379,358. It is bordered by nine countries; Egypt and Libya in north, DRC, Uganda and Kenya in the south, Chad and Central African Republic in west and Ethiopia and Eritrea in the east. Juba is the capital city of Southern Sudan, with estimated population of 400,000 with unknown poultry population and all were backyard poultry. *Avian Influenza in Sudan*. First H5N1 Virus in Sudan was identified on April, 17th in Khartoum and AI Gezira states farms. In August, 2006 H5N1 virus was identified in backyard chickens in Juba, Southern Sudan [5]. At the time, no surveillance in place for animals or humans. Field Epidemiology Laboratory and Training Program, Centers for Disease Control and Prevention, World Health Organization, Food and Agriculture Organization, UNICEF were invited by Ministry of Animal Resources and Fisheries and Ministry of Health, Government of Southern Sudan to assist in AI outbreak in Juba [5]. To assess for human cases of AI and additional AI cases in poultry in Juba, we conducted a house-to-house survey. *Objectives of the survey*. 1) To determine how widespread poultry deaths in Juba town were; 2) To identify if poultry were still dying in Juba town since the first isolated AI virus in poultry; 3) To explore potential human exposure to, and infection with AI virus.

Methods

Sampling and Data Collection. From September, 20th – 25th, 2006, a cross-sectional survey of households with or without poultry was conducted in Juba town. Survey participants were identified through convenience sampling by households: Five teams of five members each and supervisor for each team were constituted. At the designated location, the supervisors identified a household to begin the interview. The teams then moved to the next nearest tenth household in the neighborhood. To ensure potential human exposure was explored, survey members actively sought household where poultry had died. They asked from a visited household if the respondent knew of, or had heard of poultry dying in the neighborhood. The Ministry of Animal Resources and Fisheries veterinary officers were informed if the survey leaders found sick or dead poultry for sample collection and carcass disposal, and sent to CDC-Kenya, then OIE for testing [5]. Each visited household was marked with a chalk so it would not be revisited. All counties of Juba were included in the survey. Geographical Positioning System (GPS) coordinates taken for households where samples were collected, nine square kilometers, west of the Nile River, were covered. Data were entered at CDC office in Nairobi and all the questionnaires returned.

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Case Definition. We define a suspected case as someone with unexplained acute lower respiratory illness with fever (>38°C) and cough, shortness of breath or difficulty breathing, in addition to one or more of the following within 7 days prior to onset of symptoms; close contact (within 1 meter) with a person (e.g. caring for, speaking with, or touching) who is a suspected, probable, or confirmed H5N1 case; exposure (e.g. handling, slaughtering, defeathering, butchering, preparation for consumption) to poultry or wild birds or their remains or to environments contaminated by their faces in an area where H5N1 infections in animals or humans have been suspected or confirmed in the last month; consumption of raw or undercooked poultry products in an area where H5N1 infections in animals or humans have been suspected or confirmed in the last month; close contact with a confirmed H5N1 infected animal other than poultry or wild birds (e.g. cat or pig); and handling samples (animal or human) suspected of containing H5N1 virus in a laboratory or other setting [7]. The H5N1 has an incubation period of 2-8 days with a median of 3 days, but may be as many as 17 day. Respondents were asked about signs and symptoms for up to 21 days (three weeks) prior to the survey as it is easier to recall 3 weeks rather than 17 days. To determine potential human exposure the H5N1 virus, information was collected on current ownership of sick poultry and if the household had had poultry deaths within the past four months. People living in households with currently sick poultry or with recent poultry deaths were considered potentially exposed.

Laboratory Testing. All cloacal and tracheal samples collected from a number of dead and dying birds/chickens from a backyard farm in Juba were tested at the CDC Kenya laboratory and OIE reference laboratories in the United Kingdom [5]. Limitations of our study participants were from a convenience sample good or worse scenario. No baseline data to compare with the findings of our survey. Recall bias, duration for the number of poultry owned was long and likely to lead to recall bias plus those with death after confirmation of AI recall more than those before the announcement. Insecurity in East bank of the Nile prevented large portion of Juba area could not be surveyed.

Results

Study participants (n = 1,613) households were interviewed. 80% (1300/1613) of the households reported having either chickens, ducks or pigeons. 6% (47/838) currently sick chicken and 31% (32/739) currently sick duck respectively. 2% (31/1613) households responded that their poultry had all died in one day, while 5% (72/1613) said they had died within 2 week and 8%(124/1613) households said they had died within 1 week. In all, 18% (296/1613) and 14% (229/1613) of households reported having chickens and ducks that died within the last 4 months.

Serologic studies. All poultry samples (47) collected were negative for H5N1 using the PCR. One person who met the suspected case definition tested negative for H5 [5].

Figure 1. Occurrence of poultry deaths in four months prior to survey.

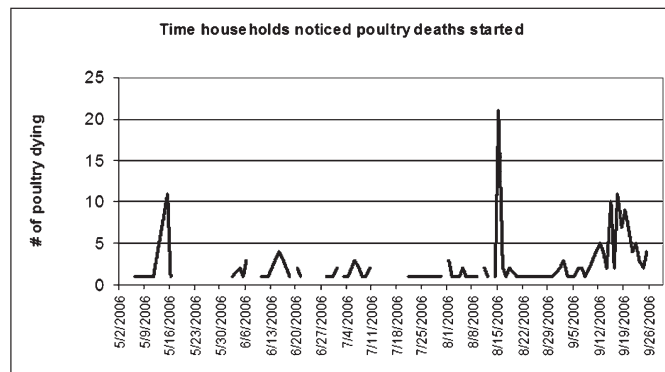
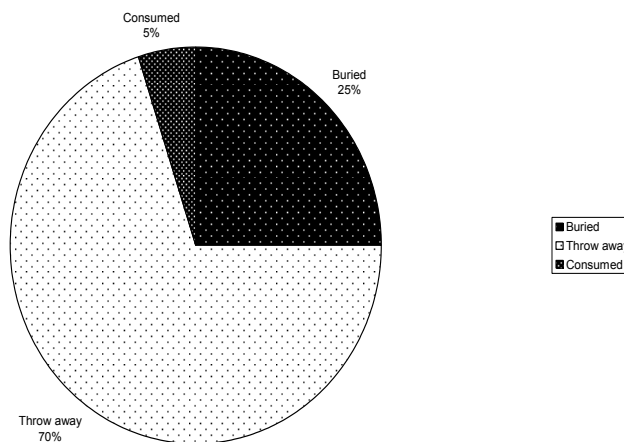


Figure 2. How did people handle dead poultry?



Discussions and Conclusions

Poultry deaths were widespread and had been occurring in and around Juba for 3-4 months prior to the first reported H5N1 case in poultry, it was not possible, to determine if these deaths represents background poultry deaths without baseline information. There were more deaths in September, but this cannot be directly attributed to avian influenza. It is more likely a recall bias as households are likely to recall recent deaths. There are two high spikes, mid-May and mid-August for which there is no apparent explanation. All poultry samples tested negative for H5N1, but we cannot conclude that there were no additional outbreaks; may be due to small sample size or because it was snapshot i.e one point in time. Our rapid assessment identify only one person who met the case definition for suspected AI and tested negative for AI. Data from the survey were used to inform culling strategies, even if it was not a perfect survey, the government was not ready to cull all chicken from Juba because no positive chickens were found. Households visited during the survey were given messages relating to handling of sick or dead poultry. Recommendation: In countries with poor surveillance, rapid assessments through door-to-door household surveys can be an effective tool in

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identifying suspected human cases and evaluating the extent of AI outbreaks in poultry.

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Proceedings Topic #4

Replication and Assembly

Oral

Presentations

Rescue of Influenza C Virus From Recombinant DNA

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We developed a reverse genetics system that allows the production of influenza C viruses entirely from cloned cDNA. For this purpose, 293T cells were transfected with plasmids that direct the synthesis of each of the seven viral RNA segments of the C/Johannesburg/1/66 (C/JHB/1/66) virus under the control of the human RNA polymerase I promoter and with plasmids encoding the viral nucleoprotein and the PB2, PB1 and P3 proteins of the viral polymerase complex. This allowed engineering of influenza C viruses harboring Thr284Ala and/or Thr465Ile substitutions in the hemagglutinin esterase fusion (HEF) protein. The mutant viruses were found to be genetically stable upon passage on SK 93/2 cells. Growth kinetics were performed on MDCK-II cells pretreated with bovine brain gangliosides (BBG) in order to provide the Neu5,9Ac₂ receptor used by influenza C virus which is lacking on these cells. Under those conditions, the Thr284Ala virus grew to titers similar to the wild-type (wt) virus whereas the Thr465Ile virus or the double mutant grew to 10-fold higher titers. Furthermore, the Thr284Ala mutant was found to grow to high titers on untreated MDCK-II cells in the presence of a high concentration of trypsin. We thus demonstrated that the reverse genetics system described here provides a means to study the effects of viable point mutations introduced into the genome of influenza C virus.

Introduction

The genome of influenza C virus, a member of the *Orthomyxoviridae* family, consists of seven segments of single-stranded RNA of negative polarity. Each genome segment is in the form of a ribonucleoprotein (RNP) which consists of the viral RNA (vRNA) molecule associated with the nucleoprotein (NP) and the polymerase complex made of the PB1, PB2 and P3 proteins [1]. In contrast to influenza A and B viruses, influenza C virus harbors a single envelope glycoprotein, the hemagglutinin-esterase-fusion protein (HEF). This protein possesses three functions, i.e. receptor recognition, viral fusion and receptor destroying function. The HEF utilizes the 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac₂) for attachment to the cell surface [2,3]. The fusion activity of the HEF, which requires activation at low pH in the endosome, is dependent on the proteolytic cleavage of the HEF₀ precursor into two subunits HEF₁ and HEF₂ [4,5]. The acetyl esterase activity of the HEF releases the O-acetyl residues from the receptor molecule [6]. Reverse genetics systems for the production of infectious virus from cloned cDNA have been previously described for the influenza type A [7,8] and B [9,10] viruses but not for type C influenza viruses. Several strategies have been used for

the reconstitution of RNPs from cloned cDNA, requiring the production of exact vRNA segments and that of the minimum set of viral proteins (PB1, PB2, PA and NP) necessary for transcription/replication of the viral RNA. Viral RNA segments are usually produced from plasmids containing the full-length cDNAs cloned under the control of the RNA polymerase I (POL-I) promoter such as to initiate transcription at the first nucleotide of the 5'-end of the vRNA [7,8,10]. Exact 3'-end extremities of the vRNAs are ensured by placing either a POL-I terminator or hepatitis delta ribozyme sequence downstream of the cDNA. The first successful rescue of a vRNA segment from recombinant plasmid DNA involved co-infection with a helper virus [11,12]. For the rescue of virus entirely from cloned cDNA, the four viral proteins required for transcription/replication of the vRNAs are produced from expression plasmids under the control of an RNA polymerase II (POL-II) promoter. The use of bidirectional RNA POL-I/II plasmids to clone the full-length cDNAs corresponding to each of the viral segments allowed to reduce the number of plasmids required for virus generation from twelve to eight [9,13]. An alternative strategy uses of the T7 RNA polymerase promoter for both expression of the vRNAs and viral proteins [14]. Here we describe the rescue of the C/JHB/1/66 virus using plasmids that direct the synthesis of the seven vRNA segments under the control of the human POL-I promoter together with the plasmids that direct the expression of the NP, PB1, PB2 and P3 proteins required for transcription/replication of the influenza C virus vRNAs [15]. This reverse genetics system was further used to engineer influenza C viruses carrying mutations at positions 284 and/or 465 in the HEF protein that could be involved in receptor binding [16] or fusion activity [17], respectively.

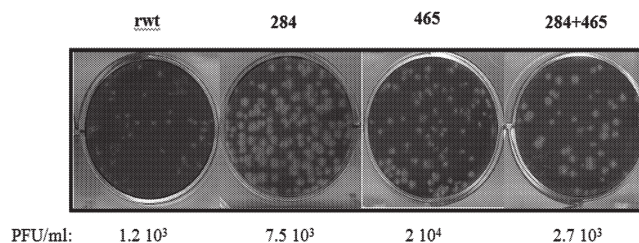
Results and Discussion

Production of plasmids that direct the synthesis of the seven vRNA segments of C/JHB/1/66 influenza virus. As a first step to produce full-length cDNAs corresponding to the seven vRNA segments of C/JHB/1/66 virus, we determined the sequences of the 3' and 5' non-coding regions of the seven vRNAs (accession numbers AF170573 to AF170576 and AM410041 to AM410043) as described previously [18]. Analysis of the sequences showed that the first 11 nt as well as nt 14 at the 3' end of the seven segments were conserved. At the 5' end, the first 12 nt and nt 15 were conserved except for the NS segment for which nt 6 was a G instead of a U as previously described for the C/California/68 virus (M10087). The length of the 3'NC regions ranged from 17 nt for the PB2 to 29 nt for the NP segment. The 5'NC regions were more variable in length, ranging from 19 nt for the PB2 segment to 102 nt for the NP segment. Comparison of the coding sequences with those available in the GeneBank database revealed no differences resulting in amino acid substitutions for the PB2, PB1, P3, NP and M segments (AF170573 to AF170576 and AM410042). For the HEF sequence, one difference at position 1414 resulted in a Thr to Ala substitution at position 465 when comparing our (AM410041) and previously published sequences of C/JHB/1/66

[19]. For the NS segment, our sequence (AM410043) revealed six nucleotide differences with that published for C/JHB/4/67 [20]. Three of these were non silent and resulted in two amino acid substitutions in NS1 (aa 74 and 228) and two in NS2 (aa 124 and 144). To generate plasmids that direct the synthesis of the seven vRNA segments of C/JHB/1/66, the corresponding full-length cDNAs were cloned in anti-sense orientation at the *Bbs*I site of plasmid vector pPR [15] between the human RNA POL-I promoter and hepatitis delta ribozyme sequences, resulting in plasmids pC/PR/X/JHB, where X corresponds to the name of each segment. By site directed mutagenesis on plasmid pC/PR/HEF/JHB, mutations were introduced in the HEF gene sequence, resulting in Thr284Ile and Thr465Ala substitutions and the introduction of *Eco*R V and *Ngo*M IV restriction sites, respectively.

Rescue of infectious influenza C virus from cloned cDNAs. A first prerequisite for the rescue of infectious influenza C virus was to identify highly transfectable cells that are permissive for the virus. The SK 93/2 cell line, which is highly permissive for influenza C virus, was poorly transfectable. We therefore developed conditions that allowed efficient growth of the C/JHB/1/66 virus on 293T cells which are highly transfectable but lowly adherent and very sensitive to the concentration of trypsin required for the cleavage of the HEF protein. When 293T cells were cultured on poly-Lysine plates in the presence of 0.25 µg/ml TPCK-trypsin viral titers up to 10^5 - 10^6 PFU/ml were obtained upon infection at an m.o.i. of 0.001 with C/JHB/1/66 at 33°C. A second prerequisite was to be able to efficiently detect and titrate the influenza C virus produced by reverse genetics. The highly permissive SK 93/2 cells were not adherent enough for use in plaque assay. We therefore developed a plaque assay on Madin darby canine kidney II (MDCK-II) cells similar to that used for influenza A viruses [21]. However, MDCK-II cells are resistant to infection by influenza C virus because they lack the Neu5,9Ac₂ receptors. This can be overcome by pretreatment of the cells with bovine brain gangliosides (BBG, 0.2mg/ml), which naturally contain Neu5,9Ac₂ for 45 min at 37°C [22]. Having defined optimal conditions for virus growth and titration, to rescue influenza C virus from cloned cDNAs, we transfected 293T cells (6×10^5 cells on 35 mm poly-D-Lysine plates) with the seven pC/PR/X/JHB plasmids together with the four expression plasmids that encode the three polymerase subunits and NP protein (0.5 µg of each plasmid) and 10 µl of FUGENE 6 (Roche). After 16 hours at 33°C, the transfection reagent was removed, the cells were washed twice and further incubated at 33°C with DMEM containing 0.25 µg/ml TPCK-trypsin (Worthington).

Figure 1. Plaque phenotype of the rescued wild-type (rwt) and mutant (284, 465, 284+465) viruses. The transfection supernatants collected at day 10 post-transfection of 293T cells were titrated by plaque assay on MDCK-II cells coated with BBG. Viral titers of the rescued viruses are indicated.



As shown in Figure 1, 10 days after transfection, rescued virus was detected in the supernatant at titers of 10^3 to 10^4 PFU/ml. Wild-type (rwt) virus as well as the single or double HEF mutants could be rescued, whereas no virus could be rescued when the HEF vRNA expression plasmid was omitted (not shown). The viruses were readily amplified on SK 93/2 cells to titers of 10^5 - 10^7 PFU/ml and successfully recovered for five consecutive passages. The presence of the mutations was assessed by RT-PCR targeting the HEF gene followed by restriction enzyme digestion. Amplicons of the expected size were obtained. These were cleaved by *Eco*R V in the case of the 284 and 284+465 mutants and by *Ngo*M IV in the case of the 465 and 284+465 mutants (not shown). Thus, as also confirmed by sequencing, the mutant viruses were successfully rescued and were genetically stable after five passages on SK 93/2 cells.

Effect of the Thr284Ile and Thr465Ala mutations in the HEF protein. We next analyzed the effects of the substitutions at aa 284 and 465 in the HEF protein on virus growth capabilities. The Thr284Ile substitution in the HEF was previously described [16,23] for a virus obtained by passaging C/JHB/1/66 virus on MDCK-II cells in the presence of a high concentration of trypsin. The Thr465Ala substitution located in the peptide fusion domain of the HEF corresponds to the only difference between our sequence and the previously published C/JHB/1/66 sequence [19]. We thus compared the ability of the rescued wt and single or double mutant viruses to grow on MDCK-II cells with or without pre-treatment with BBG and in the presence of a low (0.25 µg/ml) or high (5 µg/ml) concentration of TPCK-trypsin. As shown for one representative experiment (Table 1), all viruses were highly dependent on the presence of BBG at a low concentration of trypsin. At a high concentration of trypsin, viruses with Ile284 in the HEF were able to grow to significant titers in the absence of BBG. Furthermore, viruses with an Ala465 in the HEF grew to 10-fold higher titers than their counterparts either in the presence of BBG at a low concentration of trypsin or in the absence of BBG at a high concentration of trypsin.

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Table 1. BBG dependance and trypsin phenotype of the rescued wild type or HEF mutant viruses. Viral titers (PFU/MI) reached after 4 days of growth in the indicated conditions are shown.

Culture conditions	Viruses			
	rwT	284	465	284+465
w/o BBG			125	175
low trypsin w/ BBG	250	50		
low trypsin w/o BBG	7.8 10 ³	1.2 10 ⁴	6.2 10 ⁴	2.0 10 ⁵
high trypsin	50	7.0 10 ³	233	2.8 10 ⁴

Thus our results further confirmed that the Thr284Ile mutation in the HEF described previously [16,23] is necessary and sufficient to enable influenza C virus to grow on MDCK-II cells at a high concentration of trypsin. The Thr284Ile substitution in the HEF, located in the vicinity of the receptor binding site [24] may result in a conformational change of the receptor binding domain and increased affinity for the receptor. In addition, trypsin at high concentration could act on the MDCK-II cells and unmask Neu5,9Ac₂ sialic acids present in very low amounts that could be used by the Ile284 viruses. The Thr465Ala substitution located in the peptide fusion domain of the HEF₂ subunit increased virus growth capabilities. Since a non-polar residue replaced a polar residue, the global hydrophobicity of the fusion peptide might be increased and the fusion capabilities of the virus enhanced. Interestingly, recovery of the Ala465 viruses by reverse genetics seemed to be easier, virus yields in the transfection supernatants being 10 to 100-fold higher than for the rescued wt virus. This could prove useful to study the positive or negative effects of mutations in any of the seven segments on the growth and replication of influenza C virus. Our system for influenza C virus completes the series of reverse genetics systems for the different genera of the *Orthomyxoviridae* family. Very recently, a similar system was described for *C/Ann Arbor/1/50* using 293T cells and subsequent amplification in eggs [25]. Such reverse genetics systems, which might be further improved, provide means to study the influenza C virus life cycle, the molecular mechanisms of viral pathogenicity or to engineer influenza C viruses to generate expression vectors, which could be used as live vaccines.

Acknowledgements

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Intranuclear Dynamics of the Influenza A Virus RNA Polymerase as Revealed by Live Cell Imaging Studies

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Influenza A virus replicates and transcribes its RNA genome in the nucleus of infected cells. The viral RNA polymerase is essential to these processes and is also a host range determinant and pathogenicity factor. We have established a live cell imaging system that allows the observation of fluorescently tagged influenza polymerase subunits on their own or in combination with other viral components. We employed this system in FRAP studies to analyse the dynamic behaviour of the viral polymerase and its subunits in the nuclear environment. While the individual polymerase subunits examined displayed fast nuclear dynamics, the full polymerase complex was markedly less mobile. This suggests the polymerase complex interacts with an insoluble cellular nuclear component.

Introduction

The influenza A RNA-dependent RNA polymerase is a heterotrimer composed of the subunits PB1, PB2 and PA and is essential to viral replication [1]. Cellular pre-mRNAs are bound by the PB2 subunit and their 5' cap is cleaved by PB1 to serve as primers for viral transcription [2]. PB1 is also the polymerase that elongates the nascent RNA strand [3]. The precise role of the PA subunit is undefined. Viral RNA synthesis occurs in the nucleus of infected cells [4, 5]. This has long been attributed to a dependence on the cellular transcription machinery. It provides ready access to capped mRNA substrates [2] as well as the cellular splicing machinery [6]. RNA polymerase II activity also assists nuclear export of selected viral mRNAs [7]. Clearly, viral parasitism of host nuclear functions requires an intimate interplay between viral and host components and in recent years, multiple interactions have been defined. Several cellular interaction partners have been identified for the influenza polymerase subunits including the large subunit of cellular RNA polymerase II [8, 9] which interacts with the full viral polymerase complex only. Furthermore, virus-host cell interactions contribute to host specificity and virulence of human and avian influenza viruses and this concept applies to the polymerase, in particular the PB2 subunit [10, 11]. To further understand the interplay between influenza virus and the host cell we analysed the dynamic behaviour of the viral polymerase in the nuclear environment using FRAP experiments.

Materials and Methods

Cell culture, plasmids and DNA transfection. 293T cells were cultured and transfected with plasmids as described [7]. Plasmids expressing untagged and GFP-tagged PB2, -PB1, -PA and -NP proteins have previously been described [12, 13]. Plasmid pcDNA-PB1sdd was constructed by site-directed mutagenesis generating a double mutation in the conserved SDD motif of the polymerase active site of PB1 (PB1-D445A/D446A).

FRAP microscopy. Cells on 42mm glass coverslips were transferred to live cell chambers and maintained at 37°C in CO₂ independent medium (Gibco). Images were captured on a Zeiss LSM 510 confocal microscope using a 63X objective and a digital zoom factor of 5. GFP was excited using the 488 nm laser line of a 30mW Ar laser running at 6.1A and 1% output. Photobleaching was performed on a 1.4 μm² bleach window at 100% laser output. Five pre-bleach and 70 post-bleach images were collected at 0.39 sec intervals. Fluorescence intensities of regions of interest were obtained using the LSM510 software.

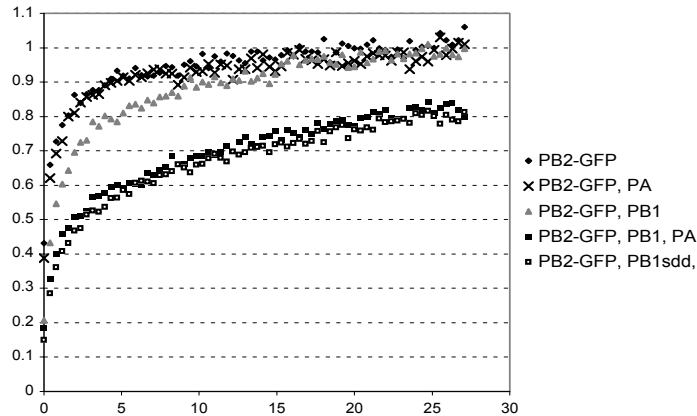
Data analysis. Background fluorescence and acquisition bleaching were adjusted for and fluorescence intensity was normalised using the following equation: $(F(t)-BF) / (R(t)-BF) \times NF$ where $F(t)$ is the observed fluorescence at time t , BF is the mean measured background fluorescence, $R(t)$ is the fitted fluorescence in the reference window at time t and NF is a normalisation factor equal to the average of the recorded values of $(F(t)-BF)/(R(t)-BF)$ before bleaching. Time to half recovery ($t_{1/2}$) was determined by adapted classical FRAP analysis as described [14].

Results

The nuclear dynamics of recombinant influenza A virus polymerase was examined in live-cell FRAP experiments. Expression vectors for C-terminally GFP-tagged PB2, as well as untagged subunits, were employed. 293T cells were transfected with plasmid mixtures expressing PB2-GFP alone, in combination with a second untagged polymerase subunit, or with both other subunits to reconstitute the full heterotrimeric polymerase. 24 h post-transfection FRAP analysis was carried out in live cells. A defined nuclear area was photobleached and recovery of the fluorescence signal over time was monitored. PB2-GFP displayed very rapid recovery kinetics and fully recovered to initial fluorescence intensity indicating the virtual absence of immobile molecules (Figure 1). When transfected in combination with pcDNA-PA no significant change to PB2-GFP dynamics were observed (Figure 1), as expected because PB2 and PA do not stably interact in the absence of PB1 [15]. Expression of PB1 in combination with PB2-GFP caused a slight reduction in the rate of fluorescence recovery, although recovery still reached 100% of initial fluorescence intensity (Figure 1). However, a much more dramatic decrease in recovery rates was observed in cells expressing the full polymerase complex and recovery was not complete, suggesting a fraction was immobilised within the nucleus (Figure 1). To determine the relevance of transcriptional competence of the viral polymerase for these slow nuclear dynamics a PB1 mutant lacking the

catalytic SDD polymerase motif was tested. In cells expressing this transcriptionally inactive polymerase complex recovery of PB2-GFP fluorescence was similar to that observed with the WT polymerase (Figure 1). Thus, the full influenza polymerase complex displays a marked reduction in nuclear mobility as compared to a single subunit or dimer combinations and this does not require a transcriptionally competent polymerase.

Figure 1. FRAP measurements in cells expressing the indicated polymerase subunits.



To quantify the nuclear dynamics of the polymerase, fluorescence recovery data from multiple experiments was fitted with an equation adapted from a previous study [14] and time to half recovery ($t_{1/2}$) was calculated (Table 1). These values confirmed the fast nuclear dynamics of PB2 alone or in the presence of non-interacting PA with average $t_{1/2}$ values of less than one second (Table 1). The slightly reduced recovery rate of the PB2-GFP - PB1 dimer was reflected in a similar increase in $t_{1/2}$ (Table 1). In cells expressing the full polymerase complex, whether in replication competent or inactive form, $t_{1/2}$ increased dramatically by at least 7-fold as compared to PB2 alone, consistent with the marked decrease in fluorescence recovery rates. Overall, assembly of all three P proteins confers slower nuclear dynamics to the full influenza polymerase complex and prevents recovery to full initial fluorescence intensity. This strongly suggests a specific interaction of the heterotrimer with a relatively immobile nuclear component.

Table 1. Nuclear dynamics of combinations of influenza P proteins.

Transfected plasmids	$t_{1/2}$ (sec) (mean \pm s.d.)	relative to PB2-GFP
pPB2-GFP	0.93 \pm 0.83	1
pPB2-GFP, pcDNA-PA	0.86 \pm 0.51	0.92
pPB2-GFP, pcDNA-PB1	1.44 \pm 0.90	1.55
pPB2-GFP, pcDNA-PB1, pcDNA-PA	6.59 \pm 4.49	7.09
pPB2-GFP, pcDNA-PB1sdd, pcDNA-PA	8.92 \pm 5.88	9.59

Discussion

The full influenza polymerase complex displays relatively slow nuclear dynamics, a property not shared by any of the individual subunits or P protein dimers tested. A size-effect alone can be ruled out since an eightfold increase in size is required for a twofold decrease in diffusion coefficient for soluble spherical proteins [16] such as the influenza polymerase [17]. As $t_{1/2}$ is in close relationship with the diffusion coefficient a mere threefold increase in mass upon full polymerase complex formation cannot account for a sevenfold increase in $t_{1/2}$. At this stage it is not apparent what this additional functionality gained by the full complex might represent, but an interaction with a comparatively insoluble cellular nuclear component seems likely. It has been suggested that influenza RNA synthesis occurs in insoluble nuclear substructures [5, 18]. However, the transcriptional competence of the polymerase alone does not account for the reduced mobility phenotype as a catalytically inactive polymerase displayed similar nuclear dynamics to the WT protein. Furthermore, no vRNA template for the viral polymerase was present in our experiments. The accumulated evidence for a direct interaction between the viral polymerase and cellular RNA polymerase II [8, 9] as well as the dependence of viral transcription on functionally interlinked cellular transcription and mRNA processing machinery [3, 7] suggests the hypothesis that the cellular transcriptosome affects the nuclear dynamics of the viral polymerase. Overall, the live-cell imaging system described here will be valuable for elucidating the intracellular dynamics of the influenza virus polymerase. Future experiments will address the involvement of cellular RNA polymerase II as well as the role played by the PB2 subunits of avian or human viruses in determining the nuclear kinetics of the influenza virus polymerase.

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Control of Influenza Viral Replication and Transcription

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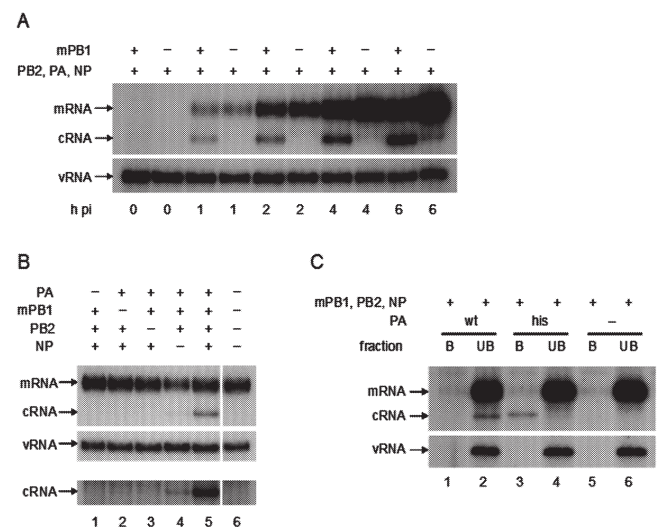
The regulated expression of genes is crucial for the fitness of all organisms in their environment, the complexity of mechanisms escalating from prokaryotes to eukaryotes. Viruses are the simplest organisms known to man, with only limited genetic resources. Paradoxically, they replicate in the complex environments of their hosts, requiring mechanisms to appropriate their host cell's synthetic machinery for viral replication while evading or neutralising the antiviral defences. Surprisingly, we show that influenza virus may forgo complex self-regulation, relying instead on adventitious regulatory mechanisms for transcription and replication of viral genes *in vivo*. Specifically, we demonstrate that influenza A virus does not switch from mRNA transcription to RNA replication by actively regulating viral RNA synthesis as previously thought [1,2]. Instead, mRNA and complementary RNA (cRNA) — the replicative intermediate — are synthesized stochastically. Our data suggest that cRNA is rapidly degraded by host nucleases unless it is stabilised by newly synthesised viral polymerase and nucleoprotein (NP).

Introduction

The influenza viral RNA polymerase, comprising PB1, PB2 and PA subunits, together with NP, binds the viral genomic RNA to form vRNPs and is responsible for both transcription (vRNA→mRNA) and replication (vRNA→cRNA→vRNA). Whereas mRNA transcription requires capped RNA primers snatched from host pre-mRNA and premature poly(A) termination of transcripts, genome replication is primer-independent and generates full-length cRNA [3]. Early experiments treating infected cells with cycloheximide, an inhibitor of protein synthesis, demonstrated that, whereas vRNPs derived from infecting virus particles are transcribed to mRNA, synthesis of cRNA and replication of vRNA is inhibited, suggesting that replication requires *de novo* protein synthesis [1,2]. It was proposed that expression of a viral protein, most likely NP, acts as a “molecular switch” to regulate the transition from transcription to replication [4,5], but the molecular mechanism for such a switch has remained elusive [6]. Given the paucity of genes in the influenza viral genome, we hypothesised that transcription and replication may be regulated stochastically; that is, random primer-dependent or independent initiation dictating the synthesis of mRNA or cRNA. We proposed that nascent cRNA is degraded unless it is bound by newly synthesised polymerase and NP to form a cRNP template for new vRNA synthesis. Our recent work [7,8], reviewed in this article, demonstrates that the polymerase that is associated with the infecting virion synthesises both mRNA and cRNA, and that the nascent cRNA is bound by free polymerase and NP.

In vivo synthesis of cRNA. In order to study the switch in influenza A virus-infected cell culture, the transition from mRNA transcription to RNA replication was inhibited using cycloheximide. It was then investigated whether inhibition of the transition could be overcome, or specifically whether the switch to cRNA synthesis could be “rescued”, by expressing a mutant viral polymerase (lacking detectable polymerase activity) and NP in the cells prior to infection with influenza virus in the presence of cycloheximide [7]. cRNA was detected in cells in which the full complement of viral polymerase and NP had been pre-expressed (Figure 1A). Omission of any one of the three polymerase subunits (mPB1, PB2 or PA) abrogated the cRNA signal (Figure 1B, compare lanes 1-3 with lane 5). However, in the absence of NP (lane 4), the cRNA signal was reduced but nevertheless remained clearly detectable above background (lane 6). This cRNA must have been synthesised by the polymerase associated with the infecting virion particle as the pre-expressed polymerase lacked any detectable activity. Similar experiments were also performed using actinomycin D, an inhibitor of mRNA transcription, instead of cycloheximide. Whereas no mRNA could be detected in these cases, cRNA was again only detected if viral polymerase and NP were pre-expressed (data not shown). Overall, therefore, these results suggested that the virion-associated RNA polymerase synthesises mRNA and cRNA stochastically early in infection.

Figure 1. Expression of influenza viral RNA polymerase and NP binds nascent cRNA synthesised by infecting virion-associated vRNPs. 293T cells were transfected with plasmids expressing viral proteins, as indicated, 12-14 h prior to infection with influenza A/WSN/33 in the presence of 100 µg/ml cycloheximide. Viral RNA species were analysed by NA gene-specific primer extension. (A) Time course of viral infection. (B) Analysis of minimal viral protein requirements for cRNA detection at 2h post-infection. The lower panel represents a longer exposure of the cRNA-specific bands. (C) Analysis of RNA species bound by histidine-tagged (his) or untagged (wt) pre-expressed RNA polymerase following separation by nickel column chromatography into bound (B) and unbound (UB) fractions. mPB1 = PB1-D445A/D446A; h pi = hours post infection; wt = wild type; his = histidine. (Adapted from [7] and [8]).



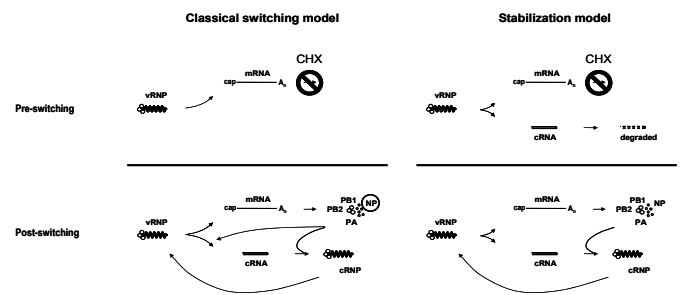
Options for the Control of Influenza VI

In vitro synthesis of cRNA. According to the above data, influenza virion-associated polymerase synthesises both mRNA and cRNA upon infection. In order to test this directly, vRNPs were extracted from purified virions and examined for *in vitro* polymerase activity in the absence of any non virion-associated proteins. In the presence of all four nucleoside triphosphates, but in the absence of any primers, full-length cRNA was synthesised [8]. This result was analogous to the *in vivo* rescue of cRNA following infection in the presence of actinomycin D. If globin mRNA was added to the *in vitro* reaction as a source of capped primer, authentic capped and polyadenylated mRNA was synthesised in addition to cRNA, analogous to the *in vivo* rescue of cRNA following infection in the presence of cycloheximide [8]. Nascent cRNA is bound by expressed polymerase *in vivo*. Based on the above results, we postulated that *in vivo* expressed viral polymerase and NP bind nascent cRNA synthesised by the virion-associated vRNPs, thereby protecting it from degradation by host nucleases. To test this hypothesis, histidine (his)-tagged PA was pre-expressed together with mPB1, PB2 and NP in the cRNA rescue assay [8]. His-tagged protein complexes were partially purified by nickel column chromatography and the co-purifying RNA in the bound and unbound fractions were analysed by primer extension (Figure 1C). Consistent with our hypothesis, whereas vRNA and mRNA were found in the unbound fraction of the nickel column (lane 4), cRNA was found exclusively in the bound fraction (lane 3). This implied that the pre-expressed his-tagged PA, presumably together with PB1, PB2 and NP, had bound nascent cRNA. This suggested that the mechanism of cRNA rescue is by the assembly of cRNP complexes.

Discussion

We have shown that influenza A virion-associated polymerase catalyses both capped RNA-dependent vRNA→mRNA transcription and primer-independent vRNA→cRNA replication *in vivo* and *in vitro*. The detection of cRNA *in vivo* is dependent on the expression of viral polymerase and NP, which binds the nascent cRNA. Based on our results, we have proposed a “stabilization” model for influenza viral infection (Figure 2) [7]. vRNPs derived from the infecting virus synthesise both mRNA and cRNA. During the early transcriptive (or pre-switching) phase, nascent cRNA is presumably rapidly degraded by host cell nucleases whereas mRNA is protected from normal cellular degradative processes by the presence of a 5' cap and a 3' poly(A) tail. Switching to the replicative (or post-switching) phase occurs when cRNA is protected by the specific binding of newly synthesised RNA polymerase and NP, leading to the formation of active and stable cRNPs suitable for replicative vRNA synthesis. Crucially, this model proposes that switching is not regulated actively at the level of mRNA or cRNA synthesis, but by newly synthesised polymerase and NP stabilizing cRNA transcripts. Our stabilization model suggests that the synthesis of mRNA or cRNA by influenza A virus polymerase perhaps occurs stochastically; that is, the random binding of a capped RNA primer or an ATP molecule dictates whether mRNA transcription or cRNA synthesis occurs, possibly by inducing subtle conformational differences in the structure of the polymerase.

Figure 2. Models for switching between influenza A viral transcriptive and replicative phases. CHX = cycloheximide. (Adapted from [7])



We suggest that this lack of regulation at the level of cRNA synthesis, whilst being “wasteful” by generating cRNA destined for degradation, reflects the limited genetic resources possessed by RNA viruses for complex regulation.

Acknowledgements

This review is based on and adapted from [7] and [8]. The work was supported by MRC grants G9523972 and G9901312 to G.G.B.

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Mitochondrial Localization of the Influenza A Virus PB2 Protein

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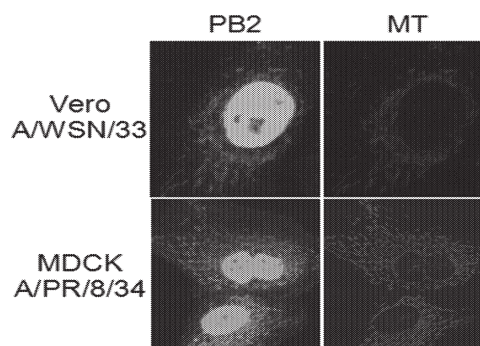
Introduction

Influenza viruses are viruses with a negative sense, segmented RNA genome that can infect a wide variety of hosts including humans, birds, and other animals. The viral genome is replicated and transcribed by the trimeric viral RNA-dependent RNA polymerase which is comprised of three viral proteins: polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA). The PB2 protein is crucial during transcription as it recognizes and binds the cap structures of host pre-mRNAs. This capped host pre-mRNA is then endonucleolytically cleaved by PB1 and the capped RNA fragment is then used as a primer which the PB1 protein subsequently elongates using the viral RNA (vRNA) as a template. The PB1 protein is also responsible for the polymerization of complementary RNA (cRNA) and vRNA during viral genome replication. The enzymatic function of PA protein is less well characterized, but is required for both replication and transcription [1]. The PB2 protein has recently also been shown to be involved in host range restriction and virulence of avian H5N1 influenza viruses [2,3], but the underlying mechanisms remain unknown. To determine how the PB2 protein affects virulence and host range, a better understanding of the PB2 protein and its function as a polymerase subunit as well as identification of unknown functions or properties, including cellular localizations, is needed. The steps of viral replication including all described enzymatic activities of the PB2 protein such as recognition and binding of cap structures are known to occur in the nucleus of the infected cell. Recently, our laboratory identified an alternate localization of the PB2 protein to the mitochondria of the host cell [4]. This article briefly reviews this finding and discusses the implications of this localization for the viral response to cellular antiviral activities.

Results and Discussion

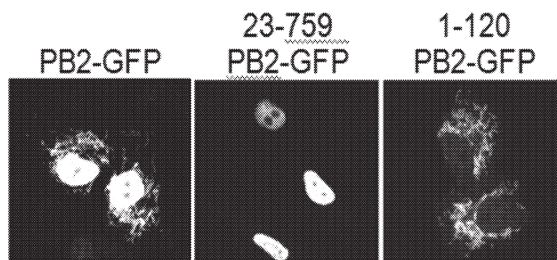
As mentioned previously, determining the cellular localization and thus potential interacting host factors of the PB2 protein may help explain its role in host range restriction and virulence. To determine where in the host cell the influenza A virus PB2 protein localized, Vero or Madin-Darby canine kidney (MDCK) cells were infected with either A/WSN/33(H1N1) or A/Puerto Rico/8/34(H1N1), respectively followed by indirect immunofluorescence. As shown in Figure 1, PB2 signals can be detected, as expected, in the nucleus, but surprisingly, this signal can also be detected in the mitochondria, thus showing an additional localization of the PB2 protein.

Figure 1. Localization of influenza A virus PB2 proteins in infected cells. Vero or MDCK cells were infected with influenza A/WSN/33 (H1N1) or A/Puerto Rico/8/34 (H1N1), respectively. Eight hours post-infection, the PB2 protein was detected via indirect immunofluorescence; Mitochondria were stained with MitoTracker®Red CMXRos (MT). (Modified figure from ref. [4]).



To identify the region of the PB2 protein responsible for its localization to the mitochondria, plasmids expressing C-terminally GFP-tagged PB2 fragments were transfected into Vero cells. Figure 2 shows that a fragment lacking the first 23 residues is unable to localize to the mitochondria, whereas a 120 amino acid N-terminal fragment is sufficient to direct GFP to the mitochondria. Taken together these data suggest that the N-terminal 23 amino acids contain the mitochondrial localization signal. Supporting this claim, a prediction of secondary structure suggests that this region forms an amphipathic α -helix containing arginine, serine, and leucine residues, features important in N-terminal mitochondrial localization signals [5]. To identify which residues of the N-terminus are required for localization, alanine mutations were constructed in the PB2 protein and the localization of these point mutants in Vero cells was assessed. Alanine or proline mutations at positions 3, 8, 12, 14, and 15 did not affect localization to the mitochondria. In contrast, L7A, L10A, and L7L10A mutations abolished the PB2 protein's localization thus identifying the importance of these residues in mitochondrial localization.

Figure 2. Localization of influenza A virus PB2 fragments. Vero cells were transfected with plasmids expressing PB2 fragments tagged with GFP at their C-Terminus. sixteen hours post-transfection, the cells were fixed and the PB2 proteins were visualized via the GFP tag. (Modified figure from ref. [4]).



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Following identification of the mitochondrial localization signal in the PB2 protein, the role of this localization was assessed. The growth of recombinant viruses containing L7A, L10A, or L7L10A mutations were tested in MDBK cells. Recombinant viruses with a single substitution (L7A, L10A) had a slight attenuation in growth and viruses with double substitutions (L7L10A) had a growth attenuation of 100-fold. To detect a disruption that the PB2 protein might cause to the mitochondria, the membrane potential of the organelle during infection was assessed. HeLa cells infected with either the wildtype A/WSN/33 or recombinant viruses were stained with TMRE, a marker of mitochondrial membrane stability and analyzed by flow cytometry. Cells infected with the wildtype virus retained mitochondrial membrane potential whereas cells with the recombinant viruses had a loss of membrane potential, suggesting that mitochondrial PB2 might prevent membrane potential loss during viral infection. However, the full functional implications of the localization of the PB2 protein to mitochondria remain unknown. The recent discovery of mitochondrial antiviral signaling protein (MAVS) (also known as IPS-1 [6], VISA [7], and CARDIF [8]) established an unexpected link between innate immunity and mitochondria [9]. It is now believed that mitochondria play an important role in the type I interferon induction pathway [10]. To date, there have been two distinct pathways found to induce the expression of the type I interferon, IFN- β , either Toll-like receptor (TLR) or retinoic acid inducible gene-I (RIG-I) mediated expression. RIG-I stimulates the expression of IFN- β in response to dsRNA, a hallmark of RNA virus infection. RIG-I contains a C-terminal RNA helicase domain which upon binding of dsRNA, undergoes a conformational change exposing two caspase activation and recruitment domains (CARDs) at its N-terminus. These CARDs subsequently signal to downstream proteins, including MAVS, causing IFN- β transcription [10]. As its name suggests, MAVS localizes to the mitochondria via its C-terminal transmembrane domain and this localization has been shown to be essential for its function [9]. The mitochondrial localization of both MAVS and the PB2 protein of the influenza A virus suggests a possible interaction. Indeed, other viruses have been shown to possess proteins which interact with MAVS, subsequently blocking IFN- β induction. Specifically, it was demonstrated that the hepatitis C virus (HCV) protein NS3/4A localizes to the mitochondria of the host cell whereupon it proteolytically cleaves the MAVS CARD domain from its transmembrane domain. This cleavage dissociates MAVS from the mitochondria, inhibiting MAVS signaling, thus blocking IFN- β induction [8]. Intriguingly, as mentioned previously, it was shown that the PB2 protein from A/WSN/33 stabilizes the mitochondrial membrane potential during influenza virus infection, and this may in turn prevent apoptosis of the infected cell. MAVS has been shown to be anti-apoptotic [9] suggesting that an interaction of the mitochondrial PB2 with MAVS could activate the anti-apoptotic signaling aspects of MAVS and inhibit the induction of IFN- β . However, the PB2 protein could modify apoptosis independently of an interaction with MAVS. In summary, the PB2 proteins of the influenza viruses A/WSN/33 and A/PR/8/34 were shown to localize to the mitochondria of

the host cell and two leucine residues at positions seven and ten of the PB2 protein N-terminus were crucial for localization. This localization seemed to stabilize the mitochondrial membrane potential during infection, and recombinant A/WSN/33 viruses with mutations in the PB2 which prevent mitochondrial localization were slightly attenuated in Madin-Darby bovine kidney (MDBK) cells. Recently, a mitochondrial protein, MAVS, has been identified which activates IFN- β expression and inhibits apoptosis. Taken together, these data suggest a possible role in IFN- β or apoptosis regulation for the PB2 protein. This would be consistent with the role that PB2 protein plays in host range restriction as well as virulence of avian influenza virus H5N1 infections in mammalian animal models [2,3]. A hallmark of highly pathogenic avian H5N1 influenza virus infection in mammals includes increased levels of apoptosis in areas of infection and dysregulation of cytokines [11]. It was also shown that IFN- β was upregulated in cells infected with two H5N1 avian influenza viruses isolated in 1997 [12]. This could suggest that PB2 proteins of avian influenza viruses are unable to regulate the host cell response to infection like human influenza viruses, potentially via mitochondrial association. Our preliminary data supports this speculation as we could not detect mitochondrial localization of PB2 proteins from avian influenza viruses. In conclusion, it will be important to determine whether or not the PB2 protein interacts with MAVS and if so, how this interaction affects MAVS signaling in mammalian cells in the presence of PB2 proteins from highly pathogenic avian influenza viruses.

Acknowledgements

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Nuclear Import and Assembly of the Influenza Virus RNA Polymerase Complex

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Influenza A virus RNA polymerase is a heterotrimeric complex composed of three subunits PB1, PB2 and PA. It catalyzes viral RNA transcription and replication in the nucleus of infected cells. Here we review our recent work on nuclear import and assembly of the newly synthesized RNA polymerase complex. We found that PB1 needs the presence of PA for its efficient nuclear accumulation whereas PB2 alone accumulates in the nucleus efficiently. An *in vitro* reconstitution experiment further showed that an active polymerase complex can be reconstituted *in vitro* between a preformed [PB1-PA] dimer and the PB2 monomer. A host nuclear import factor (RanBP5) was also identified which interacts with the PB1 subunit, facilitating the nuclear import of the [PB1-PA] dimer. Therefore, we proposed a partly cytoplasmic and partly nuclear assembly model: PB1 interacts with PA in the cytoplasm and is transported into the nucleus as a dimeric complex. Final assembly of the polymerase complex occurs between [PB1-PA] and individually transported PB2 in the nucleus.

Introduction

The eight segments of influenza virus negative-sense genome are transcribed and replicated in the cell nucleus by a virus-encoded RNA-dependent RNA polymerase complex [1]. Primary viral RNA transcription (vRNA→mRNA) is catalyzed by viral RNA polymerase complex from incoming virions. Secondary viral RNA transcription (vRNA→mRNA) and replication (vRNA↔cRNA), however, are catalyzed by newly synthesized polymerase. The three subunits of the influenza virus RNA polymerase complex are synthesized in the cytoplasm as independent proteins, and need to be transported into the nucleus and assembled into a trimeric complex during this process. The nuclear import of large proteins or complexes requires specific interaction with nuclear import receptors which direct them across the nuclear membrane [2]. This process is highly selective and energy-dependent. There are nuclear import receptor families, e.g. importin α family, importin β family, which, either alone or cooperatively, are responsible for binding specific nuclear localization sequences (NLS) in their cargos and directing them through the nuclear pore complex. In order to understand the detailed mechanisms of nuclear import and assembly of the influenza virus RNA polymerase complex, we performed intracellular localization [3], *in vitro* reconstitution [4], and protein co-purification studies [5] with individually expressed single polymerase subunits, and co-expressed dimeric complexes in human embryonic kidney

cells (293T). The data from these studies has led us propose a model for the influenza virus RNA polymerase assembly [5].

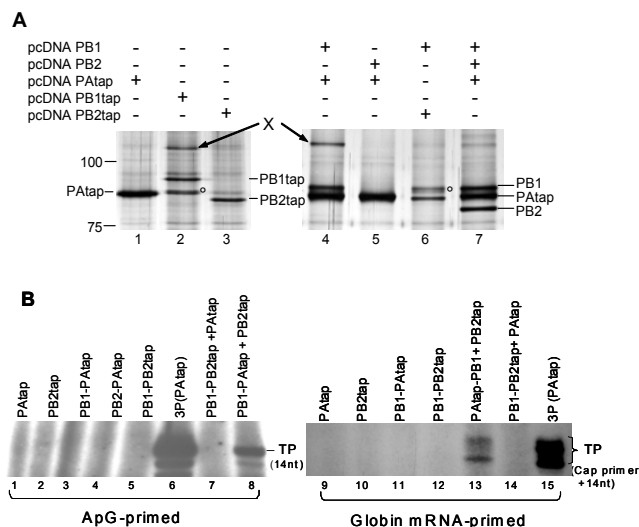
In vivo Localization Studies of Influenza Virus RNA Polymerase Subunits

In order to study the cellular localization and the assembly of the polymerase complex, green fluorescent protein (GFP)-tagged polymerase subunits derived from influenza A/WSN/33 virus were generated [3]. Then the localization of the individually expressed GFP-tagged polymerase subunits and co-expressed [PB1-PA] and [PB1-PB2] dimers were examined by fluorescence microscopy [3]. The results of these experiments showed that individually expressed PB1 and PA subunits were distributed in both the cytoplasm and the nucleus, while the PB2 subunit accumulated in the nucleus. Surprisingly, coexpression of PA with PB1-GFP led to efficient accumulation of PB1-GFP in the nucleus, whereas coexpression of PB2 with PB1-GFP did not alter PB1-GFP localization. In addition, coexpression of PB1 with PA-GFP also resulted in PA-GFP nuclear accumulation. Therefore, these data support the hypothesis that PB1 and PA are imported into the nucleus as a heterodimeric complex; in contrast, PB2 is imported as a monomer [3].

In vitro Assembly of a Functional Influenza Trimeric Polymerase Complex

In order to understand the process of viral RNA polymerase assembly, an *in vitro* reconstitution approach was used [4]. As shown in Figure 1A, partially purified individually expressed PB1, PB2 and PA monomers and co-expressed [PB1-PA], [PB1-PB2] dimers and the 3P trimeric complex using a TAP (tandem affinity purification) tagged subunit were prepared. The reconstitution of an active RNA polymerase complex was tested by mixing these monomers, or dimers with monomers in different combinations in solution. The polymerase activities were assayed by ApG and globin mRNA primed transcription which represent cap-independent and cap-dependent transcription, respectively. Short synthetic RNA oligonucleotides corresponding to the 5' end (15 nt) and 3' end (14 nt) of the wild-type influenza A virus vRNA promoter were used in these assays [4]. Figure 1B showed that, in both ApG-primed and globin mRNA-primed transcription assays, significant transcription was observed when monomeric PB2 was assembled with coexpressed [PB1-PAtap] dimer (lanes 8 and 13). No activity was observed when monomeric PA was assembled with [PB1-PB2tap] dimer (lanes 7 and 14). Furthermore, no transcription activity was detected with any monomers or the two dimeric complexes [PB1-PAtap] and [PB1-PB2tap] (lanes 1-5, 9-12), which confirmed that all three subunits were required for significant transcriptase activity. An assembly of the polymerase by mixing three individually expressed subunits showed no activity (data not shown). Interestingly, these results were entirely consistent with our cellular localization studies which indicated that PB1 and PA were transported into the nucleus as a heterodimeric complex. Thus, we proposed a model in which the trimeric polymerase complex could be assembled sequentially in the

Figure 1. PB1-PA dimers can assemble with PB2 in vitro to produce functional polymerase. (A) Purification of TAP-tagged RNA polymerase subunits from 293T cells transfected with expression plasmids for various combinations of the three polymerase subunits (PB1, PB2 and PA) analyzed by silver staining of 8% SDS-PAGE gel. Sizes of protein standards in kDa are shown on the left. Arrows indicate the position of unknown protein X co-purified with polymerase subunits. The open circles indicate the position of copurified Hsp90. (B) Lanes 1-8: in vitro ApG-primed transcription with all possible combinations of monomers, dimers and 3P complex. Lanes 9-15: in vitro globin mRNA-primed transcription with all possible combinations of monomers, dimers and 3P. The transcription products (TP) are indicated on the right. This figure is adapted from reference 4.



nucleus from a dimeric [PB1-PA] complex and individually transported PB2 [3].

Role of RanBP5 in Nuclear Import and Assembly of the Viral RNA Polymerase Complex.

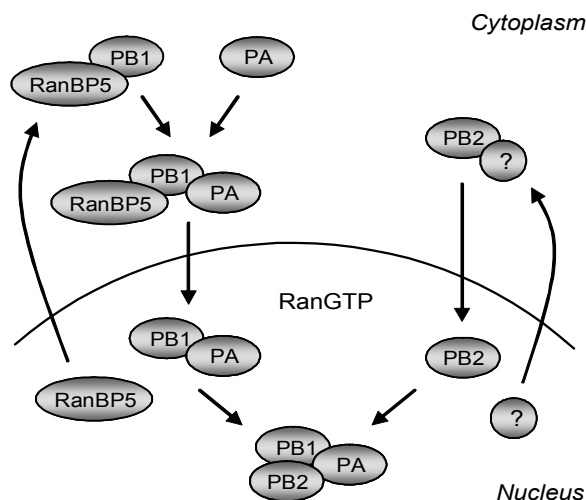
As shown in Figure 1A, band X, migrating at about 120 kDa, was found to be co-purified with PB1tap and [PB1-PA] dimer, but not with PB2tap, PA, [PB1-PB2tap] dimer. To characterize this band, standard in-gel trypsin digestion followed by liquid chromatography (LC) and MS/MS analysis (LC-MS/MS) were performed. The LC-MS/MS analysis identified band X as Ran binding protein 5 (RanBP5), also known as karyopherin β 3, importin β 3 or importin 5. A western blot using a rabbit polyclonal anti-RanBP5 antibody confirmed the identity of RanBP5 [5]. RanBP5 is a member of the importin β family of transport receptors. Authentic nuclear import complexes formed by these nuclear import receptors and their import substrates are typically characterized by their sensitivity to RanGTP. Indeed, in vitro treatment of partially purified [PB1-PA] with RanGTP resulted in RanBP5 release from the [PB1-PA] dimer [5]. We also found that the release of RanBP5 upon incubation with RanGTP facilitated the assembly of PB2 with the [PB1-PA] dimer as indicated by the increased amount of PB2 assembled with the [PB1-PA] dimer, and a corresponding increase of in vitro transcription activity [5]. Therefore, we concluded that association of RanBP5 with [PB1-PA] in the cytoplasm is

required for nuclear import of the dimer, and dissociation of RanBP5 from [PB1-PA] facilitates the assembly between [PB1-PA] and PB2 in the nucleus. The biological significance of the observed interaction between RanBP5 and PB1 was examined by siRNA-mediated knock-down of RanBP5. We observed an inhibitory effect of RanBP5 knock-down resulting in delayed levels of viral RNA at early time points post-infection [5]. This delayed accumulation of viral RNA levels was consistent with the observation of reduced accumulation of [PB1-PA] dimer in the nucleus in RanBP5 knock down cells, as examined by immunofluorescence [5]. These results further supported our assembly model and confirmed that RanBP5 plays a role in the viral life cycle.

Discussion

We used various in vivo and in vitro approaches to study nuclear import and assembly of the influenza virus RNA polymerase. The data from these studies give rise to the following model (Figure 2): PB1 and PA form a dimer in the cytoplasm where a host import factor, RanBP5, specifically interacts with the PB1 subunit, forming a nuclear import complex with [PB1-PA]. After the [PB1-PA] dimer has been transported through the nuclear pore into the nucleus, the high RanGTP concentration in the nucleus triggers release of RanBP5 from the [PB1-PA] dimer, which then facilitates the assembly of [PB1-PA] with individually transported PB2 in the nucleus.

Figure 2. Model for the transport and assembly of the influenza virus RNA polymerase complex (see text for details). This figure is from reference 5 published in *Journal of Virology*.



We still do not know which host import factor is responsible for PB2 nuclear import. Interestingly, Tarendeau et al. (2007) have reported a co-crystal structure of the C-terminal domain of PB2 with importin α 5 (karyopherin α 1), suggesting that importin α 5 could be a potential host import receptor involved in PB2 nuclear transport [6]. However, direct biological evidence is still needed to confirm this. Although our in vivo and in vitro data

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support our assembly model, we cannot exclude other assembly pathways for newly synthesized viral RNA polymerase that may operate in infected cells. Recently, Naito et al. (2007) reported that Hsp90 is involved in the assembly and nuclear transport of viral RNA polymerase subunits which led them to propose an alternative polymerase assembly pathway, in addition to the pathway proposed here [7]. However, it is likely that the model proposed here represents a major assembly pathway.

Acknowledgements

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Proceedings Topic #5

**Developments in
Diagnostic and Serologic
Techniques**

**Oral
Presentations**

Measurement of Neutralizing Antibody Responses to Influenza H5N1 Clade 1 A/Viet Nam/1194/04 And Clade 2 A/Indonesia/5/05 Hemagglutinin Using a Sensitive High Throughput Luciferase-Based Retroviral Pseudotype Assay

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We have used firefly luciferase-based retroviral pseudotypes bearing influenza H5 clade 1 and 2 hemagglutinins (HA) as safe, surrogate viruses for influenza H5N1 cross-clade neutralization assays which can be carried out at Biosafety Level 2. Using this assay, sera from patients who had recovered from infection with influenza H5N1 during the Viet Nam outbreaks in 2004 and 2005 tested positive for the presence of neutralizing antibodies to A/Viet Nam/1194/04 (Titres 1:800-1:6400) and A/Indonesia/5/05 HA (Titres 1:200-1:800). The pseudotype neutralization assay is a sensitive, low-containment assay, which can be applied in a high-throughput format for human and avian surveillance and for the evaluation of pre-pandemic and avian vaccines. It is readily adaptable to newly emerging HA antigens and therefore highly suitable for the assessment of cross-clade neutralizing antibody responses.

Introduction

Human cases of infection with avian influenza H5N1 virus were first observed during large scale poultry outbreaks in Hong Kong in 1997. Since its re-emergence in Asia in 2003, 328 laboratory-confirmed human H5N1 cases have been reported from Asia, Europe and Africa of whom 200 have died (WHO, 10th September 2007). We have previously constructed SARS-CoV and other coronavirus pseudotypes to study virus neutralization and cell entry [1, 2]. We have recently described the development of a safe, sensitive pseudotype assay for influenza H5N1 neutralizing antibodies elicited after natural infection or by immunization and shown that results obtained using this assay correlate strongly with those obtained using hemagglutinin inhibition (HI) and microneutralization (MN) assays [3]. We describe the assembly of luciferase-based retroviral pseudotypes bearing the HA from influenza H5N1 clade 1 A/Viet Nam/1194/04 and clade 2 A/Indonesia/5/05 in order to study cross-neutralization between these two antigenic clades in a novel assay.

Materials and Methods

Plasmids and Cell Lines. Plasmid pl.18/VN1194 HA has been described previously [3]. Plasmid pl.18/Indo5 HA was

constructed at Novartis Vaccines using Indo5 HA ORF DNA synthesized by GeneArt. The full length HA ORFs from A/Vietnam/1194/04 and A/Indonesia/5/05 (with the cleavage site sequence changed from RESRRKKR to RERRRKKR analogous to VN1194) were amplified by PCR and cloned into the expression vector pl.18. This backbone plasmid is a pUC-based plasmid incorporating promoter and Intron A elements from human cytomegalovirus. The MLV gag/pol construct has been described previously [4]. The firefly luciferase (Luc) reporter construct MLV-Luc has been described [5]. Human 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax and high glucose (Gibco, Paisley, Scotland, UK), supplemented with 15% fetal calf serum and penicillin/streptomycin.

Viral Vector Production and Infection of Target Cells. Confluent tissue culture plates of 293T cells were split 1:4 24hr prior to plasmid transfection. Each plate of 293T cells was transfected with 1 µg gag/pol construct, 1.5 µg of Luc reporter construct, and 1.5 µg HA-expressing construct by using the Fugene-6 transfection reagent [Figure 1]. At 24 h post-transfection, 1 U of exogenous bacterial neuraminidase (Sigma) was added to induce the release of HA-pseudotyped particles from the surface of the producer cells[6]. Alternatively, if NA is also required on the surface of retroviral pseudotypes, an NA-expressing plasmid may be added to the original transfection, obviating the need for the addition of exogenous NA [Figure 1]. Supernatant was harvested 48 h and 72 h post-transfection, filtered through 0.45-µm filters, and stored at -80°C. MLV(HA) vector titers were measured on human 293T cells and presented as relative light units (RLU) per milliliter.

Sera. From 5 patients who had recovered from influenza H5N1 during the 2004 and 2005 outbreaks in Viet Nam, heparin-anticoagulated plasma was obtained 8-27 weeks after illness onset (median 11 weeks) [3].

MLV(HA) Pseudotype Neutralization Assay. 2µl plasma samples were heat inactivated at 56°C for 30 min, 2-fold serially diluted in culture medium, and mixed with MLV(HA) virions (10000 RLU firefly luciferase) at a 1:1 vol/vol ratio. After incubation at 37°C for 1hr, 1 x 10⁴ 293T cells were added to each well of a 96 well flat-bottomed white luminometer plate. Relative light units (RLU) of firefly luciferase were evaluated 48h later by iminometry using the Bright-Glo system (Promega) according to the manufacturer's instructions. IC₉₀ neutralizing antibody titres were determined as the highest serum dilution resulting in a 90% reduction of infection (as measured by luciferase marker gene transfer) compared to a pseudotype virus only control. Titres <100 are designated negative.

Results

Production of retroviral particles pseudotyped with influenza H5N1 A/Vietnam/1194/04 and A/Indonesia/5/05 HA. Retroviral (MLV) vector particles pseudotyped with Influenza A/Viet Nam/1194/2004 and A/Indonesia/5/05 HA were assembled by co-transfection of the respective HA-expressing plasmid, pl.18-HA, with plasmids encoding MLV gag-pol and firefly luciferase

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vector genome in 293T cells (Figure 1). The infectivity titre of MLV(Viet Nam HA)-Luc and MLV(Indonesia HA)-Luc on 293T cells was 3.5×10^5 and 1.5×10^6 RLU/ml respectively.

Measurement of neutralizing antibodies against A/Viet Nam/1194/2004 and A/Indonesia/5/05 HA in sera from recovered patients. Sera from 5 patients who had recovered from infection during H5N1 outbreaks in Viet Nam in early 2004 (3 patients, VN1-3) and 2005 (2 patients, VN4-5) were evaluated for the presence of neutralizing antibodies using the MLV(Viet Nam HA) and MLV(Indonesia HA) pseudotype assays. A broad range of IC_{90} neutralizing antibody titres was observed in these sera (1:800-1:6400 against Viet Nam HA and 1:200 to 1:800 against Indonesia HA) (Figure 2).

Discussion

We have developed a low-containment retroviral pseudotype-based assay that facilitates the accurate determination of neutralizing antibody responses to influenza H5N1 clade 1 and 2 viruses. Our assay detected neutralizing antibodies to H5 HA in sera from patients who had recovered from infection with influenza H5N1 during the 2004 and 2005 outbreaks in Viet Nam. In order to achieve maximum sensitivity in influenza H5N1 serological assays, the selection of virus isolated from the same outbreak, or the use of an antigenically equivalent strain is required for optimal antigenic match. Our pseudotype assay is readily adaptable to newly emerging HA (and NA) glycoproteins. This involves direct RT-PCR from influenza viral RNA and subcloning into the expression plasmid pl.18 (or other suitable expression plasmid) followed by production of new viral stocks for use in further assays. Recent studies have shown that there is often poor or negligible antigenic cross-reactivity between H5N1 influenza viruses belonging to the Indonesian (clade 2) and Vietnamese (clade 1) sublineages, an observation that has implications for the design and production of pandemic vaccines against H5N1[7, 8]. Using our sensitive pseudotype assay we have shown that sera from patients who had recovered from infection with influenza H5N1 during the Viet Nam outbreaks in 2004 and 2005 were able to cross-neutralize MLV(Indonesia HA) but with significantly lower titers (Figure 2). These data suggest that the MLV(HA) assay using antigenically distinct HA glycoproteins may be ideally suited for the detection of cross-neutralizing activity. This assay needs to be further evaluated by the screening of large panels of sera from immunized subjects and/or animals (ferrets/birds), against pseudotyped H5 viruses belonging to the other circulating H5N1 antigenic clades.

Acknowledgements

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Figure 1. MLV(HA) pseudotype construction by 3 plasmid transfection (+exogenous NA) or 4 plasmid transfection using paired HA and NA expressing plasmids. CMV = cytomegalovirus promoter, PCS = polybasic cleavage site, LTR = long terminal repeat, HA = hemagglutinin, NA = neuraminidase, Luc = luciferase, ψ = packaging signal.

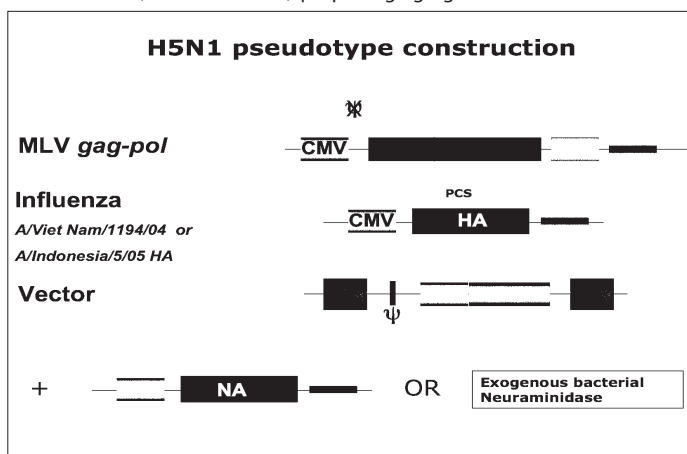
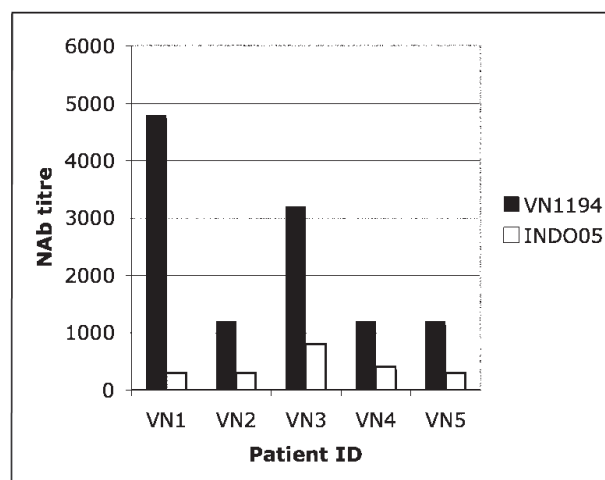


Figure 2. Neutralizing antibody (NAb) titres against A/Viet Nam/1194/04 (black vertical bars) and A/Indonesia/5/05 (grey vertical bars) HA. Patient ID = sample number.



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Use of Reverse Transcription Loop-Mediated Isothermal Amplification for Molecular Detection of Highly Pathogenic H5N1 Influenza A Viruses

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Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a simple and rapid diagnostic method that employs the amplification of target nucleic acids in an isothermal reaction. Here we report that RT-LAMP has a comparable detection limit to highly optimized RT-PCR methods. The assay was validated against all the HA subtypes (H1-H16). This included clinical specimens from a H5N1 infected patient and a panel of genetically diverse H5N1 viruses that have been isolated over the past 10 years. Clinical samples from H5N1 infected culture without viral purification also generated positive results. In this paper we discuss how the features of the RT-LAMP assay make it a potentially useful onsite diagnostic tool. Advantages such as visual detection of a positive result, low start-up costs and a single closed tube application to minimize contamination make it a useful option in resource-limited situations.

Introduction

The threat of a human pandemic emerging from a highly pathogenic H5N1 derived virus has made it eminently important for effectual surveillance and diagnostics. Highly pathogenic avian H5N1 is a virus that was originally confined to Asia, but since 2005 has spread through the Middle East to Western Europe and Western Africa. The dispersal has stemmed from migratory birds or the legal or illegal importation of animal carriers [1]. The occasional transmission of H5N1 to humans has had a significant global effect, in human healthcare, the poultry industry and several other sectors. In particular, H5N1 has resulted in the death and mass culling of millions of poultry [2]. Avian influenza has become prevalent in less economically developed countries, where the resources needed for prevention and control are not efficiently expedited. Large gaps in the global surveillance system can be introduced as a result of discrepancies in resources and expertise. As H5N1 continues to spread and infect poultry and humans, appropriate diagnostic systems must be implemented for effective containment. Presently, there are several diagnostic techniques available and they can be categorized into serological or molecular tests [3]. Molecular tests are preferred since they can provide information about subtype rapidly. Tests such as real time RT-PCR, RT-PCR and nucleic acid sequence based amplification (NASBA) can be used to confirm the presence of H5N1 within a few hours. The drawback of these methods, particularly with real-time RT-PCR is that the

start-up cost and maintenance are expensive. Therefore, despite superior sensitivity and immediate analysis of results, these systems cannot be easily introduced to a rural farm or provincial hospital. The RT-LAMP assay is a DNA amplification method where strand displacement and amplification take place under isothermal conditions [4]. 6 different regions of DNA are targeted for this process to ensure high specificity. Positive reactions generate a large amount of magnesium pyrophosphate precipitate enabling visual inspection of the results [5]. In this paper we demonstrate that the RT-LAMP can detect H5N1 from various samples, including unpurified viral culture and H5N1 spiked fecal samples.

Methods

Viruses and Samples. A heat-inactivated A/Vietnam/1203/2004 H5N1 virus was used for, and the chicken fecal swabs were taken from a Hong Kong live market.

RT-LAMP. RT-LAMP primers were designed to target the 5' end of the HA2 encoding sequence of the highly pathogenic H5N1 viruses. This region was found to be highly conserved after the alignment with 596 highly pathogenic H5N1. Furthermore, results were validated with a panel of genetically diverse highly pathogenic H5N1 viruses that have been isolated over the last 10 years as well as clinical samples. The assay was also tested against all other HA subtypes, H1-H16, for confirmation of specificity to H5N1 [6]. In a typical RT-LAMP reaction, 2 µl of RNA or heat-treated fecal sample was mixed with 2x reaction buffer (40 mmol/LM of Tris-HCl in pH 8.8; 20 mmol/L of KCl, 16 mmol/L of MgSO₄; 20 mmol/LM (NH₄)₂SO₄; 0.2% Tween 20 (v/v); 1.6 mol/L of betaine; 2.8 mmol/L each dNTPs), 50U *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA), 8U avian myeloblastosis virus reverse transcriptase (Invitrogen, Gaithersburg, MD, USA), 40 pmol/L primers FIP and BIP, 20 pmol/L primers LPF and LPR, and 5 pmol/L of primers F3 and B3. Purified viral RNA used for sample input was prepared using QIAamp® Viral RNA Mini Kit (QIAGEN). Fecal swabs were boiled at 99°C and centrifuged; supernatant spiked with purified virus was applied to the reaction. Reaction mixtures were incubated at 60°C for 120 min in a real-time turbidity meter (LA-200, Treamecs; Kyoto, Japan). The RT-LAMP primers used were F3c: 5'-TAT AGA GGG RGG ATG GCA-3'; B3 5'-CCG TCT TCC ATY TTY TTG TT-3'; F1P 5'- TCT TTG TCT GCA GCG TAY CCT TTT GGG AAT GGT AGA TGG TTG G-3'; 5'- B1P ATG GAG TCA CCA ATA AGG TCA ACT TTT TCT AAG TTR TTA AAT TCC CTT CCA AC-3'; LPF 5'- GCT CRT TGC TAT GGT GGT A -3'; LPB 5'- CAA AAT GAA CAC TCA GTT TGA -3'. RT PCR. One-step RT PCR was performed as described by the World Health Organization (H5-1: 5'-GCC ATT CCA CAA CAT ACA CCC-3'; H5-3: 5'-CTC CCC TGC TCA TTG CTA TG-3') [7].

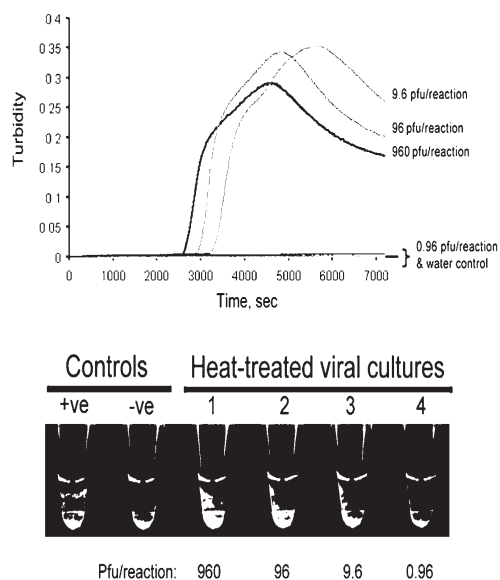
Results

RT-LAMP had a comparable sensitivity to the optimized method of RT-PCR as recommended by the World Health Organization (data not shown, see reference 6). When applied with serial dilutions of A/Vietnam/1203/2004, both had a

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detection limit of 2×10^{-3} pfu/reaction. These samples were also tested against a panel of HA subtypes (H1-H16); all were negative with the exception of the H5N1 strains (data not shown, see reference 6). To account for the genetic diversity of H5N1, 14 phylogenetically distinct strains were selected. Samples that were collected in 1997 had a positive result as did the recent Clade 2 samples Influenza A/chicken/Wajo/BBVM/2005, A/duck/Vietnam/568/2005 and A/bar-headed goose/Qinghai/5/2005 (ref 6). This indicates the assay is applicable to a range of H5N1 viruses, and reassortants with the HA from these lineages are expected to have positive results. Primers were aligned with H5 viruses of North American lineage (H5N2) and when compared with the H5N1 viruses, there were extensive mismatches. We tested the assay on clinical specimens from patients infected with H5N1. RNA purified from 2 different post-mortem lung tissue samples of a patient infected with A/HK/212/03 was positive in the LAMP assay (ref 6). In previous studies it was demonstrated that heat-treated blood samples from *P.falciparum* could be used directly in the LAMP assay [8]. To test if nucleic acid purification was necessary for H5N1 detection, viral culture was used directly as input for the assay. It was found that the LAMP reaction was resistant to PCR inhibitors in cell culture supernatant. A positive result was generated although at a lower sensitivity of 9.6 pfu/reaction (Figure 1). Figure 1 also demonstrates the obvious difference in appearance between positive and negative results, as identified by levels of turbidity.

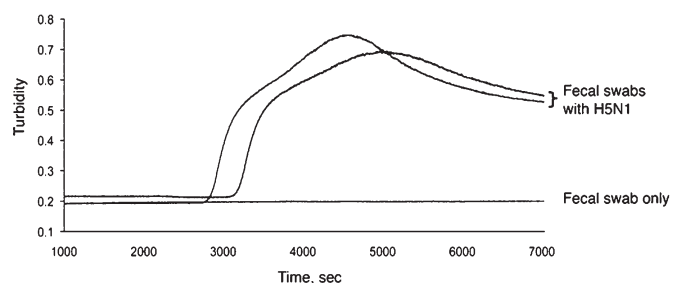
Figure 1. Detection of H5N1 by RT-LAMP using heat-treated culture supernatant. Pictures are modified from [6, 8].



Clinical samples are collected as fecal, cloacal, tracheal and blood samples. By negating the need for nucleic acid

purification, time and cost for the entire process can be reduced. To test the feasibility of the RT-LAMP assay as a field test, fecal swabs spiked with purified H5N1 virus were tested. Fecal samples were boiled and centrifuged and purified A/Vietnam/1203/04 virus was added to each reaction. These samples were found to be positive as shown in Figure 2.

Figure 2. Detection of H5N1 by RT-LAMP using fecal swabs spiked with H5N1.



Discussion

LAMP provides an option that is relatively inexpensive when compared to RT-PCR, sophisticated equipment is not required, only a water bath or incubator. The single closed tube application also reduces the possibility of contamination. Another benefit to the LAMP assay is that positive reactions generate a precipitate that can be confirmed by visual inspection, therefore downstream analysis such as gel electrophoresis is not needed. The use of ethidium bromide for visual detection of DNA in normal RT-PCR requires implementation of safe disposal due to the risk imposed. Therefore RT-LAMP can provide an option for preliminary diagnosis where laboratory facilities are not available, such as on the field, on a poultry farm or in rural clinics. The increasing incidence of H5N1 calls for improved surveillance and diagnostics and in many situations cost effectiveness is critical. By investigating alternative diagnostic techniques such as RT-LAMP, we can improve global surveillance of H5N1.

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Pandemic Influenza Preparedness: New Molecular Tools for Evaluation of Influenza Vaccines and Identification of Serological Epitopes for Avian Influenza Diagnostic Assays

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Introduction

Vaccination remains the best way to protect humans against influenza. Annual human epidemics (caused by influenza type A or type B viruses) are manifested as highly infectious acute respiratory disease with high morbidity and significant mortality. Presently, vaccination is accomplished with commercially available, trivalent, chemically inactivated (TIV) or live attenuated cold adapted (LAIV) influenza virus vaccines. Current trivalent inactivated influenza vaccines (TIV) provide limited protection against seasonal drift variants. The efficacies of the two types of vaccine have been reported to be comparable. However, about 30% of adults and 40 to 70% of the elderly (one of the high-risk groups) do not respond well to vaccination. Similarly, the response rate in the very young after TIV is much lower than in adults and requires two vaccinations. Therefore, improvement of influenza vaccine immunogenicity is of paramount importance. Of even greater concern is the recent spread of avian influenza viruses (AIV) among domestic poultry and transmission to humans that can result in a global pandemic. The H5N1 inactivated vaccine based on this viral seed has been tested in human trials and the first product was submitted for licensure recently. However, to date the immune response to this first generation H5N1 vaccine is significantly lower than those observed with the seasonal human influenza vaccines. Concerted efforts are underway to improve seasonal influenza vaccines and to generate stockpiles of effective pre-pandemic vaccines against AIVs in order to increase rates of immune response in all high-risk populations, and for broad heterosubtypic protection. At this juncture, the field is suffering from limited availability of analytical assays for qualitative and quantitative evaluation of the antibody responses elicited by the new or improved influenza vaccine candidates. If the adjuvanted or novel vaccines generate higher antibody titers in the elderly and younger populations and provide dose-sparing advantage, it will be important to determine if the antibodies generated are against the same or expanded regions of the HA1/HA2/NA. Most importantly, if broader cross-neutralization is observed *in vitro* using the currently available haemagglutination inhibition (HI) and micro-neutralization assays, or in small animal protection models, it will be very

important to map the sites recognized by the heterosubtypic neutralizing antibodies. Such information will help to generate new criteria for selection of the best candidate vaccines to move into phase III trials and for new potency assays. The new data might ultimately lead to construction of new subunit vaccines capable of eliciting broadly neutralizing antibodies against avian H5N1 subtypes, and against drifted seasonal human influenza strains. Another concern: as new avian influenza vaccines enter clinical trials around the world and get approved for general vaccination, it will be essential to differentiate between seroconversion due to the vaccines and true infections. A rapid and simple serodiagnostic test could allow the detection of new avian influenza outbreaks among previously vaccinated individuals, through the use of immunodominant epitopes in one or more internal proteins which are different or absent from the vaccine. In parallel with the intensive vaccine development efforts, there is an urgent need to improve the analytical tools for comparing immune responses generated by different vaccine candidates against both human and avian influenza viruses. To address this need we have constructed gene-fragment-phage display libraries (GFPDL) using the whole Influenza genome displaying peptides derived from the all the open reading frames of two human influenza (H1N1, H3N2) and three avian influenza (H5N1- Vietnam and Indonesia strains, H7N7) viruses. These GFPDL-libraries have been used for: a) screening and mapping of antibodies generated by new or improved (adjuvanted) licensed influenza vaccines; b) for analyses of convalescent sera from avian influenza and human influenza exposed individuals. This has led to elucidation of B-cell epitope profile following vaccination and infection as well as identification of conserved sequences involved in heterosubtypic protection, for incorporation into future vaccines.

Materials and Methods

We generated whole-genome-phage display libraries expressing all the open reading frames of two human influenza (H1N1, H3N2) and three avian influenza (H7N7 and two H5N1 clades) viruses using the method as described previously [1]. Each of these libraries contain 10^9 - 10^{10} phages, expressing influenza derived sequences ranging between 30-400 aa as fusion proteins with the bacteriophage pIII coat protein. These FLU-libraries were used to map antibodies generated by new seasonal influenza vaccines using affinity selection as described previously [1]. They are also being analyzed with convalescent sera from individuals exposed to human influenza, and for mapping the epitopes of broadly neutralizing H5N1 monoclonal antibodies.

Results

[A] *Epitope profile recognized following vaccination and infection with seasonal influenza.* Epitope mapping was performed with panels of sera after seasonal trivalent influenza vaccination using either the subunit inactivated (TIV) or the cold-adapted live attenuated (LAIV) vaccines and post- infection during the

Table 1. Compilation of epitope profiles of antibodies generated following vaccination and H3N2 infection using H3N2-GFPD-library.

GENE	ID	Functional Domain	NOT-PROTECTED	PROTECTED	POST- INFECTION
PA	1	HL S 1; Domain 2	0	25	0
	2	Domain 2	3	9	0
HA	3	Fusion peptide (FP) - F'	93	203	95
	4	FP - F' (Ag1); Esterase	0	18	0
	5	Receptor Binding	1	42	58
	6	HA2 Ectodomain	0	8	5
NP	7	Non-contiguous body domain 1	2	7	0
	8	Non-contiguous body domain 1	0	2	0
NA	9	Stalk structure; Globular head	0	2	0
	10	Globular head - Antigenic site	0	43	0
M1	12	MLS; NS2 binding	75	68	0
	13	NS2 binding domain	0	0	21
	14	C-terminal domain	0	40	113
NS1	15	RMA-binding/dimerization	0	0	28

NOTES-

The numbers in the table refer to frequency of the phage clones obtained during affinity selection using the respective serum sample.

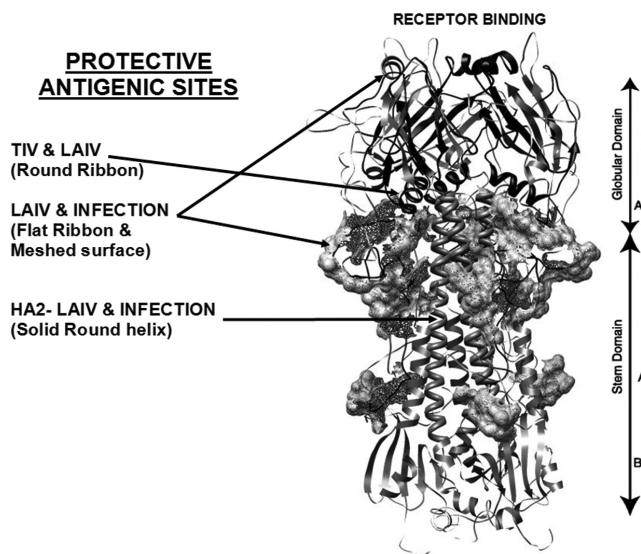
Rectangle- Sequences that could be potentially protective epitopes

Oval-Sequences that could be important for differential diagnosis of Influenza infection in the face of vaccine generated antibodies.

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2004-2005 season. Only H3N2 infections (A/California/7/2004 strain) were recorded. Post vaccination and post infection antibodies were found to target epitopes in PA, HA, NP, NA, M and NS proteins Table 1. In general, a more diverse epitope profile was generated following LAIV vaccination compared with Subunit vaccination. Protection did not always correlate with HAI titres in trial participants [2]. Comparisons between unprotected and protected H3N2 exposed individuals led to the identification of three potentially protective epitopes in HA: 1) HA fusion peptide that was elicited by both vaccination approaches; 2) HA receptor binding domain that was generated by both vaccinations and following infection; 3) HA2 ectodomain which was elicited by live-attenuated vaccination & infection only. The identified HA epitopes predictive of protection were located in previously described neutralizing regions A, B, C and E, which were designated primarily based on the sites recognized by human influenza HA-specific neutralizing monoclonal antibodies (Figure 1). Importantly, in sera from LAIV-vaccinated (protected) individuals we identified a novel highly conserved epitope located in HA2 ectodomain and an epitope in NA that was mapped to the neuraminidase catalytic site. These highly conserved epitopes could be used to design better vaccines which may provide broader cross-protection against circulating seasonal influenza strains. Comparison of the post-vaccination and post-infection reactivity profiles identified sequences in M1 and NS proteins that were only recognized by sera from infected individuals and not in either of the vaccination regimens. The discovery of such epitopes lends support to the idea that similar approach could be used for the development of a differential sero-diagnostic assay in populations that were previously vaccinated with seasonal or avian influenza vaccines.

Figure 1. Antigenic structure of haemagglutinin (HA0) identified following vaccination or infection.



Sauter et al. Biochemistry v31 pp.9609-9621.

[B] *Epitope mapping of broadly neutralizing H5N1 human monoclonal antibodies.* Epitope mapping was performed on four human monoclonal antibodies that were generated from individuals who recovered from infection with H5N1 Avian Influenza in Vietnam. These monoclonal antibodies showed a broad range of cross-protection against H5N1 isolates both in-vitro and in-vivo [3]. Our data suggested that these antibodies recognize large conformational structures on the H5N1 HA1, located mostly in the receptor-binding domain. The critical residues required for binding of MAbs, FLA 5.10 and FLD21.140 were identified using a random peptide phage display library (RPL). The corresponding chemically synthesized peptides sequences bound the respective H5 MAbs with high levels of specificity. Importantly, the critical contact residues recognized by MAbs FLA 5.10 v/s FLD21.140 could explain the differences in their breadth of cross-protection against clade 1 and clade 2 (A/Indonesia/5/05) viruses. One of the critical residues recognized by the FLA5.10 (clade 1- protection) in the Vietnam-H5-HA is substituted Leu>Ser in Indonesia-H5-HA. In contrast, the FLD 21.140 (clades 1&2 protection) recognizes a highly conserved sequence at the base of the H5N1 HA structure.

Discussion

The data using FLU-GFPD libraries with serum samples from a seasonal trivalent-vaccine trial shows the promise of this approach in identifying novel antibody epitopes elicited by vaccination or infection. More diverse epitope profile was observed following vaccination with live-attenuated cold adapted virus (LAIV) compared with inactivated vaccine (TIV). Antigenic mapping of HA1 and HA2-specific antibodies after vaccination and/or infection may predict protective epitopes for the seasonal and avian influenza. Sequences recognized by broadly neutralizing antibodies could help determine critical residues and contact spatial conformations. These epitopes will be used in the development of new assays to evaluate vaccine responses and potency. Hopefully, this approach will identify conserved sequences involved in broad cross- protection that could be incorporated into future pre-pandemic vaccines. Furthermore, unique epitopes recognized by convalescent individuals may be useful for the development of new serodiagnostic assays to monitor future outbreaks of avian influenza.

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A Semi-Automated and Cost Effective Workflow for High Throughput Avian Influenza Detection by Quantitative RT-PCR

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Introduction

Since 2003, avian influenza A (AI) H5N1 has spread in many countries, killing hundreds of people and millions of fowl. The USDA has adopted qRT-PCR using a single tube format for AI detection after RNA extraction. However the single tube format cannot handle the sheer number of samples that need to be processed during an epidemic. Here we present a semi-automated solution for high throughput sample processing and AI detection.

Method

RNA isolation was performed on a KingFisher® magnetic particle processor (Thermo Scientific) using the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambion). A Biomek® FX Laboratory Automation Workstation (Beckman Coulter) was used to assemble reagent plates. 7900HT Real-Time PCR System (Applied Biosystems) was used for detection in the 384-well format. This semi-automated approach drastically reduces cost (<\$200,000) for setting up a high throughput testing facility. RNA isolation takes just 15 minutes, detection requires 2 hours, and over 1500 samples can be processed within a single day.

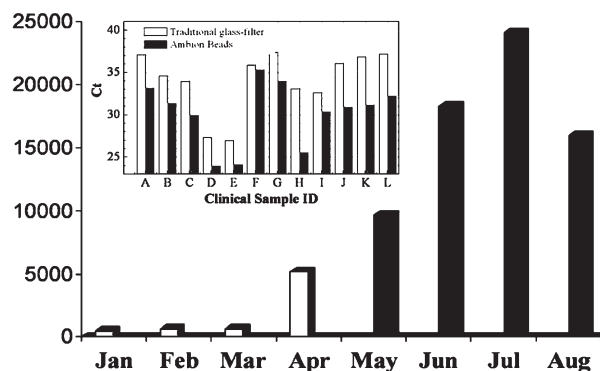
Results and Discussion

MagMAX Technology Performs More Consistently than Filter-Based Methods and Successfully Recovers Both RNA and DNA. Our data indicate that magnetic bead-based nucleic acid isolation is a very efficient and effective method to consistently isolate RNA and DNA from various biological samples. This technology eliminates common problems associated with filter-based methods including filter clogging and inconsistent recovery. It also effectively removes inhibitors that are often seen in environmental samples. Moreover, small volume elution (20 µL) provides highly concentrated nucleic acid samples that are suitable for various downstream applications. The standard MagMAX-96 Viral RNA Isolation kit includes a Lysis/Binding Enhancer to effectively lyse many pathogens to release nucleic acid, and thus has been widely used for nucleic acid isolation for the detection of both DNA pathogens and RNA pathogens. The standard kit supplies plenty wash solutions to result in very pure nucleic acids. Influenza virus is easily lysed with GuSCN-containing lysis solution, thus a simple version, MagMAX-96 AI/ND Viral RNA Isolation Kit, is created to further reduce the sample process time.

MagMAX Empowered California Animal Health and Food Safety

(CAHFS) Laboratory to combat 2002/2003 END Outbreak. The MagMAX kit was successfully employed for high throughput viral RNA isolation from swab samples for Exotic Newcastle Disease (END) detection during the 2002/2003 END outbreak in California. More than 100,000 swab samples were processed during the outbreak. Figure 1 shows that use of the MagMAX technology drastically increased throughput and enabled highly sensitive and specific detection of END. Figure 1 inset demonstrates that the MagMAX technology outperformed the glass fiber filter method. It was adopted by USDA/NVSL as the method of choice for viral RNA isolation for AI and ND detection.

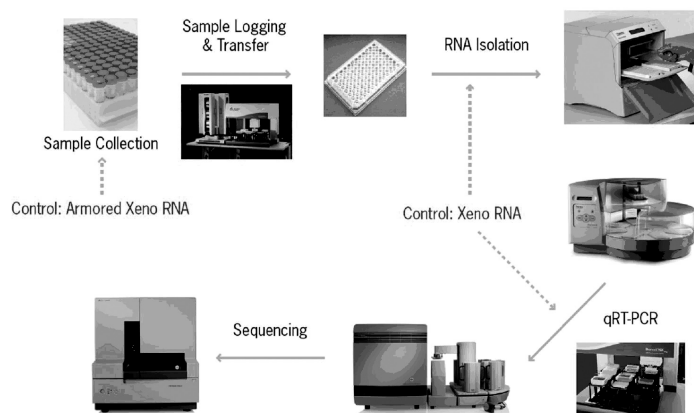
Figure 1. Samples analyzed by qRT-PCR at CAHFS laboratory during 2002/2003 end outbreak. The MagMAX™ kit protocol was introduced in April 2003. Among 78,549 documented samples, 298 of 300 virus-isolation positive samples were detected positive by RNA isolation followed with qRT-PCR, equivalent to diagnostic sensitivity of 99.33% and diagnostic specificity of 99.997%. Inset. MagMAX™ kit achieves 10X greater sensitivity than competitor's kit. Clinical tracheal and cloacal swab samples were collected from poultry infected with exotic Newcastle disease (END). RNA was isolated from 50 µL of sample using the MagMAX kit protocol or 500 µL of sample using the previously NVSL-validated protocol. The results demonstrated that when starting with 10-fold less sample, viral RNA detection using the MagMAX kit isolated RNA was as sensitive or more sensitive than the competitor's kit.



AgPath-ID™ AIV-M Reagent Kit Makes AIV Detection Easier. We have also developed a one-step qRT-PCR kit for AI detection that targets the AI matrix gene using the USDA primer/probe set. An artificial RNA is included for monitoring inhibition that is sometimes seen in wild bird fecal samples. The kit supplies master mixes to make it easier to set up reactions. The use of a complete kit instead of reagents made individually in the laboratory, ensures consistency of testing results and makes it possible to compare data obtained from different laboratories. The Core qRT-PCR master mix can also be used for effective H5/H7 detection. Protocols have been developed on general 96-well format real-time machines, such as ABI 7500 and 7900. We continue to develop ways to make our products easier to use and more effective in the hands of people who are not trained in traditional molecular biology techniques. We have new products in our development pipeline that will deliver all

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Figure 2. Semi-automated solution using: a) Biomek FX laboratory automation workstation to transfer samples into 96-well plates, and set up MagMAX reagent plates; b) KingFisher 96 magnetic particle processor to perform the MagMAX protocol for viral RNA isolation; c) Biomek FX laboratory automation workstation to set up the qRT-PCR reaction plates and transfer to a 7900HT Real time machine for AIV detection. With this approach, RNA isolation requires 15 minutes, and detection requires 2 hours. Over 1500 samples can be processed in one day with capital equipment investment of <\$200,000.



of the necessary assay reagents in a single lyophilized bead format. This format reduces the number of manual steps to run a test and improves the reliability of results. We have many collaborators around the world who have tested this new format and are extremely excited about the performance and reliability of these new kits.

Automation from Viral RNA Isolation to AIV Detection. The MagMAX viral RNA isolation method followed by AgPath-ID AIV detection can be fully automated with a sophisticated liquid handler such as Biomek® FX Laboratory Automation Workstation (Beckman Coulter) and Freedom EVO® (Tecan) integrated with a swing arm. However, such fully automated systems are very expensive and have a long turnaround time.

Acknowledgements

We thank USDA/NVSL, SEPRL, and CAHFS for their help in assay design and evaluation.

Proceedings Topic #6

Animal Influenza Ecology

Oral

Presentations

Susceptibility of Sparrows, Starlings and Pigeons to Highly Pathogenic H5N1 Avian Influenza Virus

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European starlings, house sparrows and Carneux pigeons were inoculated with highly pathogenic H5N1 influenza A viruses isolated in 2005 and 2006. Viral replication, mortality and transmission to uninfected contact birds following experimental inoculation were monitored. Sparrows were susceptible to severe infection, with 66-100% of birds dying within 4-7 days. High levels of virus were detected in oral-pharyngeal and cloacal swabs and in organs of deceased sparrows. Inoculation of starlings caused no mortality, despite high levels of virus shedding evident in oral-pharyngeal swabs. Least susceptible were pigeons, which showed no mortality and very low levels of virus in both oral-pharyngeal and cloacal swabs. Transmission to contact birds was detected only once in starlings. In summary, recent H5N1 viruses are pathogenic for small terrestrial bird species and the rate of intra-species transmission in these hosts is very low.

Introduction

Highly pathogenic avian H5N1 influenza viruses were identified in Southeast Asia in 1996, and have spread in recent years across broad regions of Eurasia and Africa. These viruses have shown high lethality in chickens and other poultry species [1-3]. Outbreaks of H5N1 and other avian influenza subtypes have caused massive losses to commercial poultry flocks in recent years [4]. Direct transmission of H5N1 virus from infected poultry is thought to be responsible for virtually all of the human H5N1 infections since 1997. Due to the burden of H5N1 influenza on human health and agriculture, and its potential to mutate and cause a global pandemic, epidemiological studies of the viruses' host range and their means of dispersal are urgently needed [5]. Highly pathogenic poultry isolates from the 1997 and 2001 H5N1 outbreaks typically cause few disease signs in experimentally infected ducks [6,7]. These viruses' low pathogenicity in waterfowl presumably facilitated efficient carriage to the highly susceptible hosts. Some H5N1 strains isolated during subsequent outbreaks are highly pathogenic in waterfowl [7,8], and some are shed by infected ducks for prolonged periods of time [9]. Together with the commercial transportation of poultry and poultry products, migratory waterfowl are likely to have played a role in the wide dispersal of highly pathogenic H5N1 viruses.

Land-based wild bird populations may also be vulnerable to lethal H5N1 infection and could contribute to the spread and interspecies transmission of the viruses. Small terrestrial birds are potentially important hosts in H5N1 ecology because many of them intermingle freely with wild and domestic populations of waterfowl and poultry. However, very limited existing data describe their susceptibility to H5N1 influenza virus infection or their potential to transmit the viruses. A study investigating the host range of A/chicken/Hong Kong/220/97 revealed that it causes lethal infection in budgerigars and finches [10]. In contrast, the same virus replicated poorly in sparrows, causing no mortality, and there was no evidence of replication by this virus upon inoculation of pigeons. A more recent chicken H5N1 isolate (A/chicken/Yamaguchi/7/04), highly lethal to chickens and quail, also replicates extensively and causes high mortality in budgerigars [11]. Since 2002, H5N1 viruses have been isolated from dead birds of several wild terrestrial species, including magpie, tree sparrow, pigeon, and large-billed crow [8,12,13]. Viruses of a novel H5N1 genotype were isolated during a survey of live tree sparrows (*Passer montanus*); these isolates were highly pathogenic to chickens [14]. Together, these reports indicate that some small land-based bird species are susceptible to infection, sometimes lethal, with highly pathogenic H5N1 viruses. In the present study, we inoculated sparrows, starlings, and pigeons with several recent H5N1 influenza viruses isolated from a variety of avian hosts. The primary aims of the study were to test the susceptibility of different species to infection, investigate the duration and routes of viral shedding from the birds, and assess the possibility of intra-species viral transmission in these hosts.

Material and Methods

Influenza A viruses. Four influenza A virus strains were studied; two from previously known susceptible hosts (duck and quail) and two from previously unknown hosts (common magpie and Japanese white-eye). The A/Duck/Thailand/144/2005 (A/DK/TH/144/05) and A/Quail/Thailand/551/2005 (A/Q/TH/551/05) viruses were isolated from Western Thailand and tested for their pathogenicity in ducks [15]. The two other viruses, A/Common Magpie/Hong Kong/645/2006 (A/CM/HK/645/06) and A/Japanese White-eye/Hong Kong/1038/2006 (A/JW/HK/1038/06) were isolated from dead wild birds collected in January and February of 2006 during the heightened Hong Kong territory-wide dead wild bird avian influenza surveillance exercise which started in October 2005. The two viruses were isolated at the Agricultural, Fisheries and Conservation Department and supplied by the University of Hong Kong, Hong Kong SAR, China. Upon arrival at St Jude Children's Research Hospital, the viruses were propagated in 10-day old embryonated chicken eggs.

Animal studies. House sparrows (*Passer domesticus*) and European starlings (*Sturnus vulgaris*), both members of the order of Passeriformes, were captured wild. Six week old White Carneux pigeons (*Columba spp.*), member of the order of Columbiformes, were purchased from Palmetto Pigeon Plant

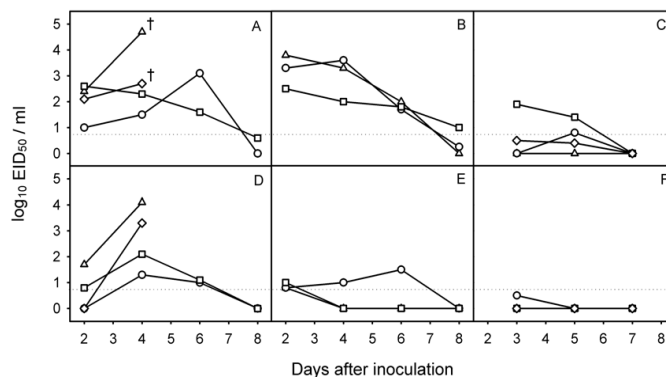
(Sumter, SC) and Double T farms (Glenwood, IA). Birds were housed in cages in the St. Jude Children's Research Hospital BSL3+ containment facility, food and water were provided *ad libitum*, and general care was provided as required by the Institutional Animal Care and Use Committee. Prior to inoculation with virus oral-pharyngeal and cloacal swabs were collected to exclude a pre-existing influenza A virus infection. Three sparrows and pigeons were inoculated intranasally with one million EID₅₀ in 50µl or 500µl PBS, respectively, for each virus. Due to their limited availability, starlings were inoculated with three viruses (one million EID₅₀ in 150µl), and group sizes were reduced (one bird for A/DK/TH/144/05, three birds for A/CM/HK/645/06 and two birds for A/JW/HK/1038/06). One day after inoculation uninfected contact birds, at a ratio of 1:1 for sparrows and starlings or 2:3 for pigeons, were housed together with inoculated animals to study intra-species transmission. Birds were monitored daily for mortality and morbidity over a period of 14 days. After inoculation oral-pharyngeal and cloacal swabs were collected on days 2, 4, 6, 8 and 11 for sparrows and starlings and days 3, 5 and 7 for the pigeons. Influenza virus was detected using 10-day old embryonated chicken eggs as previously described [7]. EID₅₀ virus titers were determined in positive swabs using the method of Reed and Muench [15]. The lower limit of quantitation of the assay is 10^{0.75} EID₅₀/ml and average virus titers in organs and swabs were calculated using the log₁₀ value of each sample.

Serology. Fourteen days after inoculation with virus, sera were collected from inoculated and contact birds, and hemagglutination inhibition (HI) titers were determined according to standard methods [16,17], using chicken red blood cells and four hemagglutinating units of virus. An HI titer >10 suggested a recent influenza virus infection, while an HI titer <10 was considered negative.

Results

The ability of four different highly pathogenic H5N1 influenza A viruses to infect and cause disease in house sparrows, European starlings, and white Carneux pigeons was determined. Experimental inoculation of sparrows caused mortality in 66-100% of the infected animals, dependent on the inoculated virus. The average time to death varied from 4.2 days for A/DK/TH/144/05 to 6.3 days for A/Q/TH/551/05 virus (data not shown). High viral loads were detected in brain and lung tissues of deceased sparrows (data not shown). In contrast, none of the starlings or pigeons died after inoculation with these H5N1 viruses. Re-isolation of virus from oral-pharyngeal and cloacal swabs obtained at various time-points after inoculation indicated that all the sparrows and starlings were productively infected by all viruses tested (Figure 1). In contrast, the frequency of virus re-isolation from inoculated pigeons varied widely between viruses. Of the four different H5N1 viruses, A/CM/HK/645/06 demonstrated the broadest host range, not only infecting sparrows and starlings, but also all of the inoculated pigeons. The A/DK/TH/144/05 virus was not re-isolated from inoculated pigeons.

Figure 1. Average viral titers in oral-pharyngeal (A-C) and cloacal (D-F) swabs of house sparrows (A, D), european starlings (B, E) and carneux pigeons (C, F) obtained at multiple days after experimental inoculation with H5N1 influenza A virus. Birds were inoculated with a/duck/Thailand/144/05 (Δ), a/quail/Thailand/551/05 (◊), a/common Magpie/Hong Kong/645/06 (◻) and a japanese white-eye/Hong Kong/1038/06 (◊). The dotted line represents the lower limit of quantification of 10^{0.75}/MI. Data are presented as log₁₀ 50% egg infectious doses per MI (Log₁₀EID₅₀/MI). = All animals succumb to infection.



Quantification of the virus titer in the swabs demonstrated that sparrows and starlings shed similar amounts of virus in the oral-pharyngeal swabs (Figure 1). Between 10³ and 10⁴ EID₅₀ per ml of isolation medium were detected on days 2 and 4 after infection in both species. However, virus titers in the cloacal swabs of sparrows were much higher than in those obtained from infected starlings. The 2005-2006 H5N1 viruses replicated relatively poorly in pigeons, as shown by low viral titers in both oral-pharyngeal and cloacal swabs on days 3 and 5 (Figure 1). The capacity of current H5N1 viruses to transmit from infected birds to same-species uninfected birds was assessed for these four H5N1 viruses. No evidence of transmission in sparrows and pigeons was found, as attempts to isolate the virus from contact birds failed (Table 1). Also no virus-specific antibodies were detected by HI in the contact birds (data not shown). In starlings, transmission of virus to contact birds was observed once for A/CM/HK/645/06 virus, but this was not seen in two further experiments.

Table 1. Transmission of H5N1 virus from infected to contact birds of the same species.

Species	Influenza A virus re-isolation from contact birds *			
	A/DK/TH/144/05	A/Q/TH/551/05	A/CM/HK/645/06	A/JW/HK/1038/06
Sparrow	0 ^a	0	0	0
Starling	0	nd [#]	33	0
Pigeon	0	0	0	0

Discussion

The susceptibility of three species of wild terrestrial birds to H5N1 influenza A virus and their ability to transmit to contact birds were assessed. Our studies reveal that major differences in susceptibility to H5N1 influenza virus infection exist among these bird species and that under our experimental conditions

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transmission occurs infrequently. Pigeons, starlings, and sparrows were found to be more susceptible to experimental infection with the recent H5N1 isolates than they were to A/chicken/Hong Kong/220/97 (H5N1) virus [6,10,18]. Although it is inappropriate to draw conclusions based on a single 1997 isolate, these data are consistent with studies that have demonstrated increased virulence or host range for recent H5N1 viruses in mammalian species, including mice, ferrets, and domestic and wild cats [20-23]. Whereas a previous study revealed that a 2003 chicken H5N1 isolate can cause severe neurological disease in pigeons, we observed no signs of disease in H5N1-infected pigeons [19]. Such a difference in pathogenicity between our study and others may be due to subspecies differences or a difference in inoculum size. A critical question concerning these small avian species is whether they can serve as intermediate hosts or reservoirs for H5N1 viruses and transmit them to poultry and mammals. Sparrows were highly susceptible to H5N1 infection, however they did not transmit to sentinel contact birds, despite a relatively low infectious dose (approximately 500 EID₅₀ for A/DK/TH/144/05 virus, data not shown) and the fact that virus was commonly detected in drinking water and faecal samples. While it is possible that the high pathogenicity of these viruses prevented bird-to-bird transmission, the data suggest that this species can act as an intermediate host and potentially transmit to both poultry and mammals, but not as a reservoir for prolonged shedding of highly pathogenic H5N1 influenza viruses. In contrast, the characteristics of H5N1 infection in starlings, i.e. non-lethal with longer-term shedding, suggest that starlings could act as an intermediate host and a reservoir for H5N1 virus. However, there was limited evidence of transmission to contact starlings, which implies that these H5N1 strains are unsustainable in a starling population. Pigeons shed only low amounts of virus upon infection and they did not transmit to contact birds, which suggest a minor role for pigeons in the ecology of H5N1 virus. Our results indicate that there are significant variations in susceptibility to H5N1 virus in different small terrestrial wild bird species. The high virulence of several recent isolates in sparrows suggests that this and other populations of small terrestrial birds may suffer significant losses during current and future H5N1 outbreaks. Further mutation of circulating H5N1 viruses might enhance their adaptation to hosts such as starlings and sparrows, further increasing virulence or allowing these species to become efficient intermediate hosts in the ecology of H5N1 influenza viruses.

Acknowledgements

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Establishment of Influenza A Virus (H6N1) in Minor Poultry in Southern China

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An H6N1 virus, A/teal/Hong Kong/W312/97 (W312), was isolated during the 'bird flu' incident of Hong Kong in 1997. Genetic analysis suggested that this virus might be the progenitor of the A/Hong Kong/156/97 (HK/97) H5N1 virus as seven of eight gene segments of those viruses had a common source. Continuing surveillance in Hong Kong showed that a W312-like virus was prevalent in quail and pheasant in 1999; however, the further development of H6N1 viruses has not been investigated since 2001. Here we report influenza surveillance data from 2000 to 2005 in southern China that shows H6N1 viruses have become established and endemic in minor poultry, and mainly replicated in the respiratory tract. Phylogenetic analysis indicates that all H6N1 isolates had W312-like hemagglutinin and neuraminidase genes. However, reassortment of internal genes between different subtype virus lineages, including H5N1, H9N2 and other avian viruses, generated multiple novel H6N1 genotypes in different types of poultry. These novel viruses are double, triple or even quadruple reassortants. Molecular analyses suggest that W312-like viruses may not be a precursor of HK/97 virus but a reassortant from HK/97-like virus and another unidentified H6 subtype virus. These results provide further evidence of the pivotal role of the live-poultry market system of southern China in generating increased genetic diversity of influenza viruses in this region.

Introduction

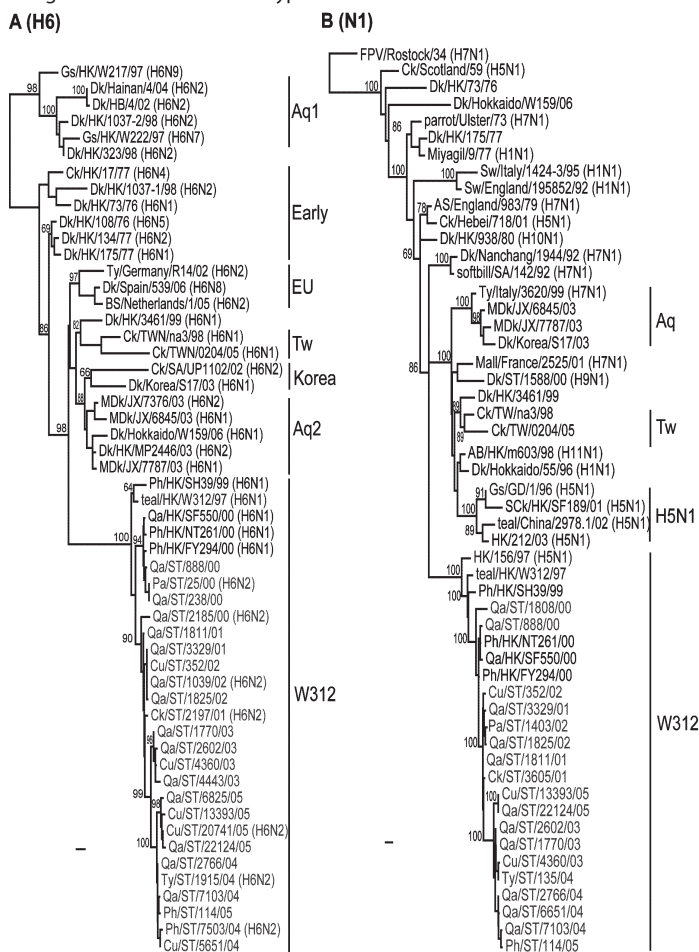
H6 influenza viruses are one of the most commonly recognized subtypes in domestic duck in southern China (4). During the Hong Kong H5N1 'bird flu' incident in 1997 an H6N1 avian influenza virus, A/teal/Hong Kong/W312/97 (W312), was isolated from live-poultry market. Genetic characterization of this virus revealed that, except for the hemagglutinin (HA) gene, its remaining seven gene segments were closely related to highly pathogenic avian influenza (HPAI) H5N1 viruses found in both poultry and humans (3). These findings suggested that a W312-like H6N1 virus might have been involved in the generation of the Hong Kong H5N1 virus (A/Hong Kong/156/97, HK/97) (2, 3). However, it is still unknown how the HK/97-like virus was generated as an H9N2 virus lineage, represented by A/quail/Hong Kong/G1/97 (G1), also shared the same internal gene complex as that of W312-like and HK/97-like viruses. As those three different subtypes of influenza viruses (H5N1, H6N1 and H9N2) were initially detected simultaneously during the Hong Kong 'bird flu' incident (2), the direction of gene flow among those viruses could not be determined.

Results

Systematic surveillance of minor poultry including quail, chukkar, Guinea fowl, partridge and pheasants in live-poultry markets from 2000 and 2005 resulted in 414 H6 subtype viruses out of a total of 11,415 samples collected (overall isolation rate, 3.6%). Most of these isolates were H6N1 subtype. Those viruses were prevalent year-round but with a higher isolation rate during the winter since 2004. However, the isolation rate for each year varied markedly from 1.7% to 6.4%. Of the minor poultry, chukkar and quail provided the main body of H6N1 isolates and had remarkably high isolation rates of 8.9% and 4.2%, respectively. In comparison, only a single H6N1 virus was isolated from 8,788 chicken and 2,530 silkie chicken specimens collected during the same period and from the same markets. A total of 402 of 414 (97%) H6 subtype viruses were isolated from tracheal swabs, and only eight from cloacal swabs and four from fecal material. These findings suggest that H6N1 viruses mainly replicated in the respiratory tract of those birds. Phylogenetic analysis of the surface genes: To understand the evolution and genesis of the H6N1, 77 of 414 (18.6%) virus isolates were sequenced and phylogenetically analyzed. Phylogenetic analysis of the H6 HA gene revealed that all viruses separated into the American and Eurasian gene pool. Within the Eurasian gene pool, four major lineages could be recognized, including Contemporary 1 and 2, Early, Aquatic. Contemporary 1 only contained viruses isolated from 1997 to 2004 in domestic ducks and geese in southern China (Fig. 1A). The second lineage (Early) mainly contained viruses isolated from 1976 to 1977, however, one virus isolated in 1998 in Hong Kong (Dk/HK/1037-1/98) also joined this lineage. The third lineage (Aquatic) consists of multiple different clades from the Eurasian influenza gene pool. Within this lineage H6 subtype influenza viruses were introduced into terrestrial poultry in several regions, e.g. Taiwan and South Africa. The fourth lineage (Contemporary 2) contains all H6 viruses that are prevalent in terrestrial poultry in southern China. These viruses were closely related to and derived from that of A/teal/HK/W312/97 and hence are referred to as "W312-like". The H6 HA gene tree clearly demonstrates that the phylogenetic position of the viruses tested corresponds to the different time points of their evolutionary pathway, and has formed a stable lineage (Fig 1A). This evolutionary pattern is different from that of the H5N1 and H9N2 viruses, which appear to have multiple evolutionary pathways with diverse co-circulating sublineages [4]. Phylogenetic analysis of N1 NA genes also shows that all H6N1 viruses isolated from minor poultry in southern China were closely related to and derived from that of A/teal/Hong Kong/W312/97 virus (Fig 1B). In concordance with the relationship of the Contemporary 1 lineage of the HA gene, the W312-like lineage appears to be derived from the Eurasian gene pool. All H6N1 viruses isolated from chicken in Taiwan also clustered with the same virus, Dk/HK/3461/99, which suggests the establishment of that virus lineage in Taiwan poultry. Phylogenetic analysis of the internal genes: In general, four different sources for the internal genes of the H6 viruses

tested were recognized, indicating that multiple reassortment events had occurred. Two of the internal gene sources were from the W312- or G1-like and Ck/Bei-like H9N2 virus lineages, while the remaining two were either from aquatic birds or from an unidentified source that was shared with current H5N1, H6N1 and H9N2 variants. For example, in the PB2 gene most of the H6N1 viruses clustered with G1-like or W312-like H9N2 viruses, while a few viruses clustered with recently identified H9N2 Ck/Bei-like variants. However, in the PA gene most H6N1 viruses were closely related to PA genes from aquatic bird isolates or novel H9N2 and H5N1 variants (data not shown). These results suggest that further reassortment between H9N2 and H6N1 viruses occurred after the Ck/Bei-like H9N2 variants were generated. It is noteworthy that the matrix (M) and non-structural (NS) genes of the H6N1 viruses showed much less genetic diversity than the other genes and belonged to either the G1-like or Ck/Bei-like H9N2 lineages.

Figure 1. Phylogenetic relationships of the H6 hemagglutinin (HA) (A) and N1 neuraminidase (NA) (B) genes of representative influenza A viruses. Trees were generated by the neighbor-joining method in the PAUP* program. Numbers above and below branches indicate neighbor-joining bootstrap values and Bayesian posterior probabilities, respectively. Analysis was based on nucleotides 49-1032, 1-1353 and 1-1229 of the H6 HA and N1 NA gene segments respectively. The H6 HA trees are rooted to A/turkey/Canada/63 (H6N2) and the N1 NA tree is rooted to A/Wisconsin/1/33 (H1N1). Virus subtypes are indicated in parenthesis, while those viruses with no subtype designations are of H6N1 subtype.



Molecular evolutionary analysis: Analysis of nonsynonymous substitutions (dN) of H6N1 influenza viruses from Hong Kong and Shantou in different years showed that the dN rate was higher in the N1 gene in comparison to all other genes, including the HA gene, but with the exception of the NS1 gene (Table 1). The dN rates of both the HA and NA genes gradually reduced over time since 1999. These findings suggest that the N1 gene may have been incorporated into the H6N1 viral particle later than the other genes.

Table 1. Rates of nonsynonymous substitutions (Dn) of H6N1 influenza virus in Southern China from 1999 to 2003.

Gene	Virus groups					
	HK/99 (HK)	HK/00	ST/00	ST/01	ST/02	ST/03
HA	4.4 ± 1.4	2.8 ± 0.6	2.9 ± 0.3	3.4 ± 0.3	2.5 ± 0.0	3.1 ± 0.4
N1	5.7 ± 1.0	4.9 ± 0.4	5.0 ± 0.3	4.4 ± 0.5	3.6 ± 0.4	4.4 ± 0.1
PB2	0.7 ± 0.6	0.7 ± 0.5	1.4 ± 0.4	1.1 ± 0.2	1.1 ± 0.1	1.3 ± 0.2
PB1	1.7 ± 0.3	0.9 ± 0.5	1.5 ± 0.9	0.8 ± 0.4	1.0 ± 0.0	1.3 ± 0.1
PA	2.0 ± 0.8	1.1 ± 0.2	1.5 ± 0.5	— ^a	—	—
NP	1.1 ± 1.0	1.6 ± 2.7	1.7 ± 0.7	—	—	—
M1	0	0	0	0	0	0.1 ± 0.2
NS1	6.7 ± 4.9	5.0 ± 4.9	6.1 ± 1.2	—	—	—

^aNot calculated as novel gene segments were incorporated from other virus lineages.

Discussion

Here we have provided the first comprehensive surveillance data for H6N1 viruses from 2000 to 2005 in poultry in this region. The findings of the present study revealed that H6N1 influenza viruses derived from W312-like viruses have become established in minor terrestrial poultry in southern China since 2000. Genetic analyses demonstrated that this virus lineage underwent broad reassortment with other influenza viruses of multiple origins, as also observed in H5N1 and H9N2 viruses, including directional gene exchange with those H9N2 and H5N1 viruses in poultry. Epidemiological data and phylogenetic analyses revealed that the H6N1 viruses have become established in terrestrial minor poultry, mainly in chukkar and quail. Those viruses have already adapted in this host, as their main replication site is the respiratory tract, similar to the adaptation of Ck/Bei-like H9N2 viruses in chickens and G1-like viruses in quail (5). The H6 HA gene tree clearly demonstrates that the phylogenetic position of all those viruses tested corresponds to the different time points of their evolutionary pathway. This evolutionary pattern is different from that of the H5N1 and H9N2 viruses, which appear to have multiple evolutionary pathways with diverse co-circulating sublineages (1, 5). The mechanism in the ecosystem for those differences remains to be explored. Phylogenetic analyses of the NA and internal genes revealed that those reassortant H6N1 viruses might have acquired their novel gene segments from the established H9N2 virus lineages and their reassortants, or vice versa. Like H9N2 Ck/Bei-like variants, some novel segments of the H6N1 virus genotypes have also been incorporated from the aquatic bird influenza gene pool. Currently, we have difficulty in identifying the sources of those G1-like or W312-like internal gene segments as their genetic origins are the same. Molecular analysis suggested that the N1 NA gene of W312-like virus had a higher dN substitution rate than the HA gene, which reduced

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gradually from 1999 to 2003. The higher dN rate of the N1 NA gene and its dynamic change indicates that this gene segment may have been incorporated latest in the virus particle, to which it has gradually adapted. Therefore, H6N1 W312-like virus may not be the precursor of the H5N1/97-like virus, but rather a derived strain which resulted from reassortment between H5N1/97-like and an unknown H6 subtype virus. Although we have systematically analyzed H5N1, H9N2 and H6N1 viruses from 2000 to 2005, the sources of some viral genes that were repeatedly detected in different subtypes, and obviously with a common origin (e.g. the NP gene), remains to be identified. This situation demonstrates a common genesis pathway for the emergence of variants of these three subtypes of influenza virus. Future studies are therefore needed that elucidate more clearly gene precursors to fully understand the ecology and evolution of influenza in southern China.

Acknowledgements

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High Pathogenic Avian Influenza (HPAI) H5N1: Causes and Consequences of Virus Introduction Into Northern Eurasia

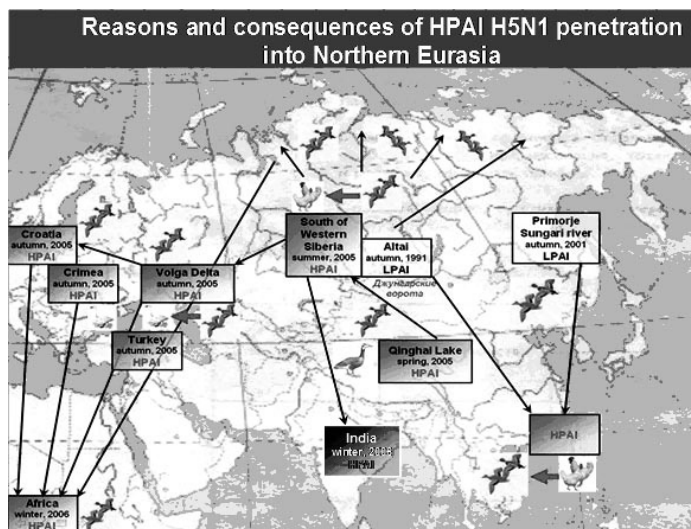
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The recent outbreaks of Asian lineage highly pathogenic avian influenza (HPAI) H5N1 in Russia and other parts of Europe demonstrate the importance for being prepared for the emergence of new pathogens into new regions by either natural or intentional causes (5). At the Ivanovsky Institute of Virology an interdisciplinary team was organized to provide a timely response to these types of outbreaks. This team included a large number of collaborating centers throughout Russia to provide accurate information on disease outbreaks and to collect field materials for testing. Low pathogenic avian influenza viruses (LPAI) are known to naturally circulate in wild bird populations, but the circulation in wild birds of HPAI presents a new challenge for our understanding of viral ecology. Past experience has shown that HPAI develop and are maintained in domestic poultry, and that spillover of HPAI was uncommon and didn't persist. However, the Asian lineage H5N1 virus appears to be an exception to this rule and has circulated in wild birds since at least 2005 (15,16). Because of the segmented nature of avian influenza viruses, that allows reassortment to occur commonly between avian influenza viruses, there is concern about reassortment between HPAI and LPAI viruses in wild birds (5). Interest in the viral ecology of avian influenza over 40 years ago gave rise to a monitoring program of virus circulation in key points of the main migration routes of birds in Northern Eurasia. This included virus isolation, sequence analysis, receptor activity, virulence, serology of wild and domestic animals and humans, action of antivirals, and evaluation of candidates for vaccine strains. In different regions of Northern Eurasia, we isolated fifteen of the sixteen known hemagglutinin subtypes of influenza A viruses including several low pathogenic H5 viruses from wild ducks in Siberia and the Far East (14). Phylogenetic analysis of the HA of early Siberian strains showed that they were similar to low pathogenic viruses that circulated in Malaysia and of the highly pathogenic avian influenza viruses that infected people and poultry in Hong Kong in 1997 (14). These Northern Asian LPAI viruses could be the precursors to the Asian lineage of HPAI that has resulted in such a catastrophic outbreak in Asia, Europe, and Africa. We predicted a risk of the spread of HPAI to Russia, especially in Siberia and the Far East by the spring migration of birds, and the outbreaks in Western Siberia in July of 2005 support this prediction. We isolated ten strains of HPAI H5N1 viruses in the Novosibirsk and Kurgan regions from sick poultry and a clinically healthy grebe *Podiceps cristatus*, deposited them into the Russian State Collection of Viruses,

and reported the full coding sequence to GenBank (6, 10, 11). The HA nucleotide sequences for the poultry and grebe viruses were practically identical. The extent of genetic identity of the isolates provided strong evidence of the link between viruses circulating among wild birds and poultry. The Russian strains were also similar to the strains from the H5N1 outbreak in wild birds in Kukurunor lake in North Western Qinghai province of China in April of 2005 (1, 2, 3, 4). The viruses from Siberia and Qinghai, China were all classified genetically as being in clade 2.2 and also had several other unique features including: 1. the cleavage site of the HA is characteristic for the HPAI phenotype; 2. A twenty amino acid deletion in the neuraminidase (NA) protein, 3. Glutamic acid in position 92 of the NS1 protein; 4. Ser in position 31 of the M2 protein which is associated with rimantadine / amantadine sensitivity; 5. Lys in position 627 position of PB2 protein (9). Results of sequence analysis correlated with biological properties (3,8). One of the isolated H5N1 viruses that was deposited in State Virus Collection was used in Russia for a large-scale production of veterinary vaccines that was used for targeted vaccination of poultry. So how and when were H5N1 HPAI viruses first introduced into Northern Eurasia and what are the possible long-term consequences (Figure 1). LPAI viruses normally circulate in Siberia and the Far East among wild birds and are probably spread during autumn migrations into Southeast Asia. These low pathogenic viruses presumably mutated to the highly pathogenic form of the virus in poultry, and the first HPAI viruses of this lineage were

Figure 1.

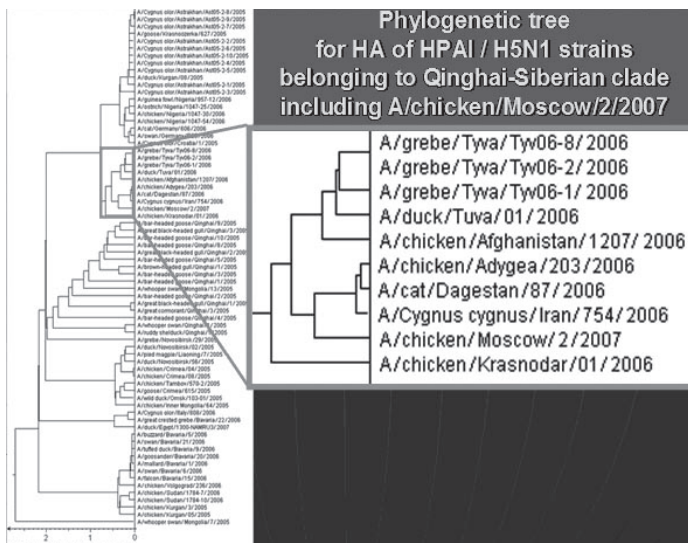


reported in 1996. The HPAI viruses were primarily reported in poultry, with only sporadic reports of wild bird infections which epidemiologically were likely a spillover from the domestic poultry reservoir. However, in April of 2005 in Qinghai province, China the first of many wild bird die offs that were believed to be the result of infection with H5N1 HPAI were reported. These Clade 2.2 lineage viruses appeared to

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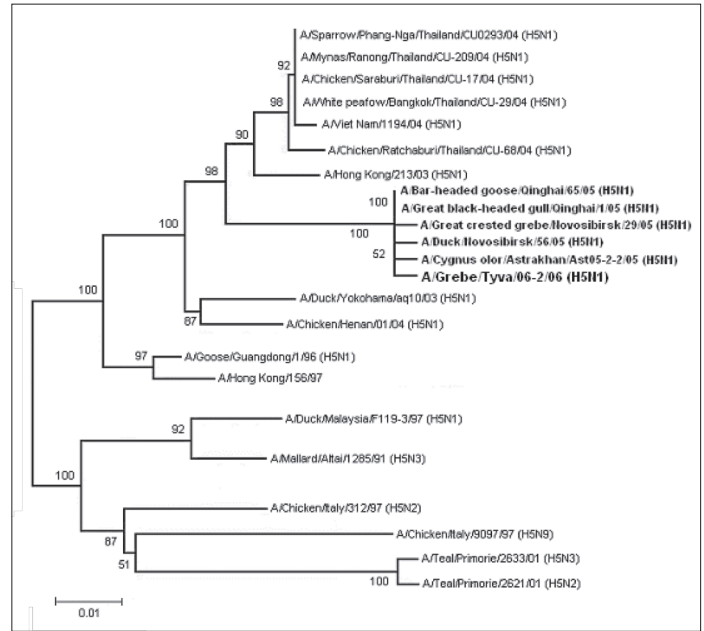
spread from China through the "Dzhungarian gate" between the Tien Shan mountains and Takla Makan desert on the West and Mongolian Altai mountains and Gobi desert on the East and penetrated into the West Siberian lowland and eventually spread to the nesting territory of Northern Eurasia on the coast of the Arctic ocean. In autumn during the Southern migration the birds flying back to the overwintering places were still carrying virus, and outbreaks were reported in most Asian and European countries and several African countries (7). In autumn, six months after (November, 2005) the West Siberian outbreak, in the Volga river delta a second outbreak with mass mortality occurred among the local population of Mute swans *Cygnus olor* and Tufted ducks, *Aythya fuligula*. Additional outbreaks were also reported in Turkey, Iran, Azerbaijan, Egypt, Nigeria and other countries. Sequence analysis of ten different viruses isolated from swans showed high sequence similarity to the Qinghai lineage of viruses. This was an unprecedented outbreak and widespread distribution of HPAI among wild birds of Northern Eurasia from spring to autumn from a HPAI virus of a single lineage (12). The next major outbreak among wild birds was reported in June, 2006, in Eastern Siberia on the Mongolian border on Ubsu-Nur lake, where it was estimated that thousands of birds died and included several different species including gulls, grebe, coots and other species of birds. Sequence analysis of seven representative strains demonstrated these viruses also belonged to the Qinghai virus lineage, and were still highly pathogenic in poultry (Figure 2A) (13). The most recent outbreak occurred in the Moscow region in February, 2007. Virus was isolated from nine different locations, and representative strains were also in the Qinghai lineage. Comparative analysis with data from seventy strains of Clade 2.2 viruses from Asia, Europe, and Africa showed the most closely related viruses

Figure 2a.



to the Moscow outbreak was from an Iranian Swan isolate, a Daghestan cat, and a chicken from Adygea. The amino-acid similarity was about 99.9% (Fig.2B), but the Moscow strain did have twelve unique amino acid substitutions. The direct origins of the Moscow virus appeared to be from the North Caucasus

Figure 2b.



region, but how it was introduced into the area is unclear but probably was related to the movement of infected poultry. The world's largest nesting area for aquatic birds is located in Northern Eurasia, and contamination of the habitat with HPAI virus is a concern. This can include the overwintering of the virus during the cold Russian winters into the spring where birds may be reinfected as they return for the nesting season. When infected and healthy birds return back through this contaminated habitat the outbreak could potentially become more severe and widespread as the virus is amplified. An unanswered question is whether the HPAI virus will revert to the low pathogenic form of the virus or eventually become naturally extinct in the wild. It is impossible to forecast how much time this process will take, months or years. The study of virus evolution in natural ecosystems should be a priority objective. It's necessary for us to predict the future of the next HPAI epornitic or potential pandemic in humans. The key is likely in the territory of Northern Eurasia, which is "a big kitchen" to Southeast Asia's "dining-room". Different species and their populations from various ecosystems have close contact in places of rest and over-winter habitats. Birds nesting in Europe and West Siberia are mostly overwintering in Southern Europe, Black and Caspian Sea basins (short-distance migrants) and in Africa, South Asia, and the Indian subcontinent (long-distance migrants). Birds from Eastern Siberia and the Far East overwintering mostly on China,

Korea, Japan (short-distance migrants), Indo-China, Pacific islands, America, Australia, and New Zealand (long-distance migrants). Populations of birds from different regions have active contacts through overlapping flyways and over-wintering territories. These are excellent opportunities for the exchange of gene segments of adapted viruses and the potential creation of a zoonotic virus that could eventually result in a pandemic outbreak (17). Last summer we sampled birds from several different natural ecosystems in Southern Europe, Siberia, and the Far East and did not find circulation of HPAI, except for the Ubsu Nur lake incident. However, LPAI H5 was detected by PCR circulating among poultry and wild birds in the Maritime province, and LPAI H5 and H7 in Jewish autonomous republic – Birobidjan. Infection rate with H5 was highest among aquatic wild birds and poultry, and for H7 influenza among local populations of wild birds, especially Corvidae and pheasants. The most recent outbreaks in Russia that are still being investigated occurred in April, 2007 in Northern Taiga with H5 positive samples among geese, gulls, corvidae, and in May, 2007 in the polar tundra positive of a yet unidentified influenza virus among geese. These investigations are in progress.

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Newcastle Disease Virus-Based Vector Vaccine Completely Protects Chickens and Mice From Lethal Challenge of Homologous and Heterologous H5N1 Avian Influenza Viruses

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We used reverse genetics to construct a Newcastle disease virus (NDV) that expressed an H5 subtype avian influenza virus (AIV) hemagglutinin (HA). Both a wildtype and a mutated HA open reading frame (ORF) from the HPAIV wild bird isolate, A/Bar-headed goose/Qinghai/3/2005 (H5N1), were inserted into the intergenic region between the P and M genes of the LaSota NDV vaccine strain. The recombinant viruses stably expressing the wildtype and mutant HA genes were found to be innocuous after intracerebral inoculation of 1-day-old chickens. A single dose of the recombinant viruses in chickens induced both NDV- and AIV H5-specific antibodies and completely protected chickens from challenge with a lethal dose of both velogenic NDV and homologous and heterologous H5N1 HPAIV. In addition, BALB/c mice immunized with the recombinant NDV-based vaccine produced H5 AIV specific antibodies and were completely protected from homologous and heterologous lethal virus challenge. Our results indicate that recombinant NDV is suitable as a bivalent vaccine against both NDV and AIV infection in poultry. The recombinant NDV vaccine may also have potential use in high-risk human individuals to control the pandemic spread of lethal avian influenza.

Introduction

H5N1 avian influenza has been a considerable problem for both veterinary and public health. The effective control of avian influenza in poultry is therefore an important issue for public health. The culling of infected poultry is the time-honored method to control or eradicate the highly pathogenic avian influenza outbreaks, and it is also the best-known way to prevent transmission to humans. However, when the viruses are spread over a wide area and have infected multiple avian species, culling and physical containment are not likely to be successful. An alternative strategy for control is the use of culling plus vaccination. Whole virus inactivated vaccines and fowlpox virus-based recombinant vaccines have been used as control strategies for highly pathogenic avian influenza in the laboratory and in poultry farms located within a limited geographic region (1, 2, 3, 4, 5, 6, 7, 8). However, the cost of

production and the laborious administration of these vaccines are limitations for their wide application in the field. In the present study, we established a reverse genetics system to generate a recombinant Newcastle disease virus (NDV) expressing an HA gene from an H5N1 avian influenza virus (AIV). We determined that our recombinant NDV-based vaccine was immunogenic and efficacious as an H5 AIV-NDV bivalent vaccine in chickens. We also found that our recombinant NDV-based vaccine elicited complete protection in chickens and mice against challenge with homologous and heterologous H5N1 influenza viruses.

Results and Discussion

Generation and characterization of recombinant NDV virus expressing H5N1 AIV HA protein. A naturally attenuated NDV virus LaSota strain was selected as the backbone for the NDV virus infectious clone construction. The wildtype and cleavage site mutated HA gene of H5N1 avian influenza virus, A/bar-headed goose/Qinghai/3/2005 (BHG/QH/05) (9), were inserted between the P and M gene of the NDV genome. The rescued wildtype NDV and the recombinant viruses were designated as rLa, rLa-H5w and rLa-H5m. The H5 HA gene expression in the recombinant NDV viruses was confirmed by western-blot and immunofluorescence assay. The mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) test of the recombinant NDV viruses was tested as recommended in the OIE Manual (10). The MDT values of the rLa, rLa-H5w and rLa-H5m viruses are greater than 120 hours, indicating that they behave as lentogenic viruses. The ICPI value for rLa was 0.4, with two out of eight chickens that received the virus rLa intracerebrally showing disease signs on day 1 p.i and dying on day two. The chickens that received the two NDV recombinant viruses, rLa-H5w and rLa-H5m, remained healthy during the observation period, and the ICPI values of the recombinant viruses were zero. When the viruses were administered intravenously, all of the chickens remained healthy during the observation period, and IVPI values of the viruses were zero.

Protective efficacy in chickens against NDV and H5N1 AIV challenges. The protective efficacy of the recombinant viruses against NDV and AIV viruses were examined and the potential of these recombinant viruses to serve as bivalent vaccines was investigated. To determine the protective efficacy of the recombinant virus against highly pathogenic NDV, we challenged chickens with 10^5 ELD₅₀ of highly pathogenic F48E9 NDV 3 weeks post vaccination (p.v). Chickens immunized with rLa and the recombinant viruses were completely protected from NDV challenge, showing no signs of disease or deaths for two weeks. In contrast, all of the chickens in the control groups died within 3 days. The BHG/QH/05 is an antigenic drift virus when compared to the H5N1 viruses that were isolated early in China. The HA gene of BHG/QH/05 shares approximately 95% identity with the HA gene of A/goose/Guangdong/1/96 (GS/GD/96) virus. To determine whether the recombinant viruses could induce protection against the homologous virus

as well as heterologous H5 influenza viruses, we challenged the vaccinated chickens with the homologous virus BHG/QH/05 and the early isolate GS/GD/96. Three weeks after vaccination, we collected sera and tested their HI antibody titers to the different challenge viruses. rLa-H5w and rLa-H5m induced mean HI antibody titers to homologous virus of $8.8\log_2$ and $8.9\log_2$, respectively. The mean HI antibody titers to the heterologous virus GS/GD/96 induced by rLa-H5m and rLa-H5w were $4.2\log_2$ and $4.3\log_2$, respectively. When we challenged the chickens with 10^3CLD50 of the different viruses, the vaccinated birds were completely protected from challenge by both homologous and heterologous influenza H5 viruses. No virus shedding, clinical signs or deaths were observed. In the control group of chickens challenged with BHG/QH/05, high titers of virus shedding were detected from both trachea and cloacae, and the animals died on or before day 3 post challenge. In the control chicken group challenged with GS/GD/96, all of the animals shed high titers of viruses and died before day 5 p.c. These data indicate that recombinant NDV based vaccines expressing wildtype and mutant influenza HA are effective against challenge by homologous and heterologous influenza viruses, and that they are also bivalent vaccines offering protection against challenge by pathogenic NDV.

Protective efficacy in mice against H5N1 avian influenza virus challenge. Groups of mice were inoculated with 2 doses of 10^8EID50 of the rLa or rLa-H5w virus intraperitoneally at the age of 3-weeks-old and then boosted with the same dose of vaccine by the same administration route at 6-weeks-old. Two weeks after the second dose of vaccine, we challenged the mice with 10^3MLD50 of highly pathogenic H5N1 viruses, BHG/QH/05 and A/duck/Fujian/13/02 (DK/FJ/02), which has been described previously (11). Virus was not detected from any organs examined from the rLa-H5w vaccinated mice that were killed on day 3 and 6 p.c. In the rLa vaccinated groups, virus was detected in the nose turbinate, lungs, kidney and brains of mice challenged with both BHG/QH/05 and DK/FJ/02 viruses on day 3 p.c., and high titers of virus were also isolated from the lungs and brains of mice challenged with both viruses on day 6 p.c. Virus was not detected from the spleens of any mice at either time point. Five mice from each group were kept for observation of clinical signs and death. In the rLa vaccinated groups, the mice started to show ruffled furs and weight loss at day 3 p.c., and all mice died within 9 days p.c. However, the mice that received the rLa-H5w virus stayed healthy and survived during the observation period. The protective efficacy of the recombinant NDV based vaccine observed in chickens against homologous and heterologous influenza challenge is also extended to mice. In summary, we generated recombinant NDV expressing either wildtype or mutant HA gene of an H5N1 avian influenza virus using reverse genetics, and evaluated its potential use as a bivalent vaccine against pathogenic influenza infection as well as against pathogenic NDV infection. After a single immunization dose, the recombinant viruses expressing wildtype or mutant HA induced a strong HI antibody response

to NDV and H5 AIV in chickens, and protected chickens from disease signs and death against highly pathogenic NDV. Most importantly, the vaccinated chickens were completely protected from homologous and heterologous H5N1 virus challenges, and displayed no virus shedding, signs of disease or deaths. We also determined that the recombinant virus expressing the wildtype HA could induce complete protection in mice against lethal doses of homologous and heterologous H5N1 virus challenges. Our results demonstrated that the recombinant viruses could work as live bivalent vaccines to provide protection against infection against highly pathogenic NDV and H5 influenza strains.

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Pathogenicity and Increased Virulence of Italian H7N1 HPAI Virus in the Mouse Model

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Introduction

Avian influenza viruses generally exhibit a relatively restricted host range, with efficient viral replication occurring in the natural host and complete or partial restriction of viral replication occurring in the other species. Previous work [1] has established the crucial role of the viral protein PB2 in determining influenza virus host range in mammals. In detail, single amino acid substitutions, from glutamic acid (E) to lysine (K) at position 627 and from aspartic acid (D) to asparagine (N) at position 701 of the PB2 protein appear to increase virulence and enable more extensive replication respectively, overwhelming the host's defence mechanisms and resulting in high mortality rates in mice [2, 3]. These mutations are believed to occur as a result of viral adaptation to the mammalian host. Sequence analysis of a selection of Italian highly pathogenic avian influenza viruses (HPAI) of the H7N1 subtype [4], revealed in an ostrich isolate a naturally occurring mutation from glutamic acid to lysine in 627 of PB2 gene. The main purpose of our work was to investigate the pathogenicity of the ostrich isolate in comparison to an H7N1 HPAI chicken isolate lacking this mutation, in the mouse model.

Materials and Methods

Viruses. Among the collection of Italian highly pathogenic avian H7N1 viruses we selected one isolate obtained from an ostrich (A/ostrich/It/2332/00, H7N1) displaying a K residue in position 627 of the PB2 gene. As a control virus we selected a chicken isolate (A/chicken/It/5093/99, H7N1), with E in the same position. Both isolates have D in 701 of PB2, which is in keeping with their avian origin. Virus stocks were propagated in the allantoic cavity of 9-11 day old embryonated SPF eggs and were titrated to determine the EID₅₀. Isolates were sequenced before and after the animal experiment. Mouse experiment. Female BALB/c mice, 6-8 weeks of age were mildly anaesthetised with CO₂ before infection. Mice were divided in two different groups of ten each and were infected intranasally with 50 µl of a solution containing 10⁵ EID₅₀ of each virus. Mice were observed twice a day for clinical signs and weighed every

day for ten days following infection. Two mice from each virus-infected group were sacrificed on day three, five, seven and ten post-infection and the following organs were collected: brain, lung-trachea, spleen, liver, kidney and intestine. The organs of the mice that died were collected immediately. Six uninfected mice were used as negative control group. Pre and post-infection. LD₅₀ of pre and post-infection isolates were also determined. Virological assay. Samples for virological examination were homogenized in 3 ml of phosphate buffer saline with antibiotics and clarified by centrifugation. The undiluted and ten-fold serially diluted supernatants were titrated for virus infectivity in 9-11 day old embryonated SPF eggs. RT-PCR and sequencing. Samples for molecular investigation were homogenised in 0.5 ml of phosphate buffer saline and RNA was extracted using a commercial kit (High Pure™ RNA extraction kit; Roche). Real-Time RT-PCR was performed for all the tissue samples using primers targeting the M gene of type A influenza virus. Full sequencing of pre- and post-infection isolates was performed. Results: Clinical signs. The uninfected control group did not show any clinical signs and no weight loss was recorded. The group infected with the ostrich isolate 2332/00 showed depression, ruffled coat and anorexia starting from day 4 post-infection. These symptoms were accompanied by severe dyspnoea, which became more and more severe and caused death of all infected mice between day 7 and day 8 post-infection. A mean weight loss of 40% was recorded in this group. The LD₅₀ for this virus was 10^{1.75} EID₅₀. The second experimental group of mice infected with the chicken isolate 5093/99 did not show any significant clinical signs or mortality. However, a weight loss of 8% was recorded. The LD₅₀ of the original isolate was > 10^{5.6} EID₅₀ while the post-infection isolate value was 10^{3.4} EID₅₀, indicating an increase in virulence. RT-PCR, sequencing and virus isolation. Among all samples collected from infected mice, only lung-trachea and brain yielded positive results in Real time RT-PCR and virological assay for 2332/00 and 5093/99. Viruses were recovered from brain and trachea/ lung of mice infected with both strains. Interestingly, sequencing of one of the CK/5093/99 isolate recovered from the brain of one mouse on day 10 pi revealed that a mutation to the PB2-627K variant had occurred. No D to N mutation in position 701 of the PB2 gene was revealed in the two strains.

Discussion and Conclusions

The Italian H7N1 HPAI epidemic affected 413 avian farms, and neither mammals or human beings appeared to be infected [5]. The results obtained from this experiment indicate that a natural isolate of HPAI H7N1, obtained from an ostrich and possessing a Lys residue in position 627 of the PB2 gene was highly virulent and lethal for experimentally infected mice. The virus caused in mice, similar clinical signs to those observed with mammalian-derived H5N1 and H7N7 viruses [6]. The replication of strain 2332/00 in the brain and respiratory tract was confirmed by virus isolation. In our experiment the D to N mutation in position 701 of PB2 did not occur, and therefore such mutation does not appear to be essential for

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efficient replication of H7N1 viruses in mice. The results of this investigation suggest that a E627K mutation in PB2 could occur in species such as an ostrich that is phylogenetically very distant from galliforms, and not necessarily as result of adaptation to mammals. However, data related to the post-infection isolate 5093/99 indicate that viral population exhibiting PB2-627 E to K mutation can be readily selected following infection of mammalian hosts. The behaviour in the mouse model of PB2 K627 avian origin strains does not differ significantly to that of mammalian adapted H5N1 and H7N7 viruses. This appears to be the first report of a H7 avian isolate exhibiting such properties and suggests that such genetic variation may occur within the avian host, possibly without an adaptive step in mammals.

Acknowledgements

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Proceedings Topic #7

Clinical Vaccine Evaluation

Oral

Presentations

Live Attenuated Vaccine in Russia: Advantages, Further Research and Development

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This paper presents a summary of the development and utilization of live influenza *ca* reassortant vaccine (LAIV) in Russia during 1990-2007 with special attention to its advantages as well as further research plans. Previous publications demonstrated that, in addition to anti-HA-antibodies, LAIV induced in humans production of mucosal IgA and stimulation of CTL-based immunity [1]. Also a 40-50% reduction of otitis media and pneumonia was reported among children vaccinated with LAIV [2].

Effectiveness against drifted virus variants. One of the advantages of LAIV is its cross-protection against drifted variants of influenza viruses. Table 1 shows the effectiveness of LAIV in reducing acute respiratory diseases (ARD) among children and adults in clinical trials performed in 1986-2004. Vaccine effectiveness varied between 30-59% and depended on the antigenic similarity between the circulated influenza epidemic strains and the LAIV vaccine components [2, 3, 4, 5].

Table 1. Efficacy of LAIV during the circulation of drift influenza viruses.

YEAR, PLACE OF STUDY	AGE GROUP	NO. OF VACCINATED	VACCINE COMPONENTS	CIRCULATED EPIDEMIC STRAIN	EFFICACY %
1986-87, Alma-Ata, Kazakhstan	Children 3-14 years old	Vaccine – 25117 Placebo – 28703	A/Brazil/11/78 (H1N1) A/Philippines/2/82 (H3N2)	A/Taiwan/1/86 (H1N1)-like	30
1988-89, Alma-Ata, Kazakhstan	Children 3-14 years old	Vaccine – 29690 Placebo – 31878	A/Taiwan/1/86 (H1N1) A/Philippines/2/82 (H3N2)	A/Taiwan/1/86 (H1N1)-like B/Victoria/2/87-like	36
1989-90, Novgorod, Russia	Children 7-14 years old	Vaccine – 4693 Placebo – 4168	A/Taiwan/1/86 (H1N1) A/Sichuan/2/87 (H3N2) B/USSR/3/87	A/Shanghai/16/89 (H3N2)-like	41
1990-91, Novgorod, Russia	Children 7-14 years old	Vaccine – 5288 Placebo – 6385	A/Taiwan/1/86 (H1N1) A/Shanghai/16/89 (H3N2) B/USSR/3/87 (B/Victoria-like)	A/Taiwan/1/86 (H1N1)-like B/Victoria/2/87-like B/Yamagata/16/88-like	32
1991-92, Novgorod, Russia	Children 7-14 years old	Vaccine – 3403 Placebo – 3022	A/Taiwan/1/86 (H1N1) A/Shanghai/16/89 (H3N2) B/Beijing/32/92	A/Beijing/32/92 (H3N2)-like	36
2000-01, St. Petersburg, Russia	Children* 7-14 years old	Vaccine – 554 Placebo – 1265	A/Beijing/262/95 (H1N1) A/Sydney/5/97 (H3N2) B/St. Petersburg/95/20	A/New Caledon./20/99 (H1N1)-like	58
2003-04, St. Petersburg, Russia	Adults 18-23 years old	Vaccine – 202 Placebo – 158	A/17/New Caledonia/99/145 (H1N1) A/17/Panama/99/242 (H3N2) B/Victoria/2/87	A/Wyoming/3/03 (H3N2)-like	59

*LAVI for adults was used.

Long-term protection. Clinical trials in humans demonstrated that LAIV provides with long-term protection against influenza-associated diseases (for 16 months after vaccination during 1991-1993 epidemic seasons). The 40% level of protection was demonstrated among children who received LAIV in the previous, but not current, epidemic season; there was no protection observed in schools where children received in the previous season inactivated vaccine [6]. **Non-adaptive immune response.** Additional benefit of LAIV relates to providing protection shortly after administration, prior to the development of the adaptive immune response. Reduction of ARD among children in the first week post vaccination was 37%-66% [3]. Also it was shown that influenza viruses are excellent inducers of IFN α/β with considerable elevation in natural killer (NK) cell

activity at the early stages of viral infection [7, 8].

Cell-grown LAIV. The LAIV grows efficiently as in eggs as in mammalian cell culture [9]. Similar protection efficacy of cell and egg derived vaccines was observed in ferrets challenged with *wt* virus. Titers of the *wt* virus isolated from lungs of the placebo group were 10^6 - 10^7 genome copies/ml while the virus was not detected in lungs of ferrets immunized with egg- or MDCK-derived LAIV [10].

Herd immunity. The indirect protective effect on non-vaccinated persons has been demonstrated among schoolchildren vaccinated with LAIV. The extent of herd immunity depended on the proportion of vaccinated children in a particular school. By contrast, herd immunity was not revealed in schools where children were immunized with inactivated vaccine [4].

LAIV and asthmatic patients. The safety of LAIV in asthmatic patients is still being discussed. In the murine model of allergic bronchial asthma developed in our laboratory, it was shown that, in contrast to infection with *wt* viruses, vaccination of mice with LAIV at the remission phase of asthma did not enhance the allergic-specific immune responses and inflammatory changes in lungs. Further research to study safety of LAIV among patients with allergy and immunodeficiency conditions will be undertaken.

Pandemic reassortant LAIV. A potential pandemic vaccine candidate (A/17/H5) obtained by classical reassortment of the A/Leningrad/134/17/57 (H2N2) cold-adapted master donor strain (Len/17) and non-pathogenic avian strain A/17/duck/Potsdam/86/92 (H5N2) has displayed high levels of protection in mice challenged with highly pathogenic (HP) H5N1 viruses [11]. Recently we investigated safety (reactogenicity) and the cross-protection efficacy of the H5N2 LAIV (Len/17/H5) against a contemporary HP avian virus A/chicken/Kurgan/02/05 (H5N1) in Java macaques. Monkeys immunized with this LAIV were monitored for 7 days after each immunization by examining morning and evening rectal temperatures, behavior and weight loss. No fever or other side effects were observed following either the first or second dose of vaccine. Virus replication was confirmed in 2 of 4 monkeys on days 3-5 after the first vaccine

Table 2. Protective properties of live attenuated influenza vaccine A/17/Duck/Potsdam/86/92 (H5N2) after challenge in java macaques by clinical, immunological and virological tests.

Preparation	Monkey #	Temperature reaction*		Rise of antibody titers in HI	Virus isolation	
		Duration, hours	Rise above normal		Duration, hours	Tissue
Vaccine	1	≤8	0.1	1	≤48	Blood
	2	≤8	0.3	8	≤24	Nasal wash
	3	≤8	0.1	2	≤24	Nasal wash
	4	0	0	8	0	
Placebo	5	≥48	0.3	8	≥72	Blood, Nasal wash
	6	≥72	0.9	8	≥96	Blood, Nasal wash
	7	≥48	0.5	8	≥48	Blood, Nasal wash

*Upper limit of normal temperature in Java macaques is 39.1°C.

dose. The titers of virus in isolates were from 1.2 to 4.2 IgEID₅₀/ml. The immune response as measured by HI antibodies was

found in one monkey after 1st dose (1:160) and in additional 2 monkeys after second dose (1:40). Protective properties of the LAIV measured by clinical and virological tests after challenge with A/chicken/Kurgan/2/05 (H5N2) are summarized in Table 2. As it is shown, 3 of 4 vaccine recipients developed low-grade fever with temperature rise above normal 0.1-0.3°C and duration for ≤8 hours. In contrast, all 3 monkeys from the control group developed fever after challenge, with rise above normal 0.3-0.9°C and duration ≥ 4-8 hours. After challenge, rise in HA antibody titers was observed in two of 4 vaccinated and 3 of 3 control macaques. Influenza virus isolation was undertaken daily using blood and nasal washes obtained from all monkeys for 7 days after challenge. Table 2 shows that the virus was isolated from 3 of 4 monkeys in the vaccine group and from 3 of 3 animals in the control group. The duration of virus isolation was longer (≥48 - ≥96 hours) in the control group compared with the vaccine group (≤24 - ≤48 hours). The *wt* virus was isolated from only one monkey in the vaccine group, while it was found in bloods of all 3 control macaques. In summary, the vaccination of monkeys with 2 doses of LAIV, A/Len17/H5 (H5N2), provided substantial 50% cross-protection from challenge with HP 2005 human influenza H5N1 virus. Clinical trials in 20 human volunteers confirmed safety and genetic stability of the A/Len17/H5 (H5N2) LAIV. The frequency of ≥4-fold HI antibody titer rises was 47% after second dose (6% after 1st dose). The percentage of volunteers with post-vaccination serum HI titers ≥1:20 was 47% and ≥1:40 was 29%. When sera from volunteers vaccinated with A/Len17/H5 (H5N2) was tested against influenza A(H5N1) antigen, ≥4-fold rises of HI antibodies were found in 29% of sera samples, thus providing with an evidence of cross-reaction between H5N2 and H5N1 antigens. Seventy percent of volunteers responded to both antigens: A(H5N2) and A(H5N1). There was no difference in results if horse or human red blood cells were used. Two doses of LAIV stimulated also seroconversion of sIgA antibodies (65%), 16-fold raise in GMT's and 2.8-fold rise in sIgA antibodies. Currently, we are investigating the interaction of *ca* viruses with *wt* viruses, including avian strains, which is a major concern about LAIV biosafety in pre-pandemic and pandemic situations. Preliminary data has shown that the simultaneous inoculation of *ca* and *wt* viruses does not lead to increasing pathogenicity compared with single infection of *wt* viruses. The phenotypic and genotypic analysis of clones isolated from lungs of mixed infected animals revealed the *ts*-phenotype of isolates and absence of reassortants between *wt* and cold-adapted viruses [12].

Summary

Multi year extensive experience with LAIV in Russia has proven its safety and efficacy even against drifted virus variants. No evidence of genetic reversion to *wt* of mutations associated with the vaccine attenuation was detected. LAIV provided with reasonably high level of immediate (first 7 days) protection. LAIV stimulated mucosal immunity and levels of functional antibodies. Vaccination of schoolchildren with LAIV provided the community with herd immunity. There were no increased allergic reactions in children or adults immunized with LAIV. Vaccines produced in

eggs or mammalian cell demonstrated similar protection efficacy in ferrets. The cold-adapted reassortant Len17/H5 demonstrated attenuated phenotype in mice, monkeys and volunteers and not infected chicken. Len17/H5 was safe and areactogenic in pre-clinical trials in monkeys and in clinical trials in volunteers. Mice and monkeys administered Len17/H5 were substantially protected from challenge with highly pathogenic A/Hong Kong/483/97(H5N1), A/Hong Kong/213/2003 (H5N1), A/chicken/Kurgan/2005 (H5N1).

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Proceedings Topic #8

Genetic and Antigenic Evolution

**Oral
Presentations**

Genetic and Antigenic Characterization of Avian Influenza A (H5N1) Viruses Isolated From Humans in Mainland China

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Influenza, a type of respiratory disease, has inflicted mankind with severe disease and periodically has caused worldwide pandemics. During the 20th century, the introduction of influenza subtypes previously not encountered by humans has resulted in four worldwide pandemics; i.e., the H1N1 (Spanish influenza) in 1918-19, H2N2 (Asia influenza) in 1957, H3N2 (Hong Kong influenza) in 1968, and reappearing H1N1 sub-type (Russian influenza) in 1977. China is often recognized by the world as a place frequently threatened by influenza of novel subtypes. In 1997, an avian influenza outbreak caused by H5N1 emerged in Hong Kong, resulting in the killing of 7,000 chickens and the infection of 18 people with 6 deaths. As of May 31st, 2007, influenza A H5N1 virus has infected humans in at least twelve countries with 310 confirmed human cases and 189 deaths (the case fatality ratio exceeds 50%)[1]. In China, 24 human cases with 15 deaths were confirmed through Chinese avian influenza (AI) surveillance network since Nov, 2005, with the first human H5N1 infection being reported in Nov, 2005 [2]. The confirmed cases were mostly centralized in nine provinces in south China, while two cases were located in north China. Since the outbreak of avian influenza (H5N1) in animals and the confirmation of the first human case, an AI (H5N1) surveillance network was built upon based on the seasonal influenza surveillance system, including 63 network laboratories and 197 hospitals at the national level, and the number of laboratories will be increased by 20 in the following year. In order to control the quality of laboratorial detection in network laboratories, a quality control system was developed, providing necessary reagents and equipment, reference reagents, etc. Moreover, periodical supervision and technical training were performed once a year. Under the help and support of local CDCs, specimens were collected and transported to CNIC, and serological study and phylogenetic analysis were performed. *Antigenic analysis.* The antigenic characteristics of human H5N1 isolates from China were compared with those from Vietnam, Indonesia and Turkey by hemagglutination inhibition (HI) assay using reference ferret serum raised against representative

H5N1 viruses. Ferret antisera were kindly provided by the Influenza Division, Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A. Survivor sera of confirmed human H5N1 infection from Anhui, Guangxi, Fujian, Sichuan, and Jiangxi provinces, were collected 4 weeks after the onset of illness. As shown in Table 1, the H5N1 human isolates from China were well inhibited by ferret antisera raised against Anhui/1/2005 and Anhui/2/2005 viruses isolated from southern China, but were not well inhibited by antisera raised against H5N1 viruses isolated from Turkey, Vietnam and Indonesia, with at least four fold reductions in levels of HI titers compared to the levels of homologous titers. These results indicated that Chinese human H5N1 isolates are antigenically closely related to each other, but are antigenically distinguished from H5N1 isolated from Vietnam, Indonesia and Turkey. Additional HI analysis using chicken antisera raised against representative H5N1 viruses also revealed antigenic differences between the two groups (Table 1). Microneutralization assays using serum samples collected from confirmed human cases were consistent with the results obtained from the HI tests (Table 2). These findings demonstrate the considerable antigenic divergence of HPAI H5N1 viruses. *Genetic analysis.* In this study, respiratory specimens were collected from patients on days 5 to 11 after onset of illness. Samples included gargle, nasal and/or throat swabs, tracheal aspirates and phlegm, saliva, as well as lung tissues from autopsies collected from different individuals following a standard protocol. The viruses were isolated and propagated in the amniotic and/or allantoic cavities of special pathogen free (SPF) embryonated chicken eggs as described under biosafety level (BSL) 3 enhanced containment in accordance with guidelines from the National Institutes of Health and the Centers for Disease Control and Prevention (Interim CDC-NIH Recommendations (<http://www.cdc.gov/flu/h2n2bs13.htm>)). Sixteen H5N1 virus isolates were obtained from 13 fatal and 3 surviving human cases. Viral RNA was extracted from isolates using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). cDNA synthesis and PCR amplification of the coding region of the 8 gene segments were carried out using the one-step RT-PCR kit (Qiagen, Valencia, CA) with gene specific primers (primer sequences available on request). The PCR products were then purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and used as templates for nucleotide sequencing. Sequencing reactions were performed using the ABI BigDye Terminator Sequencing Kit with reaction products resolved on a MegaBACE1000 DNA sequencer (Applied Biosystems Foster City, CA). Nucleotide sequences were analyzed using DNASTAR (Lasergene, Madison, WI). The phylogenetic tree was generated by the neighbor-joining method using the Mega3.1 program. Phylogenetic analysis revealed that the HA gene of the H5N1 viruses diverged into several distinct clades (Fig 1). Clade 1 contains viruses isolated from Vietnam, such as the virus, A/Vietnam/1194/2004. Clade 2 viruses segregated into 3 sub-clades that are represented by A/Bar-head goose/Qinghai/5/2005, named clade 2.2, Indonesia/5/2005, named

clade 2.1, and Anhui/1/2005, named clade 2.3. The single human H5N1 virus from North China, A/Xinjiang/1/2006, belongs to clade 2.2 and the isolates from southern China clustered together with Anhui/1/2005, and belong to clade 2.3. The other 7 segments (including NA, PB2, PB1, PA, NP, NS and M) were all sequenced and phylogenetically analyzed. A clear reassortment was observed in the Jiangxi isolate, A/JX/1/2005, which appears to have reassorted with five strains, A/VN/1194/2004, A/BHG/5/2005, A/IND/5/2005, A/AH/1/2005, and A/CK/GXNN/6/2004. In addition, according to the sequence analysis of the M and NA genes, no amino acid mutations were observed that are associated with resistance to amantadine, rimantadine, oseltamivir or tamiflu, although most patients received up to two weeks of treatment with amantidine, rimantadine or oseltamivir (at a daily dose between 40mg-150mg). One exception of a point mutation in the M2 gene at position 31 (S31N) was observed in a recent isolate from Fujian province.

Figure 1. Phylogenetic tree generated by the neighbor-joining method using the Mega3.1 program and the bootstrap value 1000. The relationships of HA genes of representative influenza A H5N1 viruses from different regions are shown. The dark dots denote viruses isolated from humans in China; light dots denote viruses isolated from Vietnam and that were recommended as vaccine strains by the WHO.

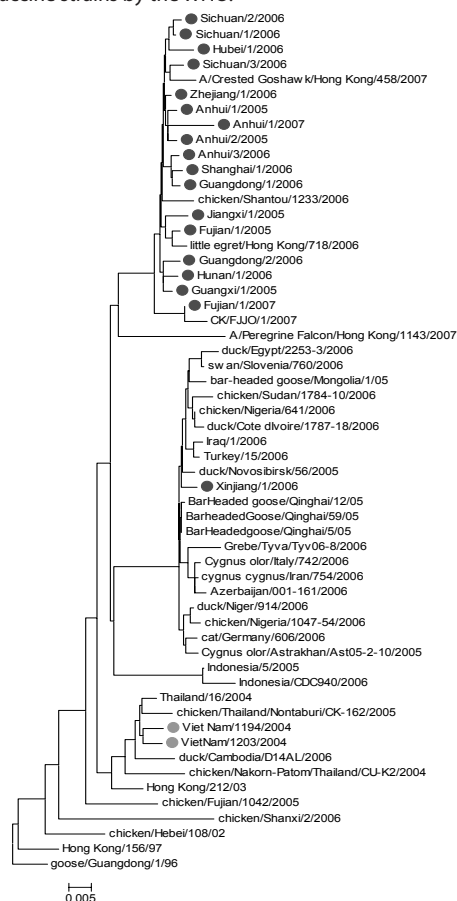


Table 1. Antigenic analysis of human H5N1 isolates by HI assay using ferret serum.

	Reference		ferret		
	VN/120	IND/	TK1	AH/	
A/Vietnam/1203/200	32	20	1	40	4
A/Indonesia/5/200	20	64	4	16	8
A/Turkey/15/200	80	32	128	80	2
A/Anhui/1/200	40	32	1	128	64
A/Anhui/2/200	20	16	5	32	32
A/Guangxi/1/200	20	80	5	64	32
A/Fujian/1/200	10	80	5	16	16
A/Sichuan/1/200	40	16	1	128	64
A/Jianxi/1/200	80	64	4	256	128

Table 2. Antigenic analysis of human H5N1 isolates by MN assay using confirmed human case serum. *Case 1 was a surviving confirmed human H5N1 case from Anhui Province (Southern China), the serum was collected 4 weeks after the illness onset. #Case 2 was a surviving confirmed human H5N1 case from Liaoning Province (Northern China), the serum was collected 4 weeks after the illness onset.

	Confirmed cases	
	case1*	case2#
A Anhui 1.2005	160	40
A Xinjiang 1.2006	40	320

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Ecology and Evolution of Influenza A (H5N1) Virus in Asia: Evidence From Systematic Influenza Surveillance

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The development of highly pathogenic avian influenza A (H5N1) viruses in poultry in Eurasia accompanied with the increase in human infection in 2006 and the recent reemergence of poultry outbreaks throughout the region, suggests that the virus has not been effectively contained and that the pandemic threat persists. Virological and epidemiological findings from our surveillance in live-poultry markets conducted over the last seven years in southern China has provided a comprehensive view of the ecology and evolution of H5N1 influenza viruses and revealed that they are endemic in different types of market poultry. Genetic and antigenic analyses have also demonstrated the dynamic evolution of these viruses in the region, with repeated introductions from southern China to neighboring regions, including Cambodia, Indonesia, Malaysia, Thailand, and Vietnam. A further dramatic spread of the virus westwards throughout Central and Southern Asia, Europe and Africa was also seen after detection of an initial outbreak in wild birds at Qinghai Lake in Central China, in line with our predictions. Genetic analyses revealed that after the H5N1 influenza viruses had established and been endemic several years, they developed into regionally distinct sublineages that allowed us to further trace their transmission pathways. However, recent surveillance data has revealed the emergence and predominance of a single H5N1 virus sublineage in poultry since late 2005 that has gradually replaced those previously detected multiple regional distinct sublineages in China. These viruses have already transmitted to neighboring regions thereby resulting in a new transmission and outbreak wave in Southeast Asia. The persistence of H5N1 virus since it first caused human disease in Hong Kong 11 years ago, as demonstrated by its endemicity over a large geographical region, along with repeated disease outbreaks in both poultry and humans indicates that it will be a long-term and difficult task to bring this virus under control in the absence of well developed disease control systems.

Introduction

Extensive surveillance and genetic studies have revealed that highly pathogenic avian influenza H5N1 viruses have become first predominant and then endemic in poultry in southern China and Southeast Asia since 2003 [1]. This resulted in the establishment of multiple distinct regional sublineages [2]. The recognition of multiple different H5N1 sublineages

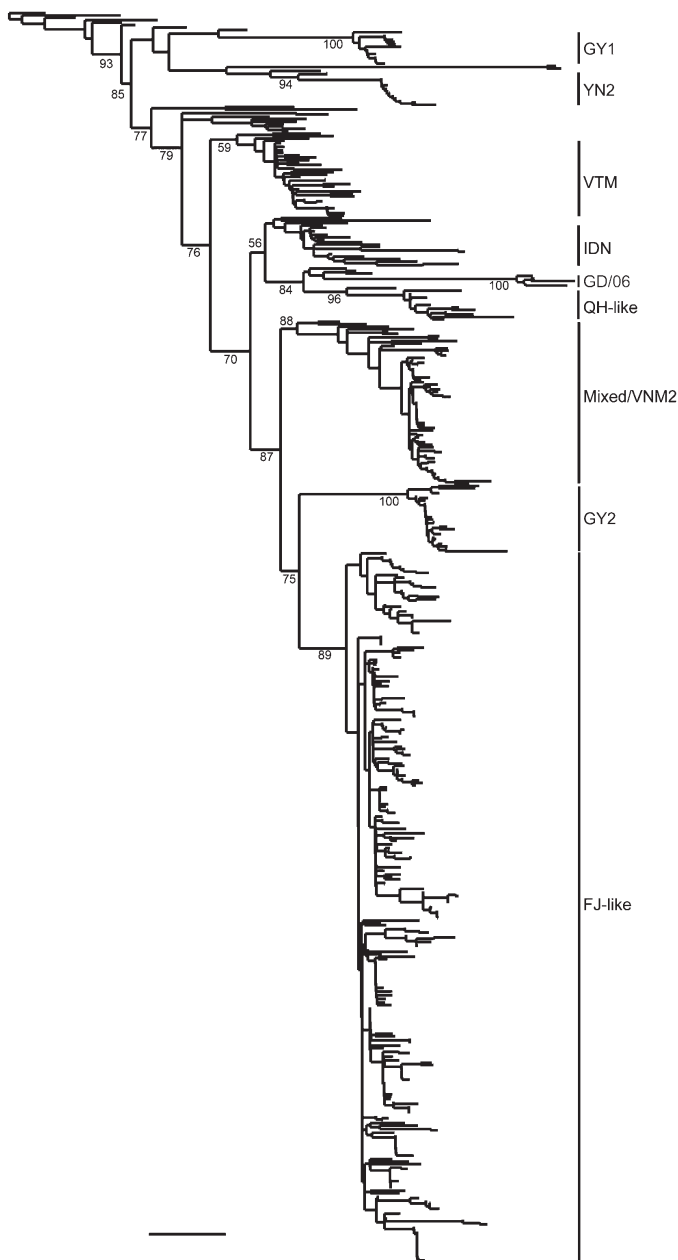
makes it possible to identify the source, and to understand the evolutionary and transmission pathways of those H5N1 viruses that have become widespread in Southeast Asia, Europe and Africa. Since H5N1 influenza virus caused the first outbreak in migratory waterfowls at Qinghai Lake in May 2005 [3], a new transmission and outbreak wave was initiated. The virus expanded its geographical distribution and caused outbreaks in poultry in over 60 countries from Central Asia, the Middle East, Europe and Africa [4]. This led directly to a marked increase in human infection cases and escalated the pandemic threat. From Jan 2006 to June 2007 the World Health Organization (WHO) confirmed 170 cases from 12 countries; while for the period 2003 to 2005 there was a total of 148 cases from just five countries [5]. Also, in Indonesia there were suspected cases of human-to-human transmission involving members of an extended family and the infection sources of other human cases have not been identified [6]. In China, despite a compulsory program for the vaccination of all poultry commencing in September 2005 [7], H5N1 influenza virus has caused outbreaks in poultry in 12 provinces from October 2005 to August 2006 [4]. At the same time, twenty-two human infection cases have been confirmed from 14 provinces since November 2005 [4, 5]. Some of those cases were residents of metropolitan areas remote from poultry farms, such as Guangzhou, Wuhan and Shanghai [4]. Furthermore, there were no obvious poultry outbreaks reported in neighboring markets or farms before or after the onset of those human infections. So whether those people were infected locally and directly from affected poultry or other sources, including humans, is still unknown. This situation directly challenges current pandemic preparedness plans, raising concern that a pandemic could emerge not only from countryside but also from an urban area, just as SARS emerged from the live-animal markets of Guangzhou and the Pearl River Delta [8, 9].

Results

Surveillance. From July 2005 to June 2006 our ongoing influenza surveillance in live-poultry markets in six provinces of southern China showed that 1,294 of 53,220 (overall isolation rate 2.4%) poultry were H5N1 positive. The main body of H5N1 isolates was from duck and goose, with only a small number isolated from chicken (chicken 0.5%, duck 3.3%, goose 3.5%). The prevalence of H5N1 viruses in southern China has increased when compared to the period July 2004 to June 2005 (overall 0.9%, chicken 0.2%, duck 1.3%, goose 2.0%). A winter-seasonal peak was observed from October 2005 to March 2006 as in previous years [1, 2]), during which H5N1 influenza viruses were isolated in each province tested. However, an extension of the peak season was observed in April to June 2006 as isolation rates remained high in these warmer months. Comparison between different types of poultry shows that H5N1 viruses were mainly isolated from domestic duck and goose wherein the viruses were prevalent year-round, while chicken tested positive mostly during the winter. It is notable that in the last 12 months, H5N1 positive chicken were detected in 11 months,

a marked increase from only four positive months in 2004/05 (data not shown). These findings indicate an escalation of H5N1 activity in poultry in 2005/06 compared to previous years. These findings suggest that H5N1 influenza viruses have not been effectively contained in this region and have maintained endemicity broadly in poultry, especially domestic duck and goose.

Figure 1. Phylogenetic relationships of the HA genes of representative influenza A viruses isolated in Asia. Trees were generated by the neighbor-joining method in the PAUP* program. Numbers above or below branches indicate neighbor-joining bootstrap values. Not all supports are shown due to space constraints. Analysis was based on nucleotides 1–1011 and the tree was rooted to A/Tern/South Africa/61. Scale bar, 0.01 substitutions per site.



Phylogenetic Analysis. To better understand the increased prevalence of H5N1 in poultry and the emergence of human infection in China, 390 (30% of total new isolates) of those avian H5N1 influenza viruses isolated from July 2005 to June 2006, plus 16 viruses isolated from smuggled poultry and dead wild birds in Hong Kong in early 2006, were sequenced and analyzed together with sequences available from public databases. Phylogenetic analysis of the hemagglutinin (HA) gene revealed that 266 of 390 (68%) of those recent H5N1 viruses from southern China, formed a distinct H5N1 sublineage (Fujian (FJ)-like, Clade 2.3.4) (Figure 1). Twenty-eight viruses isolated in Guiyang from November 2005 to January 2006 formed a sublineage (GY2, Clade 2.3.3) that is the sister-group to the Fujian-like sublineage (Figure 1). Another three sublineages from Guangdong (GD/06, Clade 2.5, n=6), Guiyang (GY1, Clade 4, n=14) and Yunnan (YN2, Clade 7, n=13) were also identified. A further 59 viruses grouped in the Mixed/VNM2 sublineage (Clade 2.3.2), while only a single virus (Gf/ST/1341/06) belonged to the QH-like sublineage (Clade 2.2) currently circulating in Africa and Europe (Figure 1). The remaining viruses analyzed in this study belonged to previously reported sublineages from China and Southeast Asia, except two isolates from Hunan (Ck/HN/2246/06 and Ck/HN/2292/06), isolated in May 2006 that do not fall with any of these sublineages. The prototype virus of the FJ-like sublineage (Dk/FJ/1734/05) was detected in March 2005. From July to September 2005, only a single strain of 33 sequenced viruses was FJ-like (Table 1).

Table 1. Number of viruses from the Fujian-like sublineage in China.

Collection date		Number of Fujian-like viruses*
2005	Jul-Sep	1/33 (3)
	Oct-Dec	72/136 (53)
2006	Jan-Mar	90/113 (80)
	Apr-Jun	103/108 (95)
Total		266/390 (68)

Remarkably, from October 2005 onwards the percentage of FJ-like viruses detected increased dramatically, until from April to June 2006, 103 of 108 (95%) of H5N1 poultry isolates tested were FJ-like (Table 1). Viruses from other sublineages were not detected in our surveillance since October 2005, November 2005, March 2006, April 2006 and May 2006, respectively. These findings reflect the process of FJ-like viruses gradually becoming predominant in this region. Phylogenetic analysis also revealed that the HA gene of five recent human H5N1 viruses from different provinces of China [4, 10, 11] belong to this FJ-like sublineage and were most closely related to poultry isolates. This suggests that H5N1 human infection from China since November 2005 were infected directly from affected poultry. Furthermore, H5N1 viruses isolated in early 2006 from neighboring regions of mainland China, including the 16 Hong Kong viruses and two poultry isolates from Laos and Malaysia, all joined the FJ-like sublineage.

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Discussion

The emergence of this FJ-like sublineage has had similar consequences to the first wave of virus transmission throughout Southeast Asia in early 2004 [1] and the second wave to Europe and Africa that followed the Qinghai Lake H5N1 outbreak [3, 4]. The findings of our study show that this virus has replaced most of those previously established regional sublineages across a large geographical area in China [2]. The predominance of this FJ-like virus appears to be responsible for the increased prevalence of H5N1 in poultry since October 2005 and recent human infection cases in China [4, 5]. Furthermore, it has already caused poultry outbreaks in Laos, Malaysia, Thailand and Vietnam [4, 5]. As such, this variant has already initiated a third wave of transmission throughout Southeast Asia, and may spread further in Eurasia. It is also probable that this virus will continue to evolve to form other regionally distinct sublineages as witnessed with the H5N1 genotype viruses in the first and second transmission waves [1, 2]. Previously we have described the establishment of multiple sublineages of H5N1 virus in southern China and Southeast Asia [2]. The emergence and replacement of these sublineages by FJ-like viruses within a short period of time highlights the difficulties faced in controlling H5N1 virus in China. A complex ecology and highly diverse virus populations make it almost impossible to capture each circulating virus sublineage even with the application of mass vaccination. This has resulted in recurrent H5N1 outbreaks in poultry in different regions and led to occasional human infection.

Acknowledgements

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Evolution of H9N2 Influenza A Viruses in Quail From Southern China

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H9N2 influenza A viruses have become established and maintain long-term endemicity in terrestrial poultry in Asian countries. Occasionally these viruses transmit to other mammals including humans. Increasing epidemiological and laboratory findings suggest that quail may be an important host as they are susceptible to different subtypes of influenza viruses. To better understand the role of quail in influenza ecology and evolution, H9N2 viruses isolated from quail during 2000 to 2005 were antigenically and genetically characterized. Our results showed that only three subtypes of influenza viruses (H5, H6, H9) could be detected in quail in southern China. Furthermore, H9N2 viruses are prevalent year-round in southern China with higher isolation rates observed in the winter, and that those viruses mainly replicate asymptotically in the respiratory tract of quail. Antigenic and genetic analysis revealed that both the G1-like (genotype A series) and Ck/Bei-like H9N2 lineages (genotype B series) were co-circulating in quail since 2000. Phylogenetic analyses demonstrated that most of the isolates tested were double or multiple reassortant variants, with four G1-like (A0-A3) and 16 Ck/Bei-like genotypes (B1-16) recognized. Non-reassortants of Ck/Bei-like viruses were not detected in quail. A novel genotype of G1-like virus, designated as genotype A3, had become predominant in quail since 2003, while multiple Ck/Bei-like genotypes were introduced to quail wherein they incorporated G1-like gene segments, but none of them became established in this host. Those Ck/Bei-like reassortants generated in quail have then been introduced to other poultry. These complex interactions form a two-way transmission system between quail and other types of poultry. The identification of HA and NP genes with high homology to Ty/WI/1/66 in some H9N2 viruses isolated from quail in 2001 suggested that those viruses had not evolved naturally. The present study provides evidence that H9N2 and H5N1 subtype viruses have also exchanged gene segments to generate currently circulating reassortants of both subtypes that have pandemic potential. Continuing influenza surveillance in poultry is critical to understanding the genesis and emergence of potentially pandemic strains in this region.

Introduction

Influenza A H9N2 viruses are present worldwide in poultry populations [1, 2]. In terrestrial poultry of southern China, two H9N2 virus lineages have become established since the mid-1990s. One virus lineage, represented by Ck/Bei/1/94 or

Dk/HK/Y280/97, is mainly prevalent in chicken, while the other one, represented by Qa/HK/G1/97, is predominant in quail [3]. Recent studies suggested that quail may have participated in the genesis of the H5N1 virus (H5N1/97-like virus) responsible for the Hong Kong 'bird flu' incident [3] and are also susceptible to different subtypes of influenza viruses [4]. Therefore, quail may play an important role in the evolution and ecology of influenza A viruses.

Results

In the present study, H9N2 influenza viruses isolated from quail from 2000 to 2005 were genetically and antigenically characterized. Our findings showed that both the G1-like and Ck/Bei-like H9N2 influenza virus lineages co-circulate in quail. Novel Ck/Bei-like genotypes were introduced into quail and further reassorted with G1-like viruses endemic in quail. Those H9N2 reassortants with G1-like gene segments have then transmitted to other poultry forming a complex system of two-way transmission between quail and other types of poultry. Genetic analysis also provides evidence that H9N2 and H5N1 subtype viruses have a two-way exchange of gene segments to generate current genotypes of both subtypes that have pandemic potential.

Prevalence of H9N2 Influenza Viruses in Quail. Systematic surveillance of market quail from 2000 to 2005 resulted in 610 influenza isolates from 4,601 samples collected (total isolation rate, 13.3%). Three influenza subtypes were identified, H9N2 (n = 414), H6N1 (n=184) and H5N1 (n=12). H9N2 influenza virus in quail was prevalent year-round but an increased isolation rate was usually observed during the winter season (October to March). Three hundred and ninety-six of 414 (95.7%) of those H9N2 viruses were isolated from tracheal swabs, and only 18 influenza viruses were isolated from the cloacal samples. This information suggests that influenza A virus mainly replicates in the respiratory tract of quail in the field.

Phylogenetic Analysis of the Surface Genes. To understand the evolution and ecology of H9N2 viruses in quail, 73 representative viruses isolated during 2000 to 2005 were genetically characterized. Phylogenetic analysis of the H9 hemagglutinin (HA) gene revealed that 33 of those viruses belonged to the G1-like lineage, while the remaining 40 isolates were closely related to Ck/Bei-like viruses (Figure 1A). The G1-like viruses gave rise to a stable lineage reflecting their long-term endemicity and evolution in this host. Among those Ck/Bei-like viruses two subgroups were recognized in quail since 2000 (Figure 1A). Subgroup 1 consisted of 19 viruses, represented by Qa/ST/243/00, and subgroup 2 consisted of 21 viruses represented by Dk/HK/Y280/97 (Figure 1a). It is noteworthy that a single virus Ck/Heilongjiang/35/2000 (Ck/HLJ/35/00) is almost identical to (99.6% homology) to Ty/Wisconsin/1/1966 (Ty/WI/1/66) [5], an early H9N2 subtype reference strain from the North American lineage. Phylogenetic analysis of the neuraminidase (NA) gene revealed a similar evolutionary patterns as the HA gene tree, with the NA genes of all but one virus corresponding with the lineage of the HA

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gene (Figure 1B). The exception was Qa/ST/4762/01 with a Ck/Bei-like HA had a G1-like NA gene, revealing reassortment between the two virus sublineages. These findings show that G1-like viruses have remained endemic in quail, and that Ck/Bei-like viruses were introduced into this species in 2000, wherein they have co-circulated with G1-like viruses.

Phylogenetic Analysis of the Internal Genes. Phylogenetic analysis of the six internal genes revealed that H9N2 viruses from quail in southern China have undergone extensive reassortment to generate multiple novel genotypes (data not shown). In the PB2 gene tree, representative H9N2 viruses clustered into three different lineages; 58 were G1-like, while 14 formed a group from an unknown avian source, likely derived from aquatic birds in the region. One virus, Qa/ST/1038/02, clustered with the H5N1/01-like viruses. Analysis of PB1 gene showed those H9N2 quail isolates formed three distinct lineages, including G1-like ($n = 59$), Ck/Bei-like ($n = 9$), and unknown avian ($n = 5$). Their PA genes also fall into three different groups, with most closely related to H5N1/01-like viruses. The NP gene of the H9N2 viruses separated into four groups: G1-like ($n = 37$), Ck/Bei-like ($n = 14$), H5N1/01-like ($n = 16$) and unknown avian ($n = 6$). It is interesting to note that the virus Ck/Shanghai/F/1998 (Ck/SH/F/98) contains an NP gene segment that was previously first detected in H5N1 virus in 2001 [6]. Furthermore, the NP gene of Ck/HLJ/35/06 is almost identical (99.9% homology) to Ty/WI/1/66. The M gene of 11 viruses grouped with Ck/Bei-like viruses and the remaining viruses were G1-like. The NS gene of 45 viruses was Ck/Bei-like, while that of the other 28 viruses were closely related to G1-like H9N2 viruses.

Genotyping. Viruses with a G1-like HA are designated as genotype A series and those with a Ck/Bei-like HA as genotype B series. Therefore, non-reassortant G1-like viruses are designated as A0, while reassortant G1-like viruses are designated sequentially as A1, A2, and so on, according to when the novel genotype was first identified. In the same manner, non-reassortant Ck/Bei-like viruses are designated as B0, and novel reassortants then numbered sequentially as B1, B2, and so on. Phylogenetic analysis revealed 20 different reassortant H9N2 genotypes isolated from 2000 to 2005 in quail. Four genotypes were G1-like (genotypes A0-A3), while 16 genotypes were Ck/Bei-like (genotypes B1-B16). In the G1-like lineage, non-reassortant G1-like virus (genotype A0) was detected from 2000 to 2002. Since 2002, H9N2 genotype A3 virus emerged and became predominant in quail and is the only G1-like virus detected in this host since 2003. For Ck/Bei-like viruses, a different complement of reassortant H9N2 genotypes were detected in quail each year, but none of them became established in this host. These genotypes were all double or triple reassortants of Ck/Bei-like, G1-like, H5N1/01-like and unknown avian viruses, with the exception of genotypes B7, B10 and B15 which are four-way reassortants.

Discussion

Characterization of H9N2 influenza viruses isolated from quail from 6 years of influenza surveillance revealed that

both G1-like and Ck/Bei-like viruses were co-circulating in this host in southern China since 2000. Genetic and antigenic studies demonstrated that a single H9N2 G1-like reassortant (genotype A3) had become established and predominant in this host since 2003, indicating these viruses were genetically stable and well adapted to quail. However, the Ck/Bei-like virus lineage (genotype B series) appeared very unstable, with new short-lived reassortants emerging each year, none of which had become established, indicating those viruses were not well adapted to this host. Our findings suggest that after introduced into quail, Ck/Bei-like viruses had further reassorted with G1-like viruses endemic in quail and subsequently transmitted to other poultry. These complex interactions formed a two-way transmission system between quail and other types of poultry. Through this system, quail served as a "mixing vessel" to facilitate many reassortment events in the current influenza ecosystem. The present study also suggests that H5N1/01-like internal genes were first incorporated into Ck/Bei-like viruses in 2001, and then into G1-like viruses in 2002 (Fig. 2). However, the phylogenetic relationships of the NP genes suggests that a H9N2 virus, Ck/SH/F/98-like, may be a possible donor of H5N1/01-like internal genes. If this is the case then the gene flow between those subtypes may be in the reverse direction. Furthermore, the dominant G1-like virus found in quail (genotype A3), along with all Ck/Bei-like viruses isolated in this study since 2003, incorporate an H5N1/01-like PA gene that is also found in current H5N1 genotype Z viruses [7]. In this regard, quail may serve as a "mixing vessel" to facilitate reassortment events between H9N2, H5N1 and H6N1 viruses to facilitate the emergence of viruses with pandemic potential in this region.

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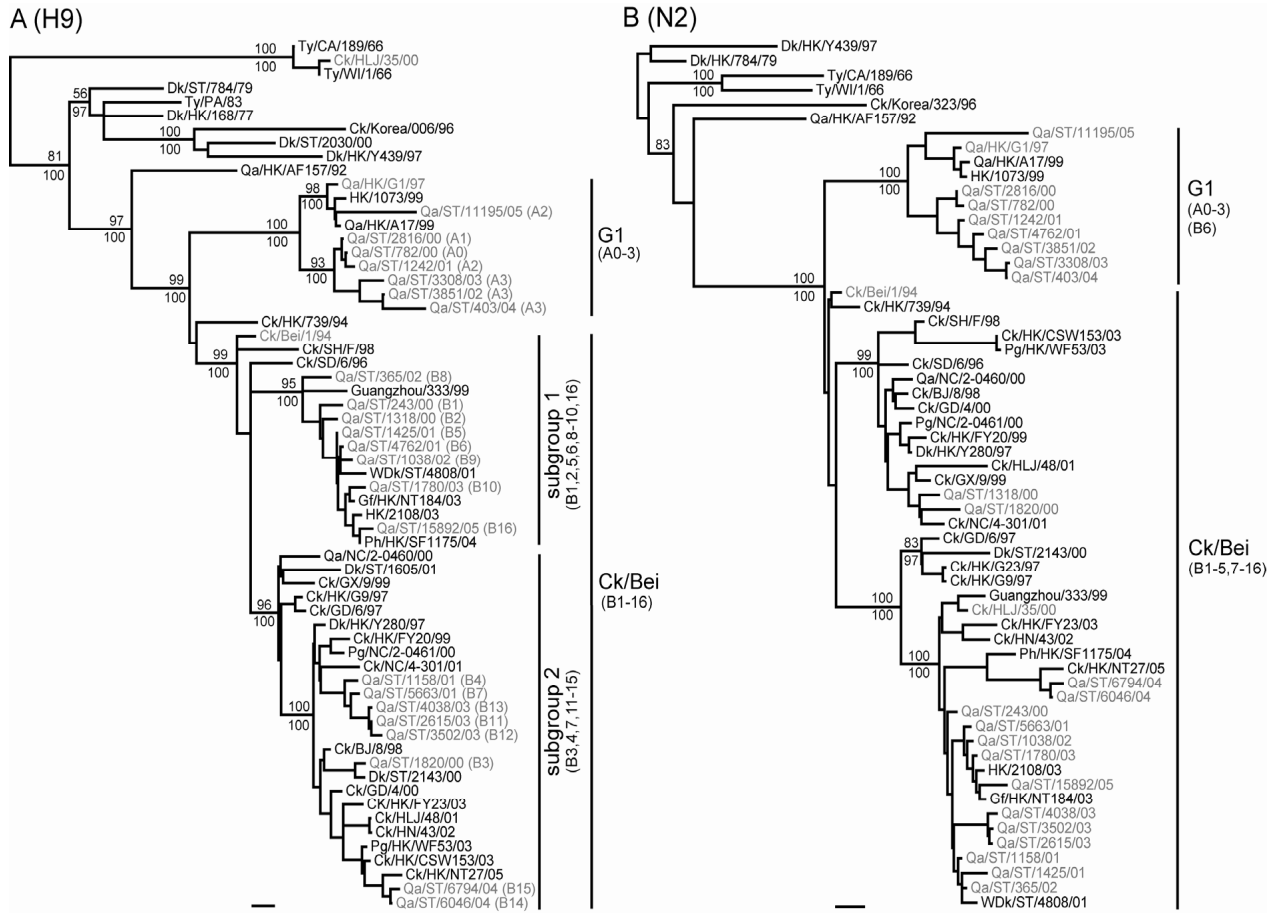
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Figure 1. Phylogenetic relationships of the HA (A) and NA (B) genes of representative influenza A viruses isolated in Asia. Trees were generated by the neighbor-joining method in the PAUP* program (Bayesian analysis revealed similar relationships.) Numbers above and below branches indicate neighbor-joining bootstrap values and Bayesian posterior probabilities, respectively. Not all supports are shown because of space constraints. Analysis was based on nucleotides 129-1042 of the HA gene and 231-1297 of the NA gene. The HA and NA trees were rooted to Qa/Arkansas/29209-1/93 (H9N2) and Ck/Pennsylvania/8125/83 (H5N2), respectively. Genotypes characterized in this study were shown brackets. Scale bar, 0.01 substitutions per site. BJ and Bei, Beijing; Ck, chicken; Dk, duck; GD, Guangdong; Gf, Guinea fowl; GX, Guangxi; HLJ, Heilongjiang; HN, Henan; HK, Hong Kong; NC, Nanchang; Pg, pigeon; Ph, Pheasant; Qa, quail; SCK, silky chicken; SD, Shandong; SH, Shanghai; ST, Shantou; Ty, turkey; WDK, wild duck.



Characterization of Low Pathogenic H5 Subtype Influenza Viruses From Eurasia: Implications for the Origin of Highly Pathogenic H5N1 Viruses

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Highly pathogenic avian influenza (HPAI) H5N1 viruses are now endemic in many Asian countries. The immediate precursor of these HPAI viruses was recognized as A/Goose/Guangdong/1/96 (Gs/Gd)-like H5N1 HPAI viruses first detected in Guangdong in 1996. However, precursors of the Gs/GD-like viruses and their subsequent reassortants have not been fully determined. Here we characterize low pathogenic avian influenza (LPAI) H5 subtype viruses isolated from poultry and migratory birds in southern China and Europe from the 1970s to the 2000s. Phylogenetic analyses revealed that Gs/GD-like virus was likely derived from an LPAI H5 virus in migratory birds. However, its variants arose from multiple reassortments between Gs/GD-like virus and viruses from migratory birds, or with those Eurasian viruses isolated in the 1970s. It is of note that unlike HPAI H5N1 viruses, those recent LPAI H5 viruses have not become established in aquatic or terrestrial poultry. Phylogenetic analyses revealed the dynamic nature of the influenza gene pool in Eurasia with repeated transmissions between the eastern and western extremities of the continent. The data also shows reassortment between influenza viruses from domestic and migratory birds in this region that has contributed to the expanded diversity of the influenza gene pool among poultry in Eurasia.

Introduction

Aquatic birds are considered the natural reservoirs of influenza A virus as all known subtypes (H1-H16 and N1-N9) have been isolated from them [1]. Genetic analyses have also suggested that all influenza viruses found in other hosts were derived from those viruses resident in aquatic birds [2]. Influenza viruses from aquatic birds have occasionally transmitted to terrestrial poultry leading to disease outbreaks, however, only some H5 and H7 subtypes influenza viruses have evolved into highly pathogenic strains that have caused significant mortality in poultry. Previous studies have demonstrated that, in the case of H5 and H7 subtypes, low pathogenic avian influenza viruses (LPAI) precursors may evolve into HPAI once transmitted to domestic poultry [3-5].

Results

Prevalence of low pathogenic H5 influenza viruses. Between July 2000 and December 2005, a total of 167,858 samples of domestic duck, geese and migratory ducks were collected in southern China and Hong Kong. Seventy-two LPAI H5 viruses were isolated on 10 sampling occasions during over-winter season (isolation rate 0.043%). The main body of those isolates was from sentry farm ducks or directly from migratory birds. Only two isolates were detected from retail market bird. In 1970s' surveillance, among 11,798 market samples 23 LPAI H5 viruses were isolated on 10 sampling occasions (isolation rate 0.2%). Most of the viruses were from domestic ducks with only one isolated from goose. It must be noted that no LPAI H5 viruses were recognized in terrestrial poultry in our surveillance.

Phylogenetic analysis of the surface genes. Phylogenetic analysis of the H5 HA gene showed that all viruses separated into the American and Eurasian lineages (Figure 1A). Within the Eurasian lineage two major sublineages of "early" and "contemporary" viruses are apparent. The first sublineage consists predominantly of older viruses from across the continent, including those viruses isolated in Hong Kong from 1976 to 1980, along with viruses from Germany, Japan and the United Kingdom. There is only one LPAI H5 virus from Italy (Ck/Italy/9097/97) that falls into this group which was introduced recently to chicken from this gene pool. Therefore, this sublineage of H5 subtype viruses probably represents the early domestic Eurasian gene pool of H5 viruses (Figure 1A). The second major Eurasian sublineage is composed of three distinct groups. Group 1 contains all but one of those recent viruses from China and Italy characterized in present study, along with other Eurasian isolates, from both domestic and migratory birds. However, four Italian viruses isolated during the outbreaks from 1997 to 1998 and one obtained from a mallard clustered together within this group, suggesting that after introduction into terrestrial poultry those viruses had undergone significant evolution. The phylogeny of this group, together with that of the "early" sublineage, indicates the continued genetic exchange within this influenza gene pool between the eastern and western extremities of the Eurasian continent. Group 2 consists exclusively of viruses from Western Europe, with the exception of the virus Dk/Chany Lake/9/03 that was isolated in Novosibirsk, Russia in Central Asia (Figure 1A). In contrast, group 3 contained viruses from the Western Pacific isolated from domestic duck and swans, along with HPAI Gs/GD-like H5N1 viruses that have subsequently spread throughout Eurasia [6]. This group therefore seems to match one of the major migratory flyways in this region. Phylogenetic analysis showed that three N2 genes from recent southern China H5N2 isolates (Gs/GY/3799/05, Dk/JX/1286/05 and Dk/JX/3345/05) were closely related to the N2 genes of viruses obtained from mallard in Italy and duck in Hokkaido, Japan (data not shown). Analysis also showed that the N3 gene from those recent H5N3 viruses isolated in southern China are closely related to H7N3 subtype viruses from domestic and

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migratory birds in Italy from 2001 to 2003 (data not shown). Therefore, both the HA and NA genes of those H5N2 and H5N3 viruses recently isolated from domestic ducks or migratory birds in southern China were from the Eurasian lineage and appear to be derived from viruses resident in migratory birds. Phylogenetic analysis of N1 NA also revealed that the NA gene of Gs/GD-like virus was most closely related to Dk/Hokkaido/55/95 (H1N1). Another H5N1 variant (Ck/Hebei/718/01) had an N1 that was almost identical to African Starling/England-Q/983/79 (H7N1), which was investigated as a vaccine candidate in China [7].

Phylogenetic analysis of the internal genes. In general, all internal gene trees, represented by the M gene, were divided into the American and Eurasian lineages, and the “early” and “contemporary” sublineages were observed within the Eurasian lineage (Figure 1B). The internal gene phylogenies further highlight the dynamic nature of the influenza gene pool in Eurasia with viruses from the east and west frequently clustering together. For the LPAI H5N2 virus, Ck/Ibaraki/1/05 all available gene segments clustered with the Mexico-like HPAI from 1994. It is noteworthy that several Gs/GD-like variants isolated from northern China from 1997 to 2004 consistently clustered within the “early” Eurasian sublineage (Figure 1B). Most of the LPAI H5 viruses from southern China clustered or grouped together with some viruses from European viruses, including those Italian HPAI and LPAI viruses. To identify the possible source of Gs/GD-like HPAI H5N1 viruses and its variants, we summarized their most phylogenetically closely related viruses for each of the gene segments (Table 1). For Gs/GD-like viruses, three of the gene segments were likely derived from migratory birds, and four of the genes are most closely related to those viruses isolated from ducks in Hokkaido, Japan. Furthermore, some of the variants isolated from our surveillance during 2001 to 2005 in southern China contain gene segments that are most closely related to gene segments from viruses in the migratory bird gene pool (Table 1). It is noteworthy that these analyses confirm that several H5N1 variants isolated from northern China from 1997 to 2004 contained many gene segments belonging to the early Eurasian gene pool identified from viruses isolated in the 1970s and 1980s. Moreover, it is surprising that one H5N1 virus (Ck/Hebei/718/01) contained an NA N1 gene with very high homology (98.3%) to that of African starling/England-Q/983/79 (H7N1).

Discussion

The findings of present study revealed that LPAI H5 viruses were isolated predominantly from migratory or sentry ducks during the winter, and were barely detected in market waterfowl and not found in terrestrial poultry in southern China from 2000 to 2005. This suggests that interspecies transmission of LPAI H5 virus from migratory birds to domestic waterfowl did occur, but that those viruses did not subsequently become prevalent in aquatic or terrestrial domestic birds. In contrast, influenza surveillance in the 1970s revealed that LPAI H5 viruses were found year-round in domestic waterfowl, indicating that there has been a change in the ecology of influenza in southern China. This likely resulted from the long-term endemicity of HPAI H5N1

viruses and extensive vaccination in poultry leading to increased host selection pressure. In the present study, the phylogenies of Gs/GD-like H5N1 virus genes either clustered with viruses from migratory birds or with those viruses isolated from ducks in Hokkaido, Japan (Table 1). As no systematic surveillance was conducted from 1980 to 1997 in southern China, we failed to identify the direct precursor of Gs/GD-like virus which emerged in 1996 [8]. Even though it is not clear whether those Hokkaido viruses were from domestic or migratory ducks, many of them clustered directly with the viruses isolated from migratory ducks at Poyang Lake, Jiangxi and Mai Po Marshes, Hong Kong since 2002. We therefore speculate that the duck viruses from Hokkaido may also be of migratory bird derivation. Thus, the available findings suggest that Gs/GD-like H5N1 virus may be derived from a LPAI H5N1 virus in migratory waterfowl along the West Pacific migratory flyway. A precedent for such an introduction and subsequent change from LPAI to HP has also been observed in Europe [3-5].

Acknowledgements

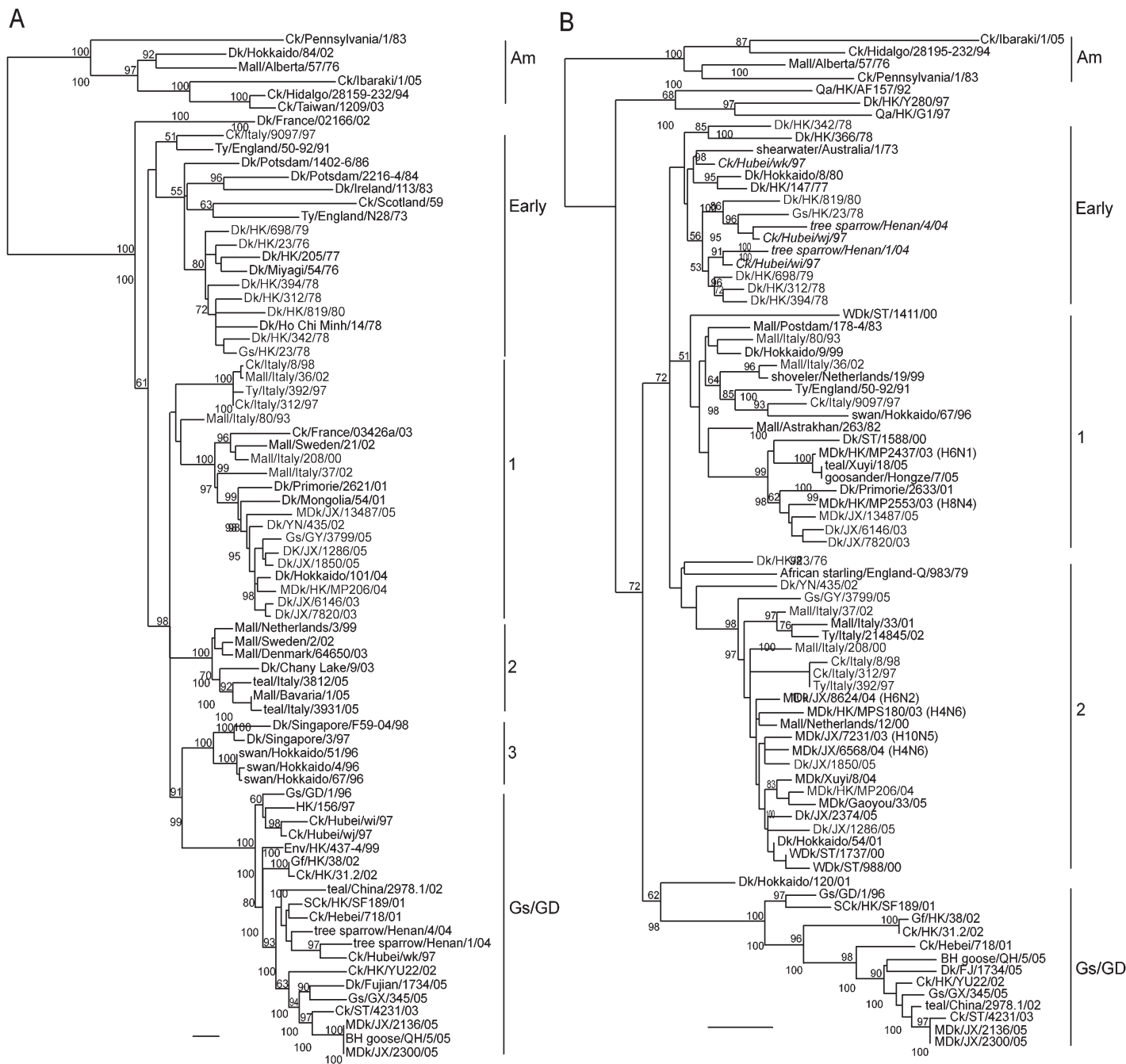
This study was supported by the Research Fund for Control of Infectious Diseases and Research Grants Council (HKU 7512/06M and HKU1/05C) of the Hong Kong SAR Government, the Li Ka-Shing Foundation, and the National Institutes of Health (NIAID contract HHSN266200700005C).

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Figure 1. Phylogenetic relationships of the HA (A) and M (B) genes of representative influenza A viruses isolated in Asia, American and Europe. Numbers above below the branch nodes indicate neighbor-joining bootstrap values >50% and Bayesian posterior probabilities >95, respectively, analyses were based on nucleotides 22-1032 of the HA gene and 26-947 of the M gene. The HA and M gene trees were rooted to A/Japan/305/57 and A/Equine/Prague/1/56, respectively. Scale bar, 0.1 nucleotide substitutions per site.



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Table 1. Possible sources of gene segments of highly pathogenic H5N1 influenza viruses isolated in China, 1996 to 2005.

Virus	Gene segment ^a									
	PB2	PB1	PA	HA	NP	NA	M	NS		
Gs/GD/1/96	MB?	Hokkaido	MB?	Hokkaido	unknown	Hokkaido	Hokkaido	MB		
Sck/HK/SF189/01	MB?	Gs/GD	Gs/GD	Gs/GD	Gs/GD	Gs/GD	Gs/GD	unknown		
Gf/HK/38/02	H9N2	MB	Gs/GD	Gs/GD	unknown	Gs/GD	Gs/GD	MB		
Ck/HK/31.2/02	MB	MB	Gs/GD	Gs/GD	MB?	Gs/GD	Gs/GD	MB		
teal/China/2978.1/02	Gs/GD	Gs/GD	MB	Gs/GD	MB	Gs/GD	Gs/GD	unknown		
Ck/HK/YU22/02	MB	Gs/GD	MB	Gs/GD	unknown	Gs/GD	Gs/GD	unknown		
Ck/ST/4231/03	MB	Gs/GD	MB	Gs/GD	unknown	Gs/GD	Gs/GD	unknown		
Gs/GX/345/05	Gs/GD	Gs/GD	MB	Gs/GD	unknown	Gs/GD	Gs/GD	unknown		
Dk/FJ/1734/05	MB?	Gs/GD	MB	Gs/GD	unknown	Gs/GD	Gs/GD	unknown		
Ck/Hubei/wj/97	Early	-	Early	Gs/GD	Early	Gs/GD	Early	Early		
Ck/Hubei/wj/97	Early	Early	Early	Gs/GD	Early	Gs/GD	Early	Early		
Ck/Hubei/wk/97	unknown	Early	Early	Gs/GD	Early	Gs/GD	Early	Early		
Ck/Hebei/718/01	-	Gs/GD	MB	Gs/GD	Early	AS/Eng	Gs/GD	Gs/GD		
TS/Henan/1/04	Early	Early	Early	Gs/GD	Early	Gs/GD	Early	Early		
HK/156/97	G1	G1	G1	Gs/GD	G1	W312	G1	G1		

AS/Eng, African starling/England-O/983/79; Early, 1970s Eurasia aquatic virus; Unknown, cannot identify source from the currently available influenza data; -, gene sequence not available; G1, Qa/HK/G1/97-like (H9N2) virus; W312, teal/HK/W312/97-like (H6N1) virus; Hokkaido, virus sequences published with the names Dk/Hokkaido/9/99 (PB1), swan/Hokkaido/4/96 (HA), Dk/Hokkaido/55/96 (NA), Dk/Hokkaido/120/01 (M).

Swine-Influenza Viruses Isolated From Humans From the US, 1990 to 2006

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Since 1990, a total of 10 cases of human infection with swine influenza A (H1N1) viruses have been identified by the Influenza Division at CDC. In most of the cases, contact with pigs prior to the illness was documented. Ten swine-like influenza A (H1N1) viruses isolated from humans were examined antigenically and genetically. Those viruses were antigenically most closely related to classical swine H1N1 viruses. All eight gene segments of six viruses isolated between 1990 and 1998 were clearly of swine origin and closely related to those of classical swine H1N1 viruses circulating among pigs in the United States. However, four recent viruses were triple reassortant viruses, each having genes of swine, human, and avian lineage. The NS, M, NA, NP, and HA genes of those isolates were of classical swine H1N1 virus origin; the PB1 gene was derived from a human influenza A virus, and the PA and PB2 genes were most closely related to those of avian influenza viruses. Although human infections with swine influenza viruses appear to be infrequent, continued isolation of triple reassortant viruses from humans demonstrates the potential for further reassortment of such virus with other subtypes which may result in novel viruses capable of efficient transmission among humans. Enhanced influenza surveillance plays a key role in the early detection and identification of novel influenza virus.

Introduction

Domestic pigs are highly susceptible to influenza A viruses and may serve as potential "mixing vessels" to facilitate the emergence of new viral gene combinations [1, 2]. Classical H1N1 swine influenza A viruses were first isolated by Shope in the 1930's [3]. From the mid-1970's to 1998, the epidemiology of swine influenza within the continental United States has remained fairly constant. Classical H1N1 viruses were the predominant type of virus isolated from pigs [4-6], although influenza of other subtypes have been isolated relatively commonly from pigs elsewhere in the world. In 1997/98, H3N2 viruses of human origin began to be isolated from pigs in the U.S. Subsequently, reassortment between classical H1N1 and human H3N2 viruses led to the appearance of H1N2 viruses. Further reassortment of the virus with an avian influenza virus resulted in the emergence of triple reassortant H3N2, H1N2 and H1N1 viruses [7-9]. In 1976, an influenza outbreak among army recruits at Fort Dix, New Jersey first focused public awareness on swine influenza virus infections in humans (10). Sporadic cases of human infection with these viruses have

been reported since then, but for the most part, humans were dead end hosts, and these isolated incidences did not result in further person-to-person transmission [4, 11, 12]. However, serological evidence from both humans and pigs suggest that virus traffic between these two species may occur more regularly [6]. In the most recent 2 decades, sporadic cases of humans becoming infected with classical swine influenza viruses have continued to occur in the U.S. [13-15]. Since 1990, a total of ten cases of human infection with swine influenza A viruses were identified by the Centers For Diseases Control and Prevention and, from each case, an influenza A (H1N1) virus was isolated and characterized. Although human cases are relatively rare, the movement of any influenza A virus across the species barrier is a public health concern, in part, due to the possibility of genetic reassortment between divergent virus subtypes. *In this report, we present a brief summary of our study on human infection by swine influenza A viruses during the past 2 decades in the U.S.*

Results and Discussion

Human infections with classical swine H1N1 virus, 1990-98. In 1990, a previously healthy 13-year old male was ill with clinical presentations of pneumonia. He had a history of recent contact with pigs prior to onset of his illness. A swine-like influenza virus, A/Oklahoma/11/90, was isolated from the patient. In 1992, a 4-year old female became ill with respiratory symptoms. She had a known history of recent swine contact and no predisposing medical conditions. Her clinical complications from the infection included pneumonia; A/Nebraska/01/92 virus was isolated from this case. In 1994, a 37-year old Indiana female with no underlying health conditions was ill with pneumonia. She had a known history of recent swine contact; A/Indiana/01/94 virus was isolated from her. In 1998, another case from Indiana was identified; the patient was a 4-year old male. He had no underlying medical conditions. However, additional clinical information about this patient could not be obtained; A/Indiana/05/95 virus was isolated from the boy. Each of these first four patients completely recovered from their illness. In 1991, a 37-year old man from Maryland became ill after contact with pigs. The patient was healthy prior to onset of the illness but died of pneumonia and other complications. A/Maryland/12/1991 virus was isolated, and a detailed study on this case was reported by Wentworth in collaboration with the CDC [13]. In 1995, a previously healthy 37-year old immunocompetent female from Minnesota became ill and the infection resulted in the death of the patient. A detailed case history of this patient has been described elsewhere [15]; A/Minnesota/18/95 virus was isolated from the patient. Antigenic analyses by comprehensive Hemagglutination Inhibition tests (HI) using post-infection ferret sera raised against representative swine and human virus strains revealed that all six of the human isolates were well inhibited only by antisera raised against classical swine influenza viruses, indicating that they were antigenically most closely related to swine H1N1 viruses (Table 1). Complete or partial gene sequencing and/or

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Table 1. Antigenic analysis of swine and swine-like influenza A viruses using hemagglutination inhibition tests.

	Ferret antisera to				Sheep Sera to	
	Classical Swine H1N1 Viruses				Human Viruses	
	NJ/8/76	NV/101/82	MD/12/91	SW/VI/97	H1N1	H3N2
Reference Antigens						
A/New Jersey/8/76	<u>160</u> ¹	320	320	320	<10	<10
A/Nevada/101/82	320	<u>640</u>	320	320	40	<10
A/Maryland/12/91	1280	320	<u>640</u>	320	40	<10
A/Swine/Wisconsin/125/97	320	640	640	<u>640</u>	10	<10
Five Human Isolates						
A/Oklahoma/11/90	320	640	320	640	20	<10
A/Nebraska/1/92	320	160	640	160	20	<10
A/Indiana/1/94	320	640	640	640	40	<10
A/Indiana/5/95	320	640	640	320	40	<10
A/Minnesota/18/95	160	640	320	320	10	<10

¹ Homologous titers for reference antigens are underlined.

PCR-RFLP were used for rapid genotyping. In all cases, the genes of the six human viruses were clearly of classical swine origin and closely related to those of classical swine H1N1 viruses circulating among pigs in the United States (Data not shown). *Human infections with swine triple reassortant H1N1 virus, 1998-2006.* In late December of 1998, an H1N1 influenza A virus, A/Wisconsin/10/98 was isolated from the nasopharyngeal swab of a 57-year old man from Marathon County, Wisconsin. Two days after the onset of symptoms, the patient presented to his physician with chills, fever, and a cough. He was diabetic, but otherwise in good health and had not recently traveled outside of Wisconsin. The man butchered a hog every one to two months, including one a few days before the onset of his symptoms. The patient was treated with amantadine and recovered without further complications. In 2005, A/Wisconsin/87/2005 virus was isolated from a 17-year old Hmong Laotian male who lives with his parents in Wisconsin. He did not have a history of recent travel, but had helped his brother butcher some pigs before onset of his illness. He recovered from the disease without further complications. In early 2006, A/Missouri/4/2006 virus was isolated from a 7-year old boy who was HIV positive and had immigrated from Kenya to the US when he was 3 years old. He had no history of exposure to pigs or livestock. He received antiviral therapy and recovered from influenza. In November 2006, a 4-year old girl from Iowa became ill and was hospitalized. She had no known direct swine exposure. An influenza A virus, A/Iowa/1/2006 was isolated from the child. She recovered from the disease without further complications. Antigenic analysis by HI using a reference panel of post-infection ferret antisera revealed that all four viruses were well inhibited by antisera raised against classical swine H1N1 influenza viruses or swine-like H1N1 viruses isolated from humans (data not shown). These results indicated that the HA glycoproteins of the recent four isolates were antigenically similar to classical swine H1N1 viruses. All eight gene segments of the 4 viruses were partially or fully sequenced. At the nucleotide level, the HA and NA genes of the viruses were found to be highly homologous to those of previously described classical swine virus lineage. The NS, M, and NP genes were also found to be most similar to those of previously described classical H1N1 swine viruses. However, their polymerase genes were highly homologous to those of previously described H1N1 triple reassortant viruses indicating

that the ultimate origin of the PB1 gene was a human H3N2 virus lineage and that the PA and PB2 genes arose from one or more avian virus lineages (Fig. 1). Phylogenetic analyses of these six genes confirmed that the polymerase genes of the 4 viruses were derived from multiple influenza lineages (data not shown).

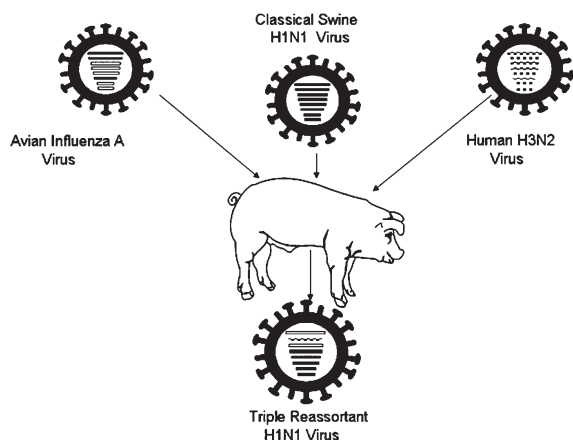
Conclusions

Human cases of swine influenza virus infection continue to occur in the United States. Contact with pigs is known to be a risk factor for human infections. As was the case in most of the ten patients described in the present study, most clinical cases of swine-influenza in humans have occurred following contact with pigs. This epidemiological pattern is consistent with the direct movement of swine viruses from porcine to human hosts. Six H1N1 viruses isolated from humans between 1990 and 1998 were classical swine H1N1 viruses containing all eight genes segments from circulating swine viruses. Four H1N1 viruses isolated from recent human cases contain genes from classical swine, avian and human influenza viruses; and are closely related genetically to swine triple reassortant viruses that are circulating among pigs in the US. Although such infections appear to be infrequent, the repeated isolation of triple reassortant viruses from humans demonstrates the potential for further reassortment of such viruses with other subtypes which may result in efficient transmission of novel viruses among humans. Strengthening influenza surveillance plays a key role in early detection and identification of novel influenza virus in humans.

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Figure 1. Genome constellation of swine influenza A (H1N1) triple reassortant virus.



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Proceedings Topic #9

Antivirals and Resistance

Oral

Presentations

Monitoring of Influenza Virus Sensitivity to M2 Blockers and Neuraminidase Inhibitors (NAIs) During Three Influenza Seasons (2004 -2007)

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The arsenal of antivirals for control of influenza virus infections is currently limited to two classes of drugs: M2 blockers and NAIs. The M2 blockers amantadine and rimantadine are ineffective against influenza B viruses lacking the M2 protein. The main tool used for M2 resistance monitoring in the present study was pyrosequencing, which allows for high throughput analysis of the M2 transmembrane domain sequence. Recent and unprecedented increase in resistance to M2 blockers among influenza A(H3N2) viruses circulating worldwide diminishes the M2 blockers effectiveness. In the US alone, resistance to M2 blockers climbed from 11% in 2004-05 season to 96% the next season and still remains high (~84%) in 2006-07. These findings underline the importance of drug resistance surveillance to NAIs. Oseltamivir and zanamivir are currently approved for the control of influenza infections; while peramivir is undergoing clinical evaluation. Virus susceptibility to NAIs is commonly assessed in an enzyme activity inhibition assay with either a chemiluminogenic or fluorogenic substrate. In the present study, we utilized a recently developed NASTar[®] kit for the detection of NAI-resistant mutants by means of chemiluminescence. In total, 1559 virus isolates collected worldwide were tested with zanamivir and oseltamivir carboxylate. The IC₅₀ values were assessed and analyzed for A(N1), A(N2), and B NA enzymes separately. The viruses exhibiting IC₅₀ values > (mean IC₅₀ + 3 SD) were re-tested and subjected to NA sequence analysis. A novel substitution Arg371Lys conferring resistance to zanamivir and oseltamivir carboxylate was detected in the influenza B virus. Three influenza A(H1N1) viruses contained His274Tyr mutation, previously shown to cause resistance to oseltamivir. Although frequency of NAI-resistance detection remains low among seasonal field isolates, a close monitoring of resistance to both classes of anti-influenza drugs is a global priority.

Introduction

The two surface viral proteins, the M2 protein and the enzyme NA, are targets for current anti-influenza drugs. Influenza B viruses are naturally resistant to M2 blockers (amantadine and rimantadine) because they lack the M2 protein. Molecular markers of resistance in influenza A viruses have been previously established and encompass residues 26, 27, 31, 32 and 34 within the transmembrane domain of the M2 protein.

Monitoring resistance to M2 blockers is primarily based on the analysis of the M gene sequences. Despite availability of amantadine and/or rimantadine for prophylaxis and treatment of influenza infections in many countries, the frequency of resistance detection remained low (<1%) among field isolates for many years. In 2003, a sharp rise in resistance to M2 blockers was detected among A(H3N2) viruses circulating in Asia and coincided with SARS and bird flu scare [1-2]. The rapid worldwide spread of resistance to M2 blockers [1-4] highlights the importance of close monitoring of resistance to a second class of anti-influenza drugs, NAIs. In contrast to M2 blockers, molecular markers of resistance to NAIs are not so well established. A majority of resistance-conferring mutations occurs in the NA active site; nevertheless, their location differs by NA type/subtype and by drug used in the selection process [5]. Therefore, detection of NAI-resistance is primarily done in the NA activity inhibition assays [6]. Other challenges in NAI-resistance monitoring include a lack of a standardized procedure and the complexity of result interpretation.

Material and Methods

Compounds. Neuraminidase inhibitors oseltamivir carboxylate and zanamivir were provided by their respective manufacturers. **Viruses.** Influenza virus isolates collected by the WHO Global Influenza Surveillance Network during three consecutive seasons: 2004-05, 2005-06, and 2006-07 were used. With few exceptions, viruses were propagated in MDCK. The following NAI-sensitive and -resistant reference strains were utilized: A/Texas/36/91 (H1N1) and its oseltamivir-resistant mutant His274Tyr; A/turkey/Minnesota/833/80 (H4N2) and its zanamivir-resistant mutant Arg292Lys; B/Memphis/20/96 and its zanamivir- and oseltamivir-resistant mutant Arg152Lys. **Pyrosequencing.** A 264 base pair product covering nucleotides 764-1027 of the influenza A matrix (M) gene was amplified according to manufacturer's protocol (Biotage, Uppsala, Sweden). A 44 base pair region, from nucleotides 764 to 827 that encodes amino acids 25-38 of the M2 protein was analyzed for markers of resistance [1-2].

NA inhibition assay. To carry out high throughput screening for drug resistance, we utilized the NASTar Influenza Neuraminidase Inhibitor Resistance Detection kit as suggested in the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Of note: NAIs and reference resistant strains are not included in the kit. The time of incubation of virus with NAI and substrate was 10 min. Luminescence was read with the use a multiplate reader equipped with injectors (Victor3 V, Perkin Elmer, USA). To determine the IC₅₀ values, we used the nonlinear curve-fitting, dose-response analysis software (Robosage, kindly provided by Drs. Lutz and Tisdale, GSK, UK). The IC₅₀ values were analyzed separately for each NA type/subtype and each drug to identify potential drug resistant mutants. The extreme outliers were excluded from statistical analysis used to determine the mean IC₅₀ value and SD. Virus isolates with IC₅₀ values greater than the mean IC₅₀ value + 3 SD were re-tested and subjected to the NA sequence analysis. To

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determine the mean and SD of the IC₅₀ values, Sigma plot, v. 10 (Richmond, CA, USA) was used.

Table 1. Trends in M2 resistance among A(H3N2) viruses collected globally over the three influenza seasons.

	2004-2005	2005-2006	2006-2007
Africa	0/4	0/2	0/6
Asia (total):	139/408 (34%)	113/144 (79%)	92/146 (63%)
Mainland China	71/76 (93%)	8/8(100%)	36/36 (100%)
SAR Hong Kong	38/52 (73%)	7/7(100%)	4/8 (50%)
Other Asia	30/280 (11%)	98/129 (76%)	52/102 (51%)
Europe	10/73(14%)	32/75 (43%)	20/52 (39%)
North America (total):	146/1346 (11%)	839/869 (97%)	255/387 (66%)
Canada	1/20 (5%)	8/8(100%)	43/136 (32%)
Mexico	5/16 (31%)	70/72 (97%)	1/1 (100%)
United States	140/1310(11%)	761/789 (96%)	211/250 (84%)
Oceania	1/8(13%)	2/3 (67%)	N/A
South America (total)	18/248 (7%)	73/76 (96%)	15/16 (94%)
Total	314/2087(15%)	1059/1169 (91%)	382/607 (63%)

Table 2. Assessment of IC50 Values in the chemiluminescent assay for influenza virus isolates circulating worldwide during the three influenza seasons. * Mild outliers, viruses with IC50 value > the mean IC50 value + 3 SD. [§]Extreme outlier(s) were excluded from the statistical analysis.

Virus	n	Oseltamivir carboxylate			Zanamivir		
		Range	Mean ± SD	Cutoff *	Range	Mean ± SD	Cutoff *
A(H1N1)	423 [§]	0.30-6.05	1.22 ± 0.65	3.17	0.49-7.30	1.54±0.81	3.97
A(H3N2)	549	0.11-5.40	0.67 ± 0.50	2.27	0.58-30.54	3.65±2.80	12.00
B	587 [§]	0.88-11.63	3.60 ±1.42	7.85	1.00-24.12	4.86±2.82	13.32

Results

Resistance to M2 blockers. Resistance to M2 blockers among A(H3N2) viruses isolated worldwide (n=2087) was an estimated 15% during the 2004-05 season; during the same period, it reached 11% in the United States. A dramatic increase in the frequency of resistance was observed in A(H3N2) viruses (n=1169) during the 2005-06 influenza season, when the resistance reached 100% in several Asian countries and peaked at 96.4 % in the United States. Analysis of the viruses collected from various geographic areas during the current 2006-07 influenza season (n=607) showed that the resistance has decreased globally (~35 %), although still remains high (84%) in the US and some other countries (Table 1). The increase in M2 blocker-resistance among influenza A(H1N1) was first detected in 2004-05, when the incidence reached 4% among isolates collected globally (n=195) [7]. In the next season, it climbed to 16% and remains at 17% in the 2006-07 season among isolates

collected worldwide. There is, however, a noticeable difference in the geographic distribution of resistant mutants (Table 1).

Resistance to NAIs. In total, 1559 influenza A and B virus isolates collected worldwide during the last three influenza seasons were tested against zanamivir and oseltamivir carboxylate with the use of the NAStar kit. A set of the well-characterized NAI-sensitive and -resistant reference strains of each NA type/subtype were included in the assay. The IC₅₀ values for each virus isolate were determined against both inhibitors and analyzed separately for each NA antigenic type/subtype (Table 2). There was little variance in the mean IC₅₀ values calculated for an individual season (results not shown). Influenza A(H1N1) viruses were the most sensitive to zanamivir and A(H3N2) viruses – to oseltamivir carboxylate (Table 2). Overall, influenza B viruses were the least sensitive to inhibition by zanamivir and oseltamivir carboxylate. The influenza A and B viruses exhibiting very high IC₅₀ values (extreme outliers) were excluded from the statistical analysis. The influenza B/Hong Kong/36/2005 virus was found to be an extreme outlier when tested against zanamivir and oseltamivir carboxylate. Sequence analysis of the NA from this virus revealed an Arg to Lys substitution at position 371 (N2 numbering). This novel mutation, associated with resistance to NAIs, occurred at the catalytic residue in the enzyme active site. The four other extreme outliers were influenza A(H1N1) viruses collected during the three seasons. Three of the A(H1N1) extreme outliers contained the substitution His274Tyr in the NA(N1) active site known to confer resistance to oseltamivir. The NA sequence analysis of the fourth outlier is underway. All viruses, exhibiting the IC₅₀ values greater than a cutoff IC₅₀ value were re-tested. Very few consistent outliers have been detected after repeated assay. The sequence analysis of the NA from those mild outliers is ongoing.

Discussion

The molecular markers of influenza A virus resistance to M2 blockers have been established in previous years. This allowed the development and successful implementation of pyrosequencing technology for high throughput screening for M2 blocker-resistance among seasonal isolates. Based on the results of this study and other reports [8], it appears that fitness of influenza A(H3N2) viruses carrying substitution S31N in M2 protein is not compromised. The same mutation, S31N, was detected in a majority of drug resistant A(H1N1) viruses detected during the last three seasons. Acquisition of resistance by A(H1N1) viruses was not a result of gene reassortment with drug resistant A(H3N2) viruses [7]. The wide spread of M2 resistance diminishes the effectiveness of the existing arsenal of anti-influenza drugs and highlights the need for close monitoring of resistance to NAIs. In the present study, we utilized the NAStar kit[®] for high throughput screening for NAI-resistance by means of the enzyme activity inhibition. In contrast to M2 blockers, molecular markers of resistance to NAIs are often NA subtype-specific and drug-specific. The other challenge in the NAI-resistance surveillance is a lack of

the clear criteria for resistance. In this study, the conclusion of virus sensitivity to NAIs was based on the IC_{50} value determined with the use of curve-fitting computer software (i.e., Robosage). We postulated that outliers with the IC_{50} values $>$ mean IC_{50} value $+ 3SD$ are considered potentially drug resistant. The NA sequence analysis of all found outliers has not yet been completed. Nevertheless, several drug-resistant mutants have been detected. The novel substitution (Arg \rightarrow Lys) at catalytic residue 371 conferred cross-resistance to zanamivir, oseltamivir carboxylate, and peramivir (results not shown). Since NAIs are the only FDA approved drugs to control influenza B virus infections, detection of isolates cross-resistant to this class of drugs raises justifiable concern. When introduced into N2 enzyme, Arg371Lys conferred resistance to zanamivir and oseltamivir carboxylate and did not significantly compromise the growth of the recombinant virus in cells [9]. The detection of influenza A and B viruses carrying Tyr at 274, a marker of resistance to oseltamivir, is another cause for concern. The NA inhibition assay is an important tool for monitoring resistance to NAIs and its standardization and other improvements are needed.

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The Antiviral Activity of Oseltamivir Against H5N1 Human A/Vietnam/1203/04 and A/Turkey/15/06 Influenza Viruses in Ferrets

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We evaluated the use of the neuraminidase (NA) inhibitor oseltamivir for early post-exposure prophylaxis and for treatment in ferrets exposed to H5N1 influenza virus. Ferrets given oseltamivir 5 mg/kg/day 4 hours p.i. were protected from lethal infection with the A/Vietnam/1203/04 (H5N1) virus but higher daily doses (25 mg/kg/day) were required for treatment when initiated 24 hours p.i. For ferrets inoculated with the less pathogenic A/Turkey/15/06 (H5N1) virus, a treatment of 10 mg/kg/day of oseltamivir reduced the lethargy of the animals, inhibited inflammation in the upper respiratory tract, and blocked virus spread to the internal organs. Importantly, all ferrets that survived the initial infection were re-challenged with homologous virus after 21 days and were protected from infection. Direct sequencing of the NA or HA1 genes in both H5N1 viruses isolated from treated ferrets showed no amino acid substitutions known to cause drug resistance in conserved residues. Analysis of individual clones detected NA amino acid changes in 1 of 10/20 clones (V116A; H274Y) sequenced from samples of ferret inoculated with A/Vietnam/1203/04 (H5N1) virus and treated with 10 mg/kg/day and in 1 of 10 clones (H274R; E277Q) sequenced from sample of ferret treated with 25 mg/kg/day of oseltamivir. However, no changes in antiviral susceptibility *in vitro* were found. These studies demonstrated that oseltamivir was effective against infection by two clades of H5N1 influenza viruses in ferrets. Early oseltamivir treatment is crucial for protection against highly pathogenic H5N1 viruses and higher dose may be needed for the treatment of more virulent viruses.

Introduction

At the present time human H5N1 influenza virus infection has been documented in 12 Eurasian countries, with a mortality rate >50% (17). Although person-to-person transmission remains limited (15), the rapid evolution, genetic diversity, unprecedented geographic spread, and changing ecology of the virus raise pandemic concerns. Strain-specific vaccines are considered the best preventive therapy, but antiviral drugs will clearly be the most important short-term resource at the start of a pandemic. Stockpiling of antiviral drugs is an essential

component of global influenza pandemic preparedness. However, limited information is available about the efficacy of antiviral drugs against potential H5N1 pandemic viruses. Oseltamivir, an NA inhibitor, was used successfully to control the transmission of highly pathogenic avian H7N7 influenza virus in the Netherlands (9). It has also been used in patients infected with H5N1 virus in Asia, but generally only 5 to 10 days after the onset of symptoms and at suboptimal doses (1, 14). It is not known whether antiviral drugs that are effective against contemporary human influenza viruses will be effective against systemically replicating H5N1 viruses. In the absence of human trials of antiviral drugs against these viruses, animal models offer the best experimental approach. Both of the NA inhibitors (zanamivir and oseltamivir) increase survival in animal models of H5N1 infection. Zanamivir protected mice against lethal challenge with A/Hong Kong/156/97 (H5N1) influenza virus and protected chickens against highly pathogenic A/Chick/Victoria/1/85 (H7N7) virus (4, 5). The orally administered NA inhibitor oseltamivir was an effective treatment for H5N1 and H9N2 influenza virus infection in mice (3, 12). Recent studies showed a significantly dose-dependent effect against A/Vietnam/1203/04 (H5N1) virus in mice and a need for higher-dose and/or more prolonged treatment for this more virulent strain (18). In the present study, we determined the dose of oseltamivir required to protect against H5N1 lethal infection and to ameliorate the duration and severity of disease with respect to virus pathogenicity, virus load, the time of initiation of treatment, and the emergence of oseltamivir-resistant variants.

Materials and Methods

Compound. The NA inhibitor oseltamivir carboxylate and oseltamivir phosphate (oseltamivir) were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

Viruses. The H5N1 influenza viruses A/Vietnam/1203/04 and A/Turkey/15/06 were grown in the allantoic cavities of embryonated chicken eggs for 32 h at 36°C. Virus titer was determined by calculating the 50% egg infectious dose (EID₅₀) per ml.

Drug susceptibility in tissue culture. MDCK cells (ATCC, Manassas, VA) were grown in minimal essential medium and drug susceptibility was determined by plaque reduction assay (6).

Assessment of drug efficacy in ferrets. Young adult male ferrets (Marshall's Farms, North Rose, NY) were anesthetized with isoflurane and inoculated intranasally with H5N1 virus in 1.0 ml PBS. For post-exposure prophylaxis, groups of 5 ferrets were inoculated with A/Vietnam/1203/04 virus at a dose of either 10 or 10² EID₅₀. Treatment with oseltamivir (5 mg/kg/day) was twice daily for 5 days began 4 hours p.i. Clinical signs of infection, relative inactivity index (13), weight, and temperature were recorded daily. For delayed treatment, groups of 5 ferrets were inoculated with either 10² EID₅₀ of A/Vietnam/1203/04 or 10⁶ EID₅₀ of A/Turkey/15/06 virus. Oseltamivir treatment (10 or 25 mg/kg/day) in twice-daily doses for 5 days was initiated 24 hours p.i. Three weeks after inoculation with H5N1

virus, surviving ferrets were re-challenged with 10^2 EID₅₀ of A/Vietnam/1203/04 and 10^7 EID₅₀ of A/Turkey/15/06 virus.

Titration of virus in upper respiratory tract and organs. On days 3 and 7 p.i., ferrets were anesthetized with ketamine (25 mg/kg) and nasal washes were collected. Organs were collected on day 6 p.i. from 2 animals in each treatment and control groups. **Virus sequence analysis.** Viral RNA was isolated by the RNeasy Mini kit (Qiagen, Valencia, CA). Samples were reverse-transcribed and analyzed by PCR using primers specific for the HA and NA genes (7). TOPO TA Cloning (Invitrogen, Carlsbad, CA) and sequencing of individual plaques obtained in MDCK cells were also performed.

Results

Efficacy of early post-exposure prophylaxis. Influenza A/Vietnam/1203/04 (H5N1) virus is extremely pathogenic to ferrets and a dose as low as 10 EID₅₀ resulted in severe disease and the deaths of 2 of 3 animals, and a dose of 10^2 EID₅₀ caused severe clinical signs and was lethal to all animals (Table 1). The ferrets that received 5 mg/kg/day of oseltamivir 4 hours after inoculation survived lethal virus challenge, although disease was not prevented (Table 1). This drug regimen effectively inhibited virus replication in the upper respiratory tract and in the lungs and small intestine. However, virus was detected in the brain, liver, and spleen of 1 of 2 animals inoculated with 10^2 EID₅₀. At the lower infectious dose, virus titers were significantly lower in all organs ($P < 0.05$), with the exception of one sample from the brain.

Efficacy of delayed treatment against A/Vietnam/1203/04 (H5N1) virus. When oseltamivir treatment initiated 24 hours p.i., all animals treated with 10 mg/kg/day experienced fever and weight loss comparable to that in control ferrets. They were extremely lethargic, and 2 of 3 animals showed severe neurological signs. All of these ferrets died between days 7 and 8 p.i. Moreover, delayed treatment with 10 mg/kg/day of oseltamivir did not inhibit virus replication in the upper respiratory tract and virus was detected in multiple organs (Table 1).

Table 1. Effect of early post-exposure (4 h p.i.) and delayed (24 h p.i.) oseltamivir treatment on the survival and clinical signs of H5N1 virus infection in ferrets.

Virus and virus dose (EID ₅₀ /ferret)	Oseltamivir regimen ^a	No. survived/total no.	No. of ferrets showing indicated clinical sign(s) of disease/total no.					Virus titer in nasal washes ^b		
			Weight loss ^c	Increase in temperature ^c	Respiratory ^d	Neurological ^e	RII ^f	3 day p.i.	7 day p.i.	
A/Vietnam/1203/04										
10	4 h delay, 5 mg/kg/day	3/3	2/3	1/3	0/3	1/3	0.17	<	<	
10	4 h delay, 0 mg/kg/day	1/3	(8.8 ± 1.9)	(5.0) ^g	2/3	0/3	1/3	0.68	2.9 ± 0.5	4.5 ^h
10 ²	4 h delay, 5 mg/kg/day	3/3	(21.4 ± 1.6)	(8.6 ± 1.2)	0/3	0/3	0.34	<	<	
10 ²	4 h delay, 0 mg/kg/day	0/3	3/3	(3.4) ^g	1/3	2/3	1.25	4.4 ± 0.8	6.5 ^h	
10 ²	24 h delay, 10 mg/kg/day	0/3	(25.2 ± 1.4)	(7.6 ± 1.4)	0/3	2/3	1.05	3.9 ± 0.6	5.5 ± 0.5	
10 ²	24 h delay, 25 mg/kg/day	3/3	(22.1 ± 2.9)	(5.7 ± 1.4)	0/3	2/3	0.30	3.1 ± 0.2	3.5 ± 0.7	
10 ²	24 h delay, 0 mg/kg/day	0/3	(13.4 ± 1.7) [*]	(6.0 ± 0.4)	1/3	3/3	1.21	4.0 ± 0.7	6.1 ± 0.5	
A/Turkey/15/06										
10 ⁶	24 h delay, 10 mg/kg/day	3/3	3/3	3/3	2/3	0/3	0.15	5.4 ± 0.4	<	
10 ⁶	24 h delay, 0 mg/kg/day	3/3	(17.9 ± 2.8)	(6.0 ± 0.9)	3/3	1/3	0.46	5.2 ± 0.4	3.3 ^h	
			(19.0 ± 1.8)	(5.7 ± 0.4)						

Importantly, when the dose of oseltamivir was increased (25 mg/kg/day), treatment resulted in 100% survival. Although

this regimen did not protect ferrets from disease, it markedly decreased its severity and the magnitude of weight loss and fever as compared to that in control animals. Treatment with 25 mg/kg/day of oseltamivir significantly decreased virus titers in the upper respiratory tract as compared to those in untreated animals ($P < 0.05$), and completely inhibited virus replication in the internal organs of 1 of 2 animals. In the other, virus was detected only in the brain.

Efficacy of delayed treatment against A/Turkey/15/06 (H5N1) virus. The ferrets that received treatment with 10 mg/kg/day of oseltamivir 24 hours p.i., stayed more active throughout the observation period, regained weight faster than untreated animals, and had a mean peak temperature increase 0.7°C lower than that in the controls (Table 1). Although virus titers were little affected by this drug regimen, the peak nasal inflammatory cell counts in nasal washes were significantly lower in treated than in control animals on days 5 and 7 p.i. ($P < 0.05$) and cell counts had returned to near-normal in the treatment group on day 5 p.i. Virus was not detected in the lungs or other internal organs of ferrets treated with 10 mg/kg/day of oseltamivir.

Re-challenge with a lethal dose of H5N1 virus. To determine whether oseltamivir treatment alters antibody production and thus immune protection against infection with a new virus, we re-challenged the surviving treated ferrets with a lethal dose of A/Vietnam/1203/04 virus. After the first challenge, ferrets showed low HI titers (1:20-1:40) against homologous antigen but this level of serum antibodies was sufficient to provide protection against lethal H5N1 virus challenge. Ferrets initially challenged with A/Turkey/15/06 (H5N1) virus showed homologous HI titers of 1:160-1:320. Animals were completely protected from re-challenge with 10^7 EID₅₀ of the virus; they showed no disease signs and none shed virus on day 3 p.i.

Emergence of oseltamivir-resistant variants. Direct sequence analysis revealed only one amino acid substitution (I418M) in a virus isolated from the brain of a single animal inoculated with 10 EID₅₀ of A/Vietnam/1203/04 (H5N1) virus and treated with 5 mg/kg/day of oseltamivir (Table 2). No changes were detected in the HA1 subunit. Plaque reduction assay did not show changes in the susceptibility to NA inhibitor. Direct sequencing of the 18 samples obtained from ferrets that received delayed treatment with 10 or 25 mg/kg/day of oseltamivir revealed no mutations in the surface glycoproteins. Analysis of virus clones showed minor population of clones carrying NA/HA mutations: 1 clone with a V116A mutation in the NA gene and 1 clone with a V178I mutation in the HA1 region out from 20 individual plaques (Table 2). We found NA amino acid changes in 1 of 10 clones (H274Y) sequenced from the lung sample of ferret treated with 10 mg/kg/day of oseltamivir and in 1 of 10 clones (H274R; E277Q) sequenced from the brain sample of ferret treated with 25 mg/kg/day of oseltamivir (Table 2). Importantly, plaque reduction assays showed no change in the susceptibility of those samples to NA inhibitor. Only one mutation was detected in the nasal wash sample of ferret inoculated with A/Turkey/15/06 virus and treated with oseltamivir. This mutation, 193R→K in the HA1 region, did

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not result in reduction of susceptibility to NA inhibitor in vitro (Table 2).

Table 2. Emergence of resistance viruses during oseltamivir treatment.

Virus/Virus dose (EID ₅₀ /ferret)	Oseltamivir regimen	Origin of sample	Amino acid change (no. clones/total no. sequenced) ^a		Virus reduction assay (nM, mean EC ₅₀ ± SD) ^b
			NA	HA (HA1)	
A/Vietnam/1203/04					
10 ²	0 mg/kg/day	Nasal wash			0.5 ± 0.1
10	4-h delay	Brain	1418M	—	0.5 ± 0.1
	5 mg/kg/day				
10 ²	5 mg/kg/day	Brain	—	—	NT
		Liver	—	—	NT
		Spleen	—	—	NT
10 ²	24-h delay	Nasal wash	V116A (1/20)	V178I (1/20)	0.5 ± 0.1
	10 mg/kg/day	Lungs	H274Y (1/10)	—	0.5 ± 0.1
10 ²	24-h delay	Brain	H274R (1/10)	NT	0.5 ± 0.1
	25 mg/kg/day		E277Q (1/10)		
A/Turkey/15/06					
10 ⁶	0 mg/kg/day	Nasal wash			32.2 ± 0.1
10 ⁶	24-h delay	Nasal wash	—	R193K	27.7 ± 0.4
	10 mg/kg/day				

Discussion

In the present study we found that a low dosage of oseltamivir protects ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus when treatment is started shortly after virus exposure. The virulence of this virus was a factor, requiring a higher oseltamivir dosage when treatment was delayed 24 hours post virus exposure. Against A/Vietnam/1203/04 (H5N1) oseltamivir was not effective at a dosage of 10 mg/kg/day (equivalent to the approved human dose of 75 mg/kg twice daily) (16) in preventing death of animals. Higher drug dosages (25 mg/kg/day) were required to protect animals from death. In ferrets inoculated with the less pathogenic A/Turkey/15/06 (H5N1) virus, oseltamivir at dosage of 10 mg/kg/day inhibited inflammation in the upper respiratory tract and blocked the spread of virus to the internal organs. The ferret model is advantageous for studies such as this one, in that it allows evaluation not only of virological parameters but also of clinical signs of infection. In our re-challenge experiments, animals were protected from lethal challenge with homologous H5N1 virus. Delayed oseltamivir treatment decreased the virus load but did not completely protect from illness. Therefore, oseltamivir treatment did not interfere with the development and maintenance of immunity to homologous H5N1 virus. The ferrets used in the experiments had serum antibodies against influenza virus of the H3 HA subtype before inoculation with H5N1 virus. It is possible that initial infection with H3N2 virus provided some cross-protection against heterologous subtypes. Emergence of resistant variants during the course of antiviral therapy has not been addressed extensively in animal models. The mouse model is the preferred choice for antiviral studies, although it may not be optimal for identifying the emergence of resistant variants due to different receptor specificity in mice and humans (8). Ferret tracheal epithelial cells express primarily sialic acid (SA) α₂,6 galactose receptor structures, and a lesser amount of SA α₂,3-galactose receptors (11). Therefore, this model more closely represents the human airway epithelium and allows

the study of NA and HA mutations that may emerge during oseltamivir treatment. Aside from the choice of model, the best method of assessing the emergence of resistant variants is unclear. Our direct sequencing of samples did not detect NA or HA amino acid changes that might confer resistance. More sophisticated analysis was required to detect a mixture of clones, one of which carry NA or HA mutations. Mutations at position 274 of NA are of major concern, as the therapy-associated emergence of oseltamivir-resistant H5N1 clones that had an H274Y NA mutation was recently described (2, 10). We identified such a mutation in 1 out of 10 clones sequenced; therefore, the clones that carried NA mutation were a small proportion of the overall virus population analyzed, and we found no change in oseltamivir susceptibility. In conclusion, these studies demonstrated that oseltamivir was effective against infection by two different clades of H5N1 influenza viruses in ferrets. A more pronounced effect was observed when treatment started early after virus exposure and thus highlighted the importance of timing in the use of oseltamivir. Therefore, strategies must be developed which can provide the earliest possible administration of antiviral drugs at appropriate dosages to individuals infected or strongly suspected of being infected with H5N1 influenza virus.

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Indonesian H5N1 Viruses Demonstrate Decreased Sensitivity to Oseltamivir

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Introduction

Two different strains of highly pathogenic H5N1 avian influenza have been circulating since 2003. Clade 1 has been found in Vietnam, Thailand, Cambodia, Laos and Malaysia. Clade 2 subsequently emerged and spread from China to Indonesia, Europe and Africa in 2004-2005. While it remains largely an infection of birds, as of July 2007 there have been 318 laboratory confirmed cases in humans, with 192 deaths. Indonesia has one of the highest fatality rates of around 80%, with 102 confirmed cases and 81 deaths. Due to its systemic availability oseltamivir is the drug of choice for treating infected humans [1]. However, several groups have reported the emergence of resistant viruses in clade 1 infected H5N1 patients treated with oseltamivir, and suggested that higher doses of oseltamivir may be needed [2-4].

Materials and Methods

Avian influenza H5N1 isolates from chickens, ducks, geese and quail were grown in eggs, and viruses were gamma irradiated to inactivate any infectivity. This does not affect the neuraminidase (NA) enzyme activity. We tested the drug sensitivity of NAs [5] from clade 1 viruses from 2004 from Vietnam and Malaysia, from 2004-5 from Cambodia and from clade 2 viruses from 2005 from Indonesia. Sensitivity was determined using the fluorescent based MUNANA (Sigma) enzyme inhibition assay [5]. Sensitivities were compared to those of a reference human H1N1 strain, A/Mississippi/3/2001, originally identified as having an H274Y mutation which confers high level resistance to oseltamivir [6]. However plaque purification of this isolate revealed it contained a mixed population of both resistant and sensitive progeny, thus providing wild type and resistant pairs as reference strains.

Results

All viruses were initially tested against both zanamivir and oseltamivir in the same assay. Despite coming from different countries and different avian species all clade 1 and clade 2 viruses had a similar sensitivity to zanamivir as the reference H1N1 NA, (Table 1). However, in this assay the sensitivities of the NAs to oseltamivir (oseltamivir carboxylate) fell into three groups (Table 1). The clade 1 isolates from 2004 were all more sensitive to oseltamivir than the human H1N1 wild type control, consistent with recent findings of Rameix-Welti et al. [7]. However the NAs of the 2005 Cambodian viruses showed a 6-7 fold decrease specifically in oseltamivir sensitivity compared to the 2004 clade 1 Vietnam and Cambodian isolates. These 2005

isolates came from the same area, as one of the more sensitive Cambodian 2004 isolates (Kandal), suggesting at least regional evolution had occurred. Of more concern was the third group.

Table 1. Mean IC₅₀'s of NA sensitivities in MUNANA based enzyme inhibition assay for H5N1 isolates from each region compared to human H1N1 wild type and resistant H274Y plaques¹.

	Zanamivir	Oseltamivir	4-Amino-Neu5Ac2en
	Mean IC ₅₀ nM	Mean IC ₅₀ nM	Mean IC ₅₀ μM
H1N1			
A/Mississippi/3/2001 [2] wt	1.18 (0.24)	2.16 (0.31)	1.12*
A/Mississippi/3/2001 H274Y [4]	1.41(0.26)	475.1 (344)	1.31*
Clade 1 H5N1 2004			
Malaysia 2004 [2]	1.21 (0.13)	0.50 (0)	
Malaysia 2004 [2]		0.44(0.11)	2.82 (0.77)
Mean		0.47 (0.07)	
Vietnam 2004 [8]	1.40 (0.44)	0.69 (0.21)	
Vietnam 2004 [4]		0.27 (0.04)	2.47 (0.42)
Mean		0.55 (0.26)	
Cambodia 2004 [6]		0.54 (0.22)	
Cambodia 2004 [4]		0.21 (0.02)	
Mean		0.41 (0.24)	

¹Results are from two independent assays, with all isolates tested in the first assay simultaneously against zanamivir and oseltamivir (top row of each pair), []= numbers tested from each region. A subset from each region was tested in the second assay against a new preparation of oseltamivir and 4-aminoNeu5Ac2en.

IC₅₀'s for each isolate were calculated using the Sigmaplot non-linear curve fitting function.

Values in brackets are standard deviations for the means of the individual IC₅₀'s for the isolates in each group.

*Several plaques were tested from the A/Mississippi/3/2001 against zanamivir and oseltamivir, but only one plaque of each was test against 4-amino Neu5Ac2en.

The NAs from all the clade 2 2005 Indonesian viruses demonstrated a 15-30-fold decrease in sensitivity specifically to oseltamivir compared to the clade 1 viruses, (Table 1). Govorkova et al. [8] also recently showed that the A/Turkey/15/06 clade 2 virus was almost 60-fold less sensitive to oseltamivir in a plaque reduction assay compared to the clade 1 A/Vietnam/1203/04. Both these results and those of Rameix-Welti et al. [7] contrast to those recently published by Hurt et al. who showed no difference between the sensitivities of clade 1 and clade 2 isolates to oseltamivir in the enzyme assay [9] despite many of the isolates being the same as tested here. The reason for the discrepancy is not known. Although both drugs are based on the transition state analogue of sialic acid, 2 deoxy-2,3-dehydro-N-acetyl neuraminic acid, (DANA) there are differences in the chemistry of the two drugs, which affect the resistance profile. There are mutations which are drug specific, conferring resistance to only one of the drugs, and subtype specific mutations, conferring resistance in either an N1 or N2 subtype [10]. Zanamivir has a single substitution of a guanidinium group at the 4' position on the sugar ring compared to DANA, whereas oseltamivir has a substitution of an amino group at the 4' position compared to DANA, but more importantly a bulky hydrophobic pentyl ether

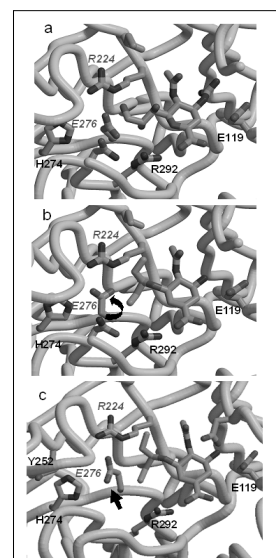
group replaces the glycerol side chain at the 6' position. In order to accommodate this bulky side chain, reorientation of E276 in the active site is necessary to create a hydrophobic pocket for oseltamivir to bind. Mutations which prevent this reorientation occurring, lead to high levels of specific oseltamivir resistance (Fig. 1 H274Y, R292K). Decreased binding to oseltamivir can also be due to altered interactions with its 4-amino group (Fig. 1 E119V)[11]. We have another inhibitor, 4-aminoNeu5Ac2en which has the conserved glycerol side chain in common with DANA and zanamivir, but it has an amino group at the 4' position, the same as oseltamivir. This allows us to determine whether altered binding is due to interactions with the 4'-amino group or the 6-pentyl ether group. We tested a subset of each group of viruses for sensitivity to 4-aminoNeu5Ac2en and simultaneously retested these against a new preparation of oseltamivir. The clade 1 and 2 viruses all had similar sensitivity to 4-aminoNeu5Ac2en (Table 1). The altered binding to oseltamivir was also confirmed as falling into the three groups as previously observed. This indicated that the decreased binding to oseltamivir was specifically due to altered interactions around the 6-pentyl ether group, thus explaining why no altered binding to zanamivir was seen. Because of the potentially important implications of our findings for public health and stockpiling strategies, and because of the apparent discrepancy to the results of Hurt et al [9] we retested two viruses from each group against an independent source of oseltamivir carboxylate (provided by Biota, Australia). Similar results were obtained, with a shift in sensitivity of around 8-fold between the clade 1 2004 isolates (mean IC_{50} 0.55 nM and 2005 Cambodian isolates (mean IC_{50} 4.5 nM) and a shift of approximately 25-fold between the clade 1 2004 isolates and the 2005 Indonesian isolates (IC_{50} 15 nM). A further 6 new Indonesian isolates have also been recently tested and these all demonstrated an approximate 20-fold decrease in sensitivity, consistent with our initial findings.

Discussion

We have shown here that compared to clade 1 isolates from 2004 some H5N1 clade 1 isolates from Cambodia in 2005 and clade 2 isolates from Indonesia in 2005 are less sensitive to oseltamivir, but not to zanamivir. We do not know whether this decreased sensitivity is seen only in the Indonesian clade 2 isolates, or is seen globally with all clade 2 isolates. However, Govorkova et al. [8] also recently reported a clade 2 virus from Turkey was less sensitive to oseltamivir compared to a clade 1 virus, hence clade 2 isolates from other regions may demonstrate this decreased sensitivity. We have not sequenced these isolates, but comparisons of the sequences in the public databases reveal several mutations in the stalk region which vary between clade 1 and clade 2 NAs. There is one mutation in the globular head which varies between all clade 1 and clade 2 isolates, H252Y. A further 3 amino acids vary between the majority of clade 1 and clade 2 NAs, S343P, E387G and G459S (N2 numbering), including the Indonesian isolates studied here. An additional V337M variation is also found in the vast majority of Indonesian isolates including the ones studied here (sequences submitted by Naomi Komadina). Others have

suggested that the amino acid at position 252 can impact on drug binding including affecting the reorientation of the E276 [7] necessary to bind oseltamivir with high affinity[12]. In clade 1 NAs this is normally H252, but all clade 2 NAs have Y252. In the recently published N1 structure although it is from a clade 1 virus, the authors had mutated the NA to a Y252 [12] making it not only similar to human N1 NAs, but also making the NA clade 2 like at this position. Comparisons of the structures of the N9 NA with zanamivir (Fig. 1a) and oseltamivir (Fig. 1b) bound, and the N1 NA with oseltamivir bound (Fig 1c), show that the E276 in the N1 structure does not appear to have undergone full reorientation compared to the N9 NA upon oseltamivir binding. This could correspond with reduced oseltamivir binding seen in the clade2 Indonesian isolates here. Whether the other sequence differences between clade 1 and clade 2 isolates also contribute to the altered binding requires mutagenesis studies.

Figure 1. Comparisons of structures of influenza N9 NA with zanamivir (a) and oseltamivir (b) bound compared to the N1 NA with oseltamivir bound (c). Curved arrow (b) shows the reorientation of the E276 in the N9 NA which generates the hydrophobic pocket necessary to accommodate the bulky side chain of oseltamivir. Straight arrow (c) shows that only partial reorientation occurs in the N1 NA with Y252.



Since none of the sequence variations correlates with any mutation known to confer oseltamivir resistance [12] this suggests that the decrease in sensitivities may be due to drift mutations rather than from exposure to oseltamivir. Recent results reveal that human isolates can also demonstrate decreased sensitivity to oseltamivir and zanamivir with drift mutations in the NA remote from the active site [6]. The specific decrease in sensitivity to oseltamivir of both 2005 Cambodian clade 1 and especially the Indonesian Clade 2 H5N1 isolates is disturbing, especially since they maintain their pathogenicity and transmissibility in birds, and are clearly pathogenic in humans. This is in contrast to recent observations that mutations conferring zanamivir resistance in human strains have poor

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viability and are not genetically stable [13]. Such a decrease in oseltamivir sensitivity could lead to suboptimal drug dosing in treating humans infected with these isolates which is thought to facilitate selection of high level resistant viruses [14]. Since the Indonesian clade 2 viruses studied here have a significant decrease in sensitivity compared to the clade 1 viruses this suggests the standard dosing of oseltamivir may be even less effective in clade 2 H5N1 treated patients. Many laboratories are developing rapid PCR sequencing methods for detecting the known mutations (H274Y, N294S) conferring oseltamivir resistance in H5N1 viruses. However we have shown here the importance of phenotypic testing of isolates in an enzyme assay rather than just genotypic screening [15]. Since the clade 2 virus is now spread through parts of Europe and Africa it is critical that there is continued global collaboration and phenotypic testing of drug sensitivity of circulating highly pathogenic avian isolates for NA inhibitor sensitivity. This knowledge is essential for developing appropriate management strategies for pandemic planning. Given that there was no altered sensitivity to zanamivir this further supports the hypothesis of minimalist drug design [16], of maintaining the inhibitor as close as possible to the natural substrate to minimize the emergence of resistance. Our results suggest zanamivir may also play an important role in pandemic stockpiles.

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Naturally Occurring Antiviral Drug Resistance in Avian H5N1 Virus

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Resistance to the neuraminidase inhibitor, oseltamivir, was found in H5N1 virus isolated from infected patients in Vietnam but no recent avian H5N1 isolates have been reported to possess known neuraminidase inhibitor resistance mutations. It is still not clear whether the NA1 gene neuraminidase mutation, His274Tyr, detected in H5N1 infected human cases originated during disease treatment or came from the avian virus source. A 2002 HK chicken H5N1 isolate was found to contain the His274Tyr mutation on NA1 and resistance to oseltamivir was confirmed using a cell-based assay. To investigate if His274Tyr might naturally occur at low levels mixed with wild type in H5N1 poultry infections we examined isolates from different hosts and geographical locations using a differential RT-PCR assay. The His274Ty quasi species was more frequently recognized in isolates from infected chickens than in isolates from ducks and geese, but no geographical difference was observed. Close surveillance of mutants in the virus population, combined with pursuit of alternative therapies, is essential to H5N1 pandemic containment strategies.

Introduction

Outbreaks caused by avian influenza H5N1 virus have been reported in poultry in nine southeast Asian countries since 2003 [1,2,3]. Increasing evidence shows H5N1 virus is endemic in poultry in this region; it may not be eradicable from avian hosts by regular control measures. The outbreak in migratory birds at Qinghai Lake in 2005 led to further expansion of the geographical distribution of H5N1 virus from Asia to Europe, the Middle-east and Africa [4,5]. Meanwhile, over 300 human infections by H5N1 virus have occurred in affected countries since 2003 [6]. There is concern that the H5N1 virus, or a derivative of it, may stand to be the next pandemic influenza virus [7]. It may still be some time before an effective vaccine, in adequate quantities, is available. This leaves protection in the face of a pandemic largely at the therapeutic level through antiviral treatment, placing emphasis on stockpiling antiviral drugs, particularly those for which resistance is not regularly detected [8]. Oseltamivir, a neuraminidase inhibitor, blocks exit of maturing virus particles from infected cells. In vivo and in vitro studies on H5N1 isolates from Vietnam and index strains from Hong Kong (1997 and 2003) have shown that oseltamivir effectively inhibits virus replication [3, 9-11]. However, resistance to oseltamivir has been observed, albeit rarely, during treatment of H3N2 and H5N1 influenza virus infections [12, 13-14]. This study examines H5N1 sequence data to investigate the occurrence and distribution

of oseltamivir resistant mutants isolated in Asia since 1997. We found that oseltamivir resistant mutant strains do occur and their prevalence varies with host-species. This information is relevant to influenza pandemic preparedness, particularly with regard to choice of suitable pharmaceutical agents for stockpiling, and emphasizes the need for alternative therapies, including novel drugs and an effective vaccine.

Materials and Methods

Sequence analysis. Sequences of H5N1 viruses sampled from 1997 onwards were selected from our collection and public databases. Sequence data were aligned with and residue analysis performed using BioEdit (Version 7) [15]. Residues Glu 119, Arg 292, His 274 and Arg 152 of the NA were selected to screen for predicted oseltamivir resistant mutants.

Detection of H274Y mutant with specific PCR. The His274Tyr mutation on subtype 1 neuraminidase (N1) is caused by a C to T substitution at nucleotide position 763. Differential PCR was performed with two forward primers, primer C (5' GAATTGGATGCTCCTAATTATC 3') and primer T (5' GAATTGGATGCTCCTAATTATT 3') that differ by one nucleotide at the 3' end, and a single reverse primer (5' AGAGGACACCGGACCACAACACTAC 3'). The primer pairs were tested with His 274 and Tyr 274 reference templates to optimize conditions for primer specificity in mixtures of the two templates (95°C 10 min, 1 cycle; 95°C 1 min, 62°C 1 min, 72°C 1 min, 35 cycles; 72°C 10 min, 1 cycle; Fig. 1). RNA extraction and cDNA synthesis from virus samples were performed as described previously [2].

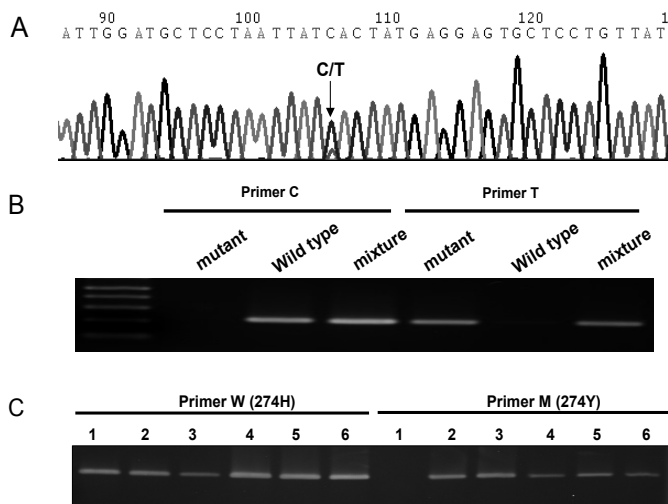
Inhibition of H5N1 virus infection in MDCK cell culture. Virus stocks were propagated in embryonated chicken eggs and TCID₅₀ in MDCK cells was determined. Oseltamivir carboxylate (Roche) was tested at concentrations of 0.1 nM to 100 uM in a cell-based virus reduction assay, modified from that of Yen and co-workers [11]. Briefly, triplicate MDCK monolayer cultures in 96-well format were infected with 100 TCID₅₀ virus doses (1 hour, 37°C, 5% CO₂), then the inoculum removed and cell layers washed with culture media (MEM, Gibco BRL). Two hundred microlitres of culture media containing the appropriate dilution of oseltamivir was added to wells, and cultures incubated for 3 days (37°C, 5% CO₂). The HA titers of individual well supernates were tested in duplicate.

Quantification of wild type (C type) and mutant (T type) NA gene by quantitative PCR. Quantitative PCR (qPCR) reactions were performed with a Roche Lightcycler system (Roche) using a Lightcycler Faststart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. For better discrimination between the wild type and mutant NA genes, Locked Nucleic Acid (LNA) primers (Prologo) were used. Wild type and mutant NA genes were cloned into the TOPO PCR 2.1 vector (Invitrogen), sequenced and used as standards for quantification. Mutant and wild type NA gene copy number were calculated with Lightcycler software using the wild type and mutant plasmids as a standard. PCR specificity was checked by melting curve analysis and gel electrophoresis.

Results

Detection of an oseltamivir resistant H5N1 isolate. To date, four neuraminidase residue changes associated with resistance to oseltamivir have been characterized; Glu119Val, Arg292Lys, His274Tyr and Arg152Lys. These appear to be NA subtype specific; Arg292Lys and Glu119Val to N2 and His274Tyr to N1 [16-18]. Published sequences of avian H5N1 virus isolates do not indicate the presence of resistance-associated mutations and the isolates tested are sensitive to oseltamivir inhibition both in vitro and in vivo [10,11]. However, the isolation of oseltamivir-resistant H5N1 viruses from humans in Vietnam indicates that oseltamivir resistance could be an emerging problem [13,14]. As oseltamivir is so important to pandemic preparedness, we wished to investigate if resistant mutant H5N1 viruses might be present in other species and areas in Asia, with a view to determining the potential of a naturally-resistant pandemic strain arising in the future. Available sequences of H5N1 viruses isolated since 1997 were examined. One isolate, A/chicken/Hong Kong/3123.1/02, was found to bear the His274Tyr mutation which conveys resistance to neuraminidase inhibitor drugs in N1 subtype viruses [16-18]. Drug sensitivity tests revealed that this isolate possessed a high degree of resistance to oseltamivir inhibition, with an EC50 of 124.9uM. In comparison, the A/chicken/Vietnam/37/04 isolate, containing the wild type residue at position 274, has an EC50 of 0.4uM oseltamivir.

Figure 1. Sequencing traces of the neuraminidase gene showed a mixture of wild type (C) and H274Y (T) mutant in an H5N1 ISOLATE (A). A C/T Mutation at this position confers resistance to oseltamivir in H5N1 subtype viruses. Primers for wild type (C) and mutant (T) were designed with one nucleotide difference at the 3' end of each primers were used with a common reverse primer derived from a non-variable region. Conditions allowing specific detection of wild type or mutant were potimized with templates containing only mutant or wild type and mixtures of these templates (B). Detection of wild type (primer W) AND his274tyr TYPE (PRIMER m) in H5N1 isolates and one original swab by PCR (C). 1: A/chicken/Vietnam/37/04, 2: A/chicken/Hong Kong/518.1/02, 3: A/chicken/485.2/02, 4: A/chicken/Vietnam/272/05, 5: A/chicken/Hong Kong/WF75/02, 6: A/chicken/Hong Kong/WF75/02 (original swab).



Presence of His 274Tyr mutant mixed with wild type virus in H5N1 isolates. The human H5N1 virus isolated from patients treated with oseltamivir has been identified as consisting of mixed populations of His 274 wild type and Tyr 274 oseltamivir-resistant virus [13,14]. As direct PCR product sequencing is the most regularly used method of determining influenza virus sequence, and sequence traces are rarely consulted, only the dominant sub-population at each nucleotide is represented in analyzed sequences, potentially obscuring the presence of low-frequency variant populations (Figure 1A). A protocol was developed and verified for rapid screening of H5N1 isolates which may contain both mutant Tyr 274 and wild type His 274 NA1 (Figure 1B). Where available, original cloacal swabs corresponding to isolates identified as containing the His274Tyr mutant were compared with viral stocks to confirm that His274Tyr mutant virus was present in the original samples prior to viral propagation in embryonated eggs (Figure 1C). Overall, 54.3% of tested isolates contained detectable levels of His274Tyr variant virus, but the distribution was skewed, with variant detected most frequently in chicken isolates, less often in human and more rarely in duck and goose isolates (Table 1). His274Tyr mutant virus was not detected in a sample of nine H1N1 isolates tested using a similar procedure (data not shown). Species-specific analysis revealed that variant His274Tyr virus was not detected in chickens in the 1997-1999 period (data not shown). Following emergence of the mutant in 2000, prevalence in chickens then rose rapidly, with just under 10% of 2000-2001 viruses testing positive by PCR screening, and variant frequency plateauing at a high of 74% over the 2002-2005 period. Geographical distribution of His274Tyr mutant virus was also analysed, but no distinct pattern could be discerned (data not shown).

Table 1. Specific PCR screening for presence of His274Tyr mutation in H5N1 viruses isolated from various hosts.

Species	Total isolates	Positive	% positive
Human	19	8	42.1
Chicken	195	125	64.1
Duck/Goose	97	36	37.1
Total	311	169	54.3

Quantitative PCR was applied to quantify the relative abundance of His274Tyr mutant and the wild type in virus stocks. In essentially all isolates containing mixed species of wild type and mutant, the mutant represented less than 1% of the virus population, and in many cases less than 0.1% (data not shown).

Discussion

Preparedness for a potential H5N1 influenza pandemic caused by currently-circulating H5N1 viruses has been recommended by the WHO. One well-publicized focus is the stockpiling of antiviral drugs, particularly inhibitors of viral neuraminidase. One

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such neuraminidase-inhibitor, oseltamivir, has been considered as one of the few options available for the containment of human-to-human transmission. This study demonstrates that oseltamivir resistant strains bearing the His274Tyr mutation are present in some H5N1 virus populations, albeit at low levels, in the absence of evidence of exposure to the drug. Oseltamivir is not known to have been introduced into the Hong Kong market prior to 2002, but we have detected His274Tyr mutants in virus populations isolated as early as 2000, which suggests that this mutant occurs naturally. Previous studies have shown that oseltamivir-resistant mutants have a growth and virulence disadvantage [19-22]. Given the continuous circulation of H5N1 viruses in poultry and wild birds in the southeast Asia region, there is a chance that His274Tyr mutants could gain the ability to increase in abundance if they obtain other adaptive changes. Even if variant viruses do not become dominant in mixed populations, they may still hamper clinical treatment with oseltamivir by increasing the overall resistance of the population. Isolation of one resistant mutant strain, A/chicken/Hong Kong/3123.1/02, from an infected chicken suggests the possibility that such mutants may possess the potential to become the main population, at least in chickens. Major H5N1 outbreaks in poultry in 1997 and 2001-2002 in Hong Kong were controlled in a timely manner and this might have stopped further expansion of such mutants [23,24]. Antiviral drugs, if appropriately applied and tightly controlled, remain important components of pandemic preparedness. However, the presence of drug-resistant mutants in the pool of potential pandemic H5N1 strains provides additional "options" for the predicted pandemic strain, especially with regard to oseltamivir-resistance. In anticipation of this possible scenario, further control options must be implemented, including stockpiling a wider range of drugs (zanamivir in particular), development of alternative pharmaceuticals, emphasis on vaccine development and, most importantly, increased surveillance for the presence of His274Tyr mutants mixed with wild type strains among new H5N1 isolates.

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Proceedings Topic #10

Innate and Adaptive Immunity

**Oral
Presentations**

TRAIL Deficiency Increases the Severity of Influenza Virus Infections

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Clearance of primary influenza virus infections is largely thought to be mediated through actions of influenza-specific CD8⁺ T cells, which promote recovery from infection by killing infected cells through perforin- and FasL-mediated mechanisms. Interestingly, recent studies have suggested that CD8⁺ T cells can also trigger apoptosis of virus-infected cells through the expression of TNF-related apoptosis-inducing ligand (TRAIL). While the ability of TRAIL to induce apoptosis through interactions with its receptor (DR5) have been best described in the field of cancer therapy, recent studies describing upregulation of TRAIL after influenza virus infection implied a role for TRAIL in the immune response to influenza virus infection. Supplementing and expanding these previous studies of TRAIL in influenza infections, we found that TRAIL is upregulated on CD8⁺ T cells in an antigen-specific manner during influenza infection. Further, infection of TRAIL-deficient mice results in increased morbidity, increased antigen load, and decreased killing capacity of CD8⁺ T cells when compared to TRAIL-sufficient mice. Together, these results suggest that TRAIL expression is an important component of immunity to influenza virus infections and that TRAIL deficiency might alter CD8⁺ T cell-mediated cytotoxicity thereby increasing the severity of influenza virus infections.

Introduction

The increasing threat of epidemic and pandemic influenza underscore the need to better evaluate the immune response to influenza virus infections and to better understand the factors that contribute to the virulence of influenza infection. A hallmark of the adaptive immune response to primary influenza virus infections is the induction of influenza-specific CD8⁺ T cell responses. These T cells target and kill influenza-infected pulmonary epithelial cells, therein clearing the virus and allowing recovery. Recently, reports have demonstrated that CD8⁺ T cells can utilize TNF-related apoptosis-inducing ligand (TRAIL), in addition to perforin and FasL, to mediate the apoptosis of virally infected cells. While the roles for perforin and FasL in clearing influenza virus infections are well established, little is currently known about the role of TRAIL in CD8⁺ T cell-mediated killing of influenza virus-infected cells. *TRAIL and apoptosis.* TRAIL is a TNF superfamily member capable of inducing apoptosis, and has received great attention in the field of cancer therapy. The extracellular domain of TRAIL is most homologous to Fas ligand (28% a.a. identity), but also has significant identity to TNF (23%), lymphotoxin (LT)- α (23%), and LT- β (22%)[1]. Though the homology of TRAIL to other TNF family members may be considered low, the crystal structure of

monomeric TRAIL is very similar to that of TNF, TNF- α , and CD40 ligand[1]. TRAIL monomers contain two antiparallel β -pleated sheets that form a β -sandwich core framework, and these monomers interact with other TRAIL monomers in a head-to-tail fashion to form a bell-shaped trimer[1]. This oligomerization enhances TRAIL activity, as studies with recombinant soluble TRAIL found that the most biologically active form was multimeric rather than monomeric[2]. Early studies of TRAIL indicated that it induced apoptosis in tumorigenic or transformed cells, but not normal cells[2]. In contrast, other TNF superfamily members are equally cytotoxic to both tumor and normal cells. To date, little information exists to explain the resistance to TRAIL-induced apoptosis observed in normal cells; however, the ability of TRAIL to selectively induce apoptosis in tumor or transformed cells has fueled the development of TRAIL as a therapeutic molecule. Consistent with apoptosis induced by other TNF family members (i.e. FasL and TNF), cells undergoing TRAIL-induced death exhibit many of the hallmarks of apoptosis, including expression of pro-phagocytic signals (i.e. phosphatidylserine) on the cell membrane, cleavage of multiple intracellular proteins by caspases, and DNA fragmentation[2-4]. Soluble TRAIL is tumoricidal on over 75% of the more than sixty hematopoietic and non-hematopoietic tumor cell lines tested *in vitro*, suggesting that TRAIL could be used as a broad-spectrum, anti-tumor molecule *in vivo*[2-5]. Peripheral blood human T cells express TRAIL after CD3 crosslinking and type I IFN stimulation, perhaps contributing to T cell AICD[6]. Human NK, M ϕ , and dendritic cells also express TRAIL following cytokine stimulation, transforming them into potent killers of tumor cells[3, 7, 8].

Regarding TRAIL deficiency. After its initial characterization by numerous *in vitro* studies, the ability of TRAIL to act as a potent inducer of tumor cell apoptosis quickly became clear, but there was nothing known regarding the normal physiological activities of TRAIL *in vivo*. To address this, Sedger *et al.*[9] generated TRAIL-gene targeted (-/-) mice. These mice develop normally and display no defects in lymphoid or myeloid cell homeostasis or function. The one abnormality observed in these mice, though, was that they were more susceptible to tumor burden. Interestingly, while the TRAIL deficiency resulted in a significant biological disadvantage for controlling the growth of TRAIL-sensitive tumors *in vivo*, the mice did not have an increased tendency to spontaneously develop tumors compared to normal mice. Thereafter, a series of studies was published that clearly and elegantly demonstrated the importance of TRAIL-expressing NK cells in the elimination of tumors *in vivo* in natural tumor immunosurveillance[10-14]. *TRAIL and viral immunity.* Complex cellular mechanisms operate during host defense against viral infections, during which the innate immune response cooperates with the adaptive immune response to eradicate the pathogen. Inducing apoptosis of virus-infected cells and viral resistance to apoptosis-inducing ligands are important factors that can determine the outcome of virus infection *in vivo*. In fact, many viruses have incorporated open reading frames encoding potent regulators of cell death[15, 16], and viruses with targeted disruptions or naturally occurring mutations in

these genes often exhibit replication defects *in vitro* as well as reduced virulence *in vivo* [17-19]. Within the context of viral immunity, reovirus, measles virus, Newcastle disease virus, respiratory syncytial virus (RSV), human cytomegalovirus (hCMV), and encephalomyocarditis virus all induce TRAIL expression on immune system effector cells, primarily through the induction of type I and II IFN [20-26]. Further, normal cells infected with RSV, hCMV, or encephalomyocarditis virus become susceptible to TRAIL-mediated killing [21-24]. While the mechanisms required for normally TRAIL-resistant cells to become susceptible to TRAIL are not well understood, the observed upregulation of TRAIL and increased sensitivity to TRAIL indicate that understanding the roles of TRAIL and TRAIL-induced apoptosis during viral infections is essential to understanding what determines the outcome of viral infection.

Influenza virus immunity and cell death. Protective immunity to primary influenza virus infection involves the clearance of infected epithelial cells [27, 28] by CD8⁺ T cells through either Fas- or perforin-dependent direct killing mechanisms [29]. This T cell-mediated killing of infected cells is thought to occur through either Fas- or perforin-dependent direct killing mechanisms [29]. Influenza-specific CD8⁺ T cells first appear in the lungs around day 4-post infection [30-32] where their continued expansion and accumulation corresponds with the early stages of viral clearance [31, 32]. The importance of CD8⁺ T cells in protection from influenza infections is further highlighted by the fact that CD8⁺ T cells mediate resistance and protection from lethal influenza virus infections in the absence of B cells, CD4⁺ T cells, and neutralizing antibody [33, 34]. The idea that CD8⁺ T cells utilize perforin and Fas ligand to mediate killing of influenza-virus infected targets is most strongly supported by the work of Topham *et al* [29], who showed that pulmonary influenza-virus titers were maintained for longer durations when either perforin^{-/-} or Fas^{-/-} hosts were examined. Furthermore, when chimeric mice were made that possessed perforin^{-/-} CD8⁺ T cells and Fas^{-/-} respiratory epithelium substantial pulmonary titers were maintained even out to 14 days post infection relative to wildtype control mice. Importantly, however, despite the loss of perforin and Fas, some of these mice showed lower virus titers on day 14 than on day 10 post infection [29]. This outcome could suggest that an additional mechanism might be employed by CD8⁺ T cells to kill influenza infected target cells.

Influenza virus immunity and TRAIL. In addition to the Fas-FasL and perforin-Granzyme B pathways for killing, CD8⁺ T cells have also been shown to utilize a TRAIL-DR5 dependent mechanism to eliminate infected cells during other types of virus infections. TRAIL induces apoptotic cell death [2, 4, 35, 36] by recruiting and aggregating caspase 8 upon binding to its receptor, DR5 [36-40]. This aggregation in turn leads to a caspase cascade and eventually to apoptotic death of the DR5⁺ cell [36-39, 41]. Importantly, expression of TRAIL and DR5 are upregulated on CD8⁺ T cells, NK cells, and infected cells following virus infection [20, 23, 42] or during increases of IFN- γ or TNF [11, 23]. In turn, IFN- γ and TNF downregulate TRAIL receptor expression on uninfected cells [23]. Overall, these results suggest that TRAIL/DR5 may play

an important role in the specific elimination of virus-infected cells. Specifically regarding influenza virus infections, a recent report has suggested that TRAIL may play an important role in the clearance of influenza virus from the lungs during influenza infection. In this study, Ishikawa and colleagues [42] show that TRAIL expression is increased on a fraction of CD8⁺ and CD4⁺ T cells, as well as NK cells, following influenza virus infections. Interestingly, the blockade of TRAIL with antibody delayed the clearance of influenza virus from the lungs. This suggests that TRAIL might be playing a significant role as an effector molecule during the immune response to influenza virus infection.

Results and Discussion

The results in the aforementioned study [42] suggest a possible role for TRAIL dependent CD8 T cell mediated apoptosis of virus-infected cells following influenza virus infections. However, these studies did not directly examine if TRAIL expression correlated with or was limited to influenza-specific T cells or if the increase in pulmonary influenza virus titers was directly related to decreased CD8⁺ T cell cytotoxicity (i.e. induction of apoptosis of infected cells) [42]. Thus, we examined what role TRAIL might play in immunity to influenza virus infections. Our data show that both TRAIL and DR5 (TRAIL receptor) are significantly upregulated in the lungs of wildtype mice following influenza virus infection. (data not shown) This expression correlates with the influx and expansion of TRAIL⁺IFN γ ⁺ influenza-specific CD8⁺ T cells and the subsequent elimination of virus from the lungs, suggesting that TRAIL/DR5 interactions (i.e. CD8⁺ T cell/infected cell interactions) could be an integral part of productive immunity to influenza virus. Further, TRAIL deficiency increases influenza-associated morbidity and disease severity. This increase in disease severity also correlates with increased influenza virus titers and decreased levels of influenza-specific cytotoxicity, which occurs despite the induction of equivalent numbers of pulmonary IFN γ ⁺ influenza-specific CD8⁺ T cells in both TRAIL-sufficient and TRAIL-deficient mice. Together, these results suggest that TRAIL expression is an important component of immunity to influenza virus infections and that TRAIL deficiency might alter CD8⁺ T cell-mediated cytotoxicity thereby increasing the severity of influenza virus infections. Establishing a better understanding of the role of TRAIL in influenza-specific immunity will improve not only our understanding of the cellular and molecular immune response to influenza, but will also broaden our understanding of how TRAIL expression/deficiency may alter the virulence of influenza virus infections. This knowledge will not only be relevant to our understanding of the mechanisms allowing elimination of virus infections of the lungs but also in the design of strategies to combat epidemic or pandemic influenza outbreaks.

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Continued Proliferation of Influenza-Specific T Cells in the Lungs During the Early Stages of Influenza Virus Infections

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Upon activation in the lymph nodes, influenza-specific CD8 T cells are thought to undergo programmed proliferation and differentiation to effector cells before migrating to the lungs to mediate viral clearance. While it is known that CD8 T cells undergo several rounds of division in the LN before migrating to the lung, it remains unclear if CD8 T cells continue to proliferate once arriving in the lungs. To address this question, we developed a dual-label system that utilizes intranasal CFSE administration to label all lung-resident CD8 T cells and intranasal BrdU incorporation to identify those cells undergoing active cell division. With this technique, we were able to identify influenza-specific CD8 T cells, which have undergone at least one round of active cell division in the lungs.

Introduction

It is generally accepted that, during an influenza infection, CD8 T cells in the lymph nodes (LN) are activated and subsequently undergo several rounds of division. However, it is unknown if all of this cell division occurs in the LN or if further cell division occurs after the T cells have arrived in the lungs. With the techniques that are currently available for examining CD8 T cell division, such as dilution of CFSE or BrdU incorporation, it is difficult to differentiate between division that occurs in the LN from that which occurs in the lungs. In order to better determine the sites of CD8 T cell expansion, we have developed a dual-label system that utilizes intranasal CFSE administration to label all lung-resident CD8 T cells and intranasal BrdU incorporation to identify those cells undergoing active cell division. Our results demonstrate that some division of influenza-specific CD8 T cells occurs in the lungs of influenza-infected mice.

Materials and Methods

Mice. 6-12 week old BALB/c mice were purchased from the National Cancer Institute. All experiments were approved by the University of Iowa Animal Care and Use Committee. **Virus Infection.** Groups of BALB/c mice were anesthetized by isoflurane inhalation and infected intranasally with a 0.1 LD₅₀ dose of mouse-adapted A/JAPAN/305/57 in 50 µl of Iscove's media as previously described (1, 2).

Influenza-specific T cell analysis. Lungs were removed, minced and single cell suspensions prepared as described (3). The cells were then stained with antibodies to CD3ε (145-2C11), CD8α (53-6.7) and BrdU (3D4 and B44) as well as MHC I tetramers containing HA₂₀₄ (H-2K(d)/LYQNVGTYV), HA₅₂₉ (H-2K(d)/IYATVAGSL); and NP₁₄₇ (H2K(d)/TYQRTRALV). Cells were

then analyzed using a BD FACS Calibur and FlowJo software. Tetramers were obtained from the NIH MHC Tetramer Core Facility.

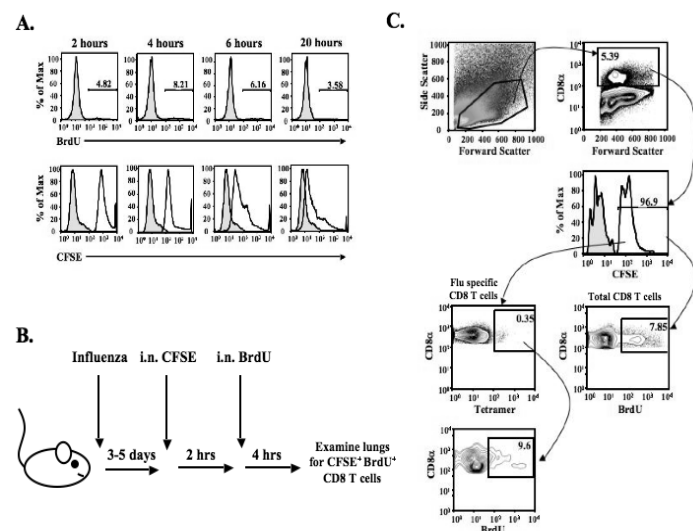
Assay for BrdU incorporation. To assess the proliferation of CD8 T cells present within the lungs, intranasal CFSE labeling was used in conjunction with BrdU incorporation. Mice were administered intranasal CFSE as previously described (3). 2 hours later, the mice were again anesthetized and administered 0.8 mg BrdU in 80 µl sterile PBS intranasally. Four hours later, the lungs were harvested and single cell suspensions were stained for BrdU according to manufacturers instructions (BD Pharmingen).

Results

BrdU incorporation is a common measure used for identifying actively dividing cells *in vivo*. When given i.p. or via drinking water, one can successfully label lymphocytes undergoing division in the spleen, LN and many peripheral organs. However, studies by Lawrence *et. al.* (3) determined that BrdU labeling via i.p. injection or in the drinking water significantly underestimated the numbers of cells that had proliferated in the lungs of influenza infected mice. Instead, the authors demonstrated more successful labeling of this population of cells by i.n. BrdU administration. BrdU is known to have a particularly short half-life *in vivo* and is degraded or diluted by rapidly dividing cells (4, 5). Therefore, in order to first optimize the labeling of proliferating CD8 T cells in the lungs, we undertook preliminary studies to determine the kinetics of intranasal BrdU incorporation after influenza infection. Mice were infected, administered BrdU intranasally and their lungs were harvested at 2, 4, 6 and 20 hours post BrdU labeling. As seen in Figure 1A (top panel), BrdU⁺ CD8 T cells can be detected as rapidly as 2 hours post labeling and continue to increase until 4 hours post labeling. However, the frequency of BrdU⁺ cells among CD8 T cells rapidly decreases from 8.21% BrdU⁺ cells at 4 hours post labeling to only 6.16% BrdU⁺ CD8 T cells at 6 hours post labeling, suggesting that the BrdU has been diluted or degraded. Given this kinetics, we chose to examine the proliferation of antigen-specific CD8 T cells in the lungs at 4 hours after BrdU labeling. Although BrdU given intranasally successfully labels proliferating cells in the lungs, it has been observed that treatment can to a lesser degree also result in labeling of proliferating CD8 T cells in the mediastinal LN (3). Therefore, by using only a single labeling technique, it would be likely that some of the BrdU⁺ CD8 T cells were incorporating the label in the LN before migrating to the lungs. To rule out this possibility, a second label would be required to identify the cells present in the lungs at the time of BrdU administration. Therefore, we chose to pair intranasal BrdU treatment with intranasal CFSE labeling. We have previously shown that intranasal CFSE administration specifically labels cells present in the lungs and not the lung draining LN (1). However, similar to BrdU dilution, CFSE labeling is diluted in dividing cells eventually resulting in the loss of labeling. In order to optimize the kinetics of CFSE labeling, we next performed preliminary

studies similar to those performed with BrdU. Infected mice were administered CFSE intranasally, and then the lungs were harvested at 2, 4, 6 and 20 hours post labeling and the CD8 T cells examined for their CFSE profiles. We observed clear CFSE⁺ populations out to 6 hours post-labeling; however, by 20 hours post-labeling, it was difficult to differentiate between CFSE⁺ and control CFSE⁻ populations (Figure 1A, bottom panels). Given these results, we chose to utilize a 6-hour time point of CFSE labeling in subsequent studies. Utilizing the experimental design outlined in Figure 1B, we next proceeded to determine the extent of proliferation of CD8 T cells in the lungs. Utilizing the gating strategy outlined in Figure 1C, total and antigen-specific CFSE⁺ CD8 T cells were examined for BrdU incorporation on days 3, 4 and 5 p.i. As figure 2 demonstrates, small numbers of CFSE⁺BrdU⁺ CD8 T cells can be identified in the lungs as early as day 3 post infection (p.i.), around the timeframe when antigen specific CD8 T cells are first arriving in the lungs (2, 6). We observed no significant differences in the frequency of total (Figure 2A) or influenza-specific (Figure 2C) CD8 T cells that had incorporated BrdU on days 3, 4 or 5 p.i. This suggests that the rate of CD8 T cell proliferation in the lungs may remain fairly constant from days 3-5 p.i.. Not surprisingly, given the continued proliferation and migration of T cells into the lungs, the numbers of CD8 T cells that are BrdU⁺ continues to increase with a 5 fold increase in total CD8 T cells (Figure 2B) and a nearly 9 fold increased in influenza-specific CD8 T cells (Figure 2D) from days 3 to 5 p.i. Overall, our results suggest that, after their division program is initiated within the lung-draining LN (2, 6), CD8 T cells continue to proliferate following their arrival in the lungs during influenza virus infections.

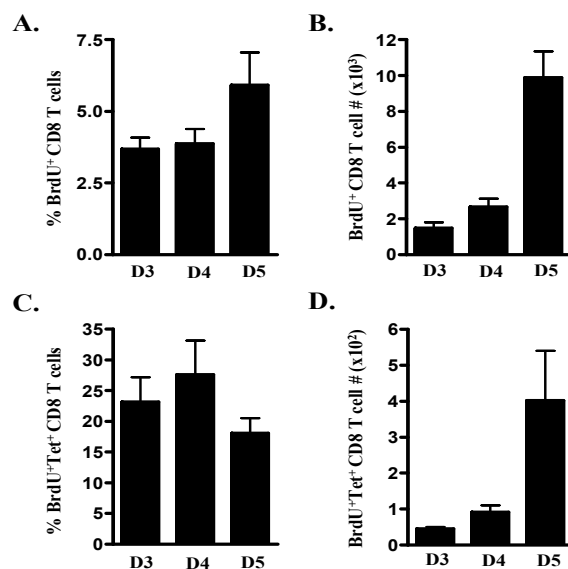
Figure 1. Dual labeling of proliferating CD8 T cells in the lungs of influenza infected mice. **A.** Mice were infected with influenza and then administered i.n. BrdU (top panel) or i.n. CFSE (bottom panel) on day 4-p.i.. At 2, 4, 6 and 20 hours post-labeling, the mice were sacrificed and CD8 T cells in the lungs were examined by flow cytometry. n= 3 mice/time-point **B.** Experimental design for dual-labeling experiments. **C.** Gating strategy for examining proliferation of total and antigen-specific CD8 T cells in the lungs.



Discussion

In this paper, we have shown that influenza-specific CD8 T cells continue to proliferate after arriving in the lungs. Interestingly no significant differences were observed in the frequency of BrdU⁺ cells among the influenza-specific T cells in the lungs on days 3, 4 or 5 p.i.. This constant frequency of BrdU cells may suggest a near uniform division rate of T cells during the early stages of influenza infection. If this is indeed the case, it will be important to determine in future studies if this frequency continues at the same rate throughout both the early and late stages (i.e. day 8-12 p.i.) of T cell accumulation in the lungs (6). If the rate of CD8 T cell proliferation within the lungs during influenza infections were instead based upon when the T cells arrived in the lungs during the course of infection, we would have instead expected to observe a significant change in the frequency of BrdU⁺ cells among tetramer⁺ cells based upon the day analyzed. It is possible that the T cells are simply “finishing-up” the division program that was initiated in the LN (7), with 1 to 2 more divisions in the lungs. If this is the case, the constant frequency of BrdU⁺ T cells likely reflects a constant rate of migration of CD8 T cells into the lungs rather than continued and steady proliferation. This model would suggest that most of the cells that incorporated BrdU would be recent immigrants into the lung. It is therefore intriguing that a large number of tetramer negative CD8 T cells incorporate BrdU (Fig 2B, D). This result could potentially reflect the division of recently arriving

Figure 2. Proliferation of influenza-specific CD8 T cells in the lungs of influenza infected Mice. Utilizing the experimental design outlined in Figures 1B-C, the frequency and number of total (A, B) and antigen specific (C, D) CD8 T cells undergoing division in the lungs of influenza infected mice was determined by flow cytometry. n=2-4 mice/group, representative of 3 separate experiments.



antigen specific T cells which are known to emigrate into the lungs in a tetramer negative state (8) – a determination that awaits further study using TCR-transgenic models. Finally the

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constant frequency of BrdU+ cells could instead reflect an incomplete penetrance of BrdU into dividing cells. This result appears unlikely given the preferential incorporation of BrdU into influenza-specific T cells relative to total CD8 T cells (Fig 2A, C) but awaits confirmation using other markers of division such as Ki67. In conclusion, we have developed a novel intranasal dual-label system that allows us to examine proliferation of CD8 T cells in the lungs during an influenza infection. With this technique, we have successfully identified antigen-specific CD8 T cells that have undergone at least one round of division after migrating to the lungs. However, this technique is limited in that it currently does not distinguish between division of cells that have been present in the lungs for an extended time period and those cells that have only recently arrived (i.e. immediately before the 6 hour CFSE labeling) or allow determination of how many rounds of division the T cells undergo in the lungs. Regardless, the results described herein demonstrate that some division of influenza-specific T cells does occur within the lungs.

Acknowledgements

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Proceedings Topic #11

Mathematical Modeling

Oral

Presentations

Viral Resistance May Limit the Benefit of Neuraminidase Inhibitors Therapy During an Influenza Pandemic

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Introduction

With an influenza pandemic seemingly imminent, most industrialized countries have stockpiled large quantities of neuraminidase inhibitors (NAIs), i.e. oseltamivir or zanamivir, to use for treatment or contact prophylaxis. *De novo* resistance to these drugs is rare. The neuraminidase inhibitor susceptibility network has reported that 0.2-0.4% A/H3N2 isolates from untreated patients have mutations on the neuraminidase (NA) genes conferring phenotypic resistance, defined as a ten fold (or more) decrease of susceptibility^{1,2}. The incidence of oseltamivir resistance in clinical trial samples was 0.33% in adults, 4% in children and 1.3% overall³. Higher rates of resistance have been observed in treated children, with values ranging from 16% to 18% reported at end-of-treatment^{3,4}, although these high rates may have been the results of underexposure to treatment in a immunologically naïve population. Resistant strains have also arisen in treated patients infected with avian influenza A/H5N1^{5,6}. In vitro assays have shown different patterns of susceptibility of influenza viruses to NAIs and evidence of cross resistance between different NAIs^{7,8}. Most identified mutations decrease the replication efficiency of the mutant strains⁹, thus theoretically affect the transmissibility potential of these viruses, but it is possible to generate mutations that do not compromise the growth of a recombinant virus in vitro¹⁰. With the exception of Japan, the use of NAIs still remains very poor in seasonal influenza epidemic, thus, to date, there is no real selection pressure. During a pandemic, the impact of a huge use of NAIs on the selection of resistant strains is unknown. Mathematical modelling studies designed to consider resistance are still rare and used homogeneous deterministic compartmental models¹¹⁻¹⁴. None addressed the potential advantages of using two antiviral treatments. Using an individual-centered model, our primary objective was to explore how resistance could limit the benefits of NAIs. We limited our analysis to scenarios combining one or two NAIs for treatment.

Material and Methods

A full description of our model has been described elsewhere¹⁵. Briefly, we used the standard Susceptible-Exposed-Infectious-Recovered (SEIR) framework to model the natural history of influenza infection, with a fixed latent period of 0.5 day, and an infectious period of 3.5 to 4 days on average. We assumed that infectivity varied with time from infection, and was proportional to viral kinetic profiles observed in experimental studies of

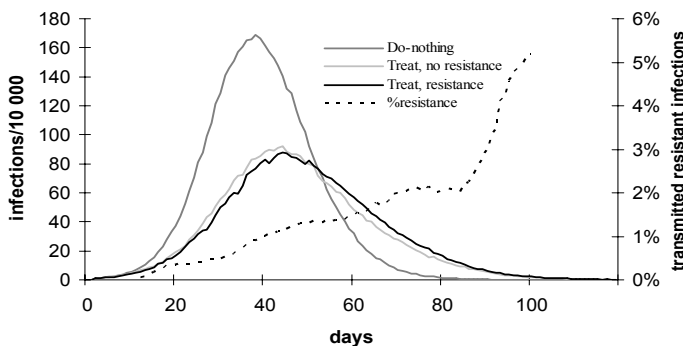
adult volunteers challenged with a wild-type influenza virus. We also assumed that infectivity and susceptibility were greater in children than in adults or the elderly^{16,17}. A community model was built to describe the spread of influenza. We first generated individuals based on a particular demographic profile and affected them to households and living places (e.g. a school for children, or workplace for working adults). We assumed that every household member made daily meeting with other household members. For schools, workplaces or other locations, meetings between individuals were modelled with mathematical random graphs, which have been shown to be adapted for describing social networks. Data on real pandemics were used to calibrate the model. The “do-nothing” scenario assumed a fully susceptible population and no pharmaceutical or non pharmaceutical intervention, except confinement to home of 80% of clinical cases. We calculated an empirical value of the basic reproductive ratio (R0) equal to 2, which means that a typical index patient transmitted influenza to 2 contacts; and a mean generation time of 2.4 days. Two hundred realisations were simulated for each scenario in which a single infected individual was entered in the network. The “treatment, no resistance” scenario assumed 80% treatment of patients who had a physician visit (70% within the first two days of illness), with a 5-day treatment regimen. We assumed that “on-treatment”, the infectivity of patients decreased by 80%¹⁸. The “treatment, resistance” scenario assumed a *de novo* resistance rate of 0.2%, and a relative transmissibility of a resistant as compared to a sensitive strain of 100%. We explored different rates of *de novo* resistance (0-5%) and decrease of transmissibility (50%-100%) in sensitivity analyses. In a second part, we explored strategies based on two different NAIs (NAI1 and NAI2). For sake of simplicity, we assumed that NAI1 and NAI2 had similar efficacy in reducing infectiousness. We assumed that the rate of *de novo* resistance to each NAI was 3% and that 1/3 of resistant strains that emerged during therapy were resistant to both NAIs (cross-resistant). The high value of 3% *de novo* resistance rate was chosen for obtaining a better chance to highlight potential advantages of using two NAIs by comparison with a single NAI. We simulated various scenarios: “random” allocation, meaning that a simulated patient had 50% chance to be treated with NAI1 or 50% chance to be treated with NAI2; “sequential” use, meaning that during alternating periods of time (15 days), a single NAI (either NAI1 or NAI2) was used; “spatial” allocation, meaning that 50% areas used NAI1 and others, NAI2. We also explored “combined therapy” scenarios where patients received dual regimens until a time point in the course of the epidemic during which a switch to mono-therapy for every new patients (random allocation) occurred.

Results

In the “do-nothing” scenario, 57% of simulations led to an explosive outbreak, lasting a mean of 82 days (SD 12 days) (figure 1). The cumulative incidence proportion of influenza infection was 46.8% and was higher in children (76%) than

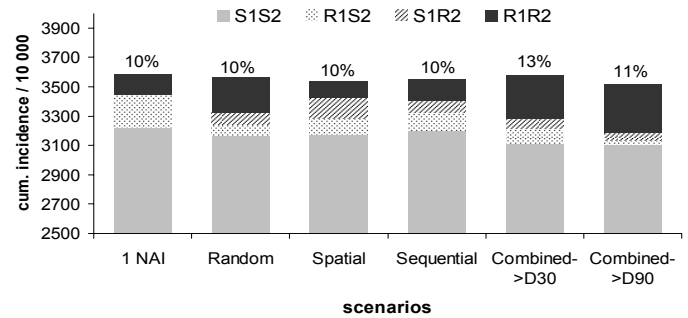
in adults (40%) or the elderly (25%). The mean clinical attack rate was 33% (SD 1%), 1.7% (SD 0.16%) of the population was hospitalized, and 0.36% (SD 0.07%) died from influenza. In the “treatment, no resistance” scenario, the cumulative incidence proportion of influenza infection decreased to 34.4%, and the epidemic lasted a mean of 102 days. When a *de novo* resistance rate of 0.2% under treatment was introduced, the cumulative incidence proportion increased slightly to 34.7%. The proportion of transmitted resistant strains was 1.1% on average but showed substantial variations: in 7% simulations, 5% to 20% of all infections were caused by a resistant strain. Moreover, the proportion of resistant infections increased with time from the onset of the epidemic to reach 2 to 5% of transmitted infections during the third month.

Figure 1. Epidemic curves (plain lines – left Y axis) for « Do-nothing”, “Treatment, no resistance” and “Treatment, resistance” (with a *de novo* rate of 0.2%) scenarios, and proportion of transmitted resistant strains (dashed line – right Y axis) in the “treatment resistance” scenario.



When the rate of the *de novo* resistance increased to 3%, or 5%, the cumulative incidence proportion increased slightly to 35.9% and 36.9%, while the proportion of transmitted resistant infections increased substantially to 10.2% and 17.3%, respectively. A decrease in the relative transmission fitness of the resistant strain decreased the proportion of transmitted resistant infections, even for high values of resistance rate: with a *de novo* rate of 3%, the cumulative incidence proportion and the proportion of resistant infections were 34.8%, 4.5%; and 34.3%, 0.4% for relative transmissibility of 80% and 50%, respectively. Finally, an increase of the treatment coverage from 80 to 100% led to a moderate decrease of the cumulative incidence as the reduction of infections with a sensitive strain was offset by the increase of infections with a resistant strain: with a resistance rate of 3% and treatment coverage of 100%, the proportion of transmitted resistant infections was 18.5%, and the cumulative incidence proportion of influenza infection, 33.1%. When using two neuraminidase inhibitors, the cumulative incidence proportions were moderately affected, with values ranging between 35.2% and 35.8% compared with a scenario in which only one NAI was used (35.9%) – figure 2.

Figure 2. Cumulative incidence of influenza infections for various scenarios exploring strategies based on 1 (1 NAI) or 2 NAIs, alternatively (“Random”, “Spatial”, or “Sequential” – see material and methods for complete description), or “combined” (D30: until day 30 in the time-course of the epidemic, D90: until D90 in the time course of the epidemic). The values above bars indicate the proportion of transmitted resistant infections. Infections are categorized in four classes according to their sensitivity to NAIs (S1, S2: sensitive to NAI1 and 2; R1, R2: resistant to NAI1 and 2 – e.g. R1R2 correspond to cross-resistant infections).



Overall, the proportion of transmitted resistant infections did not vary greatly, between 9.9% and 13.1% compared with 10.2% if only one NAI was used. It should be noted however that prolonged combined therapy over the first three months of the epidemic course selected a higher proportion of cross-resistant strains (80% of all resistant strains vs 36% if only one NAI was used). On the other hand, “spatial” and “sequential” allocations of NAIs were associated with lower proportions of cross-resistant strains (31% and 41%, respectively) than “random” allocation (61%). To conclude, this is the first report of an individual centered-modelling of influenza spreading where resistance to neuraminidase inhibitor is simulated across a plausible social network. With a *de novo* resistance rate of 0.2%, the size of a pandemic would not be greatly affected by emerging resistance. But huge use of NAIs for treatment could lead to substantial selection of resistant strains at the end of the first wave, which in turn may compromise future use of NAIs. A key parameter is the relative transmissibility of the resistant vs the sensitive strain. Finally, cross-resistance between NAIs, if any, may limit the benefit of using two NAIs, individually or combined.

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Modeling Preventive Measures During an Influenza Pandemic in Italy: A Real Time Simulation Strategy

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We developed a stochastic individual-based discrete-time model to assess the impact of pandemic influenza containing strategies. The spread of influenza was then modeled with specific levels for households, schools/workplaces, and random contacts. We considered different R_0 values (1.4, 1.7, 2), evaluating the impact of combined preventive measures (vaccination, antiviral prophylaxis (AVP), border restrictions and increased social distancing). For each measure, various scenarios were considered, assuming different target populations, timing and duration of interventions. The model took about 10 minutes to run on a standard computer (Pentium D 3.20GHz, 4 GB RAM). With R_0 values 1.4-1.7, the use of combined measures would reduce attack rates (AR) from 34-56% using XX to 4-10% using YY. Assuming $R_0=2$, AR can be reduced from 69% to 20% only if vaccination starts 4 months after isolation of the pandemic strain, in combination with a 99% reduction in airport traffic, closure of schools/workplaces for 4 weeks and AVP of close contacts of clinical cases. The results show that this individual-based model is a convenient tool allowing simulation of influenza spread, and estimation of the impact of various control measures.

Introduction

Different mathematical models have been recently implemented to examine the feasibility of pandemic containment using different strategies [1-7]. In particular, individual-based models can provide reliable estimates about the worldwide spread of influenza [1, 2]. These models have shown that the worldwide spread of influenza would occur over a period of 2 to 6 months, depending on the basic reproductive number (R_0), and that, to reduce transmission, it would be necessary to implement multiple control measures [2-4,6,8]. In this study, we evaluate the diffusion of pandemic influenza in Italy and the impact of various control measures (antiviral prophylaxis, social distancing measures, including travel restrictions, and vaccination, both pandemic and pre-pandemic vaccine), assuming different R_0 values. In order to reduce the computational time, we modeled the random spread of infection, using national commuting data. Since it has been shown that seasonal influenza vaccine effectiveness is higher in adults than in elderly and children [9-11], we also assumed that both pandemic and pre-pandemic vaccine effectiveness varies by age.

Methods

The worldwide spread of pandemic influenza and the consequent importation of cases in Italy were modelled using a global deterministic SEIR model [7]. We assume that infectious individuals are all symptomatic and no longer travelling and that exposed individuals are asymptomatic and possibly travelling before the infectious phase. To estimate the number of imported cases we couple the results of the global SEIR model with 2003 data on arrivals and departures in Italy's 38 international airports [13]. The national impact of pandemic influenza and of various control measures were predicted using a stochastic individual-based model (IBM) [1]. All parameters implemented in the model were based on published studies [1, 2, 6, 8]. We considered different transmission rates to obtain R_0 values of 1.4, 1.7, and 2, which in the IBM corresponded to cumulative infected attack rates (AR) of 34.0, 55.6, and 68.7%, respectively, indicating a mild, moderate and severe scenario [12]. Data for the Italian population was obtained from the 2001 Census, which includes information on age structure, household size, household composition (e.g., singles or couples with or without children), school attendance, employment categories, municipality of residence, and data on the population that commutes daily within national borders [14]. Based on previous studies showing that work commutes are a good predictor of the spread of influenza [1, 2,15], we used data on commuting, available from 2001 Census data [14]. Schools and workplaces were generated using previously reported methods [1]. Infection can be transmitted within households, schools/workplaces, and among random contacts. While we assumed homogeneous mixing in households, schools and workplaces, random contacts depend on each individual's specific social network. We modeled random contacts on the basis of commuting data [14]. The R_0 of the simulated epidemics were estimated according to a previously published model [16]. *Control measures: Social distancing.* We considered the nationwide closing of all schools and some public offices not providing essential services. We assumed that school and office closings begin after the onset of the first 20 symptomatic cases in Italy and that this measure be maintained for 4 weeks. We considered travel restrictions that would reduce incoming international flights by 90% or 99% for two months after the first national case. *Antiviral prophylaxis.* AVP for uninfected individuals (household contacts and contacts in school or workplace) was assumed to reduce susceptibility to infection by 30% and infectiousness by 70% [3]. We assumed that AVP be provided to 90% of close contacts of clinical cases (50% of all infected individuals). We did not consider the therapeutic use of antiviral, since it has been demonstrated that it does not affect virus transmission [18]. *Vaccination.* Vaccine effectiveness (VE) was assumed to be 70%. It was also assumed that VE varies by age: i) 59% in 2-18 year-old individuals [10]; ii) 70% 40-64 year-old individuals [9]; and iii) 40% in ≥ 65 year-old individuals [11]. We assumed that an adequate supply of vaccine could be reached at 4, 5 and 6 months after the first world case. We assumed that we would administer two vaccine doses, one month apart. Vaccination coverage was fixed at 60% of the target population, basing on 2005-2006 National

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influenza coverage [17]. The target population was divided into 4 categories: i) personnel providing essential services (15% of the 25-60-year-old workers) [25]; ii) ≥ 65 year-old individuals; iii) 2-18 year-old individuals; and iv) 40-64 year-old individuals.

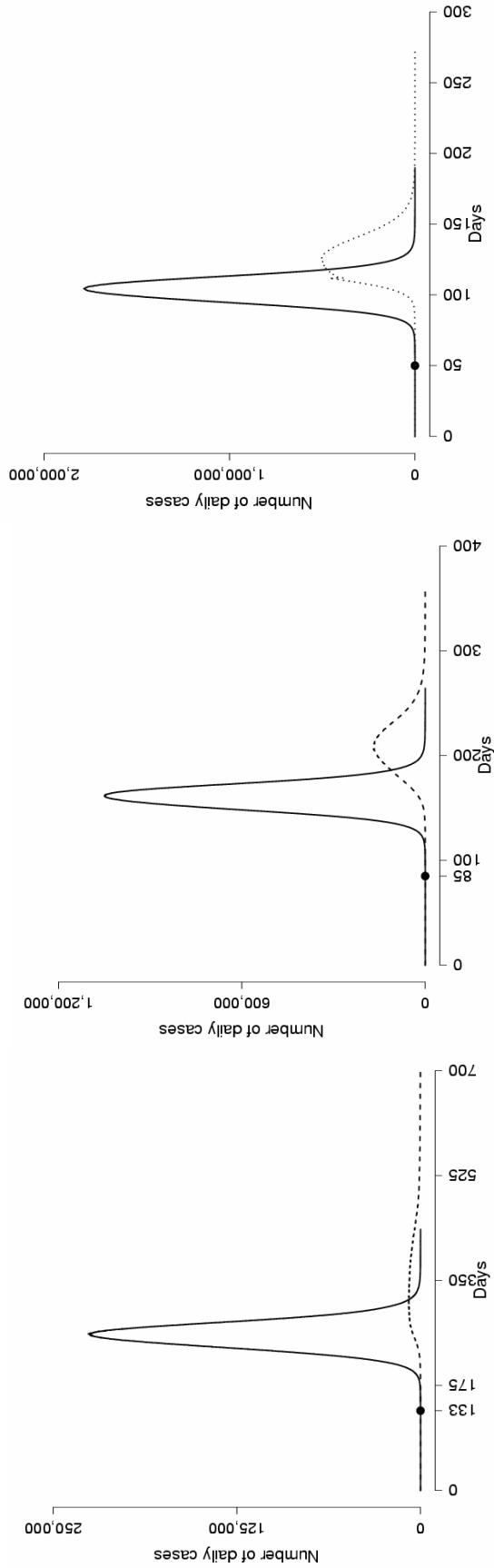
Results

Our model took about 10 minutes for a single run, allocating about 3GB of RAM memory on a Pentium D 3.2GHz workstation, allowing for real-time simulations using a standard computer. For different R_0 scenarios, the results of the global SEIR model showed that the number of imported cases would be 160,000, 226,000 and 315,000, with the first Italian case appearing, respectively, after 133, 85 and 50 days from the first world case. The epidemic peak would be reached after 259, 162 and 104 days, respectively, for the three different scenarios. The cumulative infected AR would be 34%, 55.6% and 68.7%, for the three scenarios. Border restriction, antiviral prophylaxis and social distancing measures are effective in delaying the epidemic's arrival and dampening the peak, allowing us to gain time for developing vaccines and implementing preparedness measures. However, none of these measures singly implemented is sufficient to contain a moderate to severe epidemic. Our results show that applying all containment measures to a mild to moderate epidemic, AR decreased from 34% to 4.4% and from 56% to 10%, respectively (Figure 1). In the severe scenario, only the immediate vaccine availability (4 months from the first world case), along with severe border restrictions (90% of airport traffic reduction for two months), social distancing measures (closure of schools and workplaces for 4 weeks), and antiviral prophylaxis of close contacts of the clinical cases can reduce the impact of the epidemics (with attack rates decreasing from 69% to 20%) (Figure 1). The assumed VE (i.e., 70% for all target categories or different VE for different target categories) would not substantially affect the cumulative infected AR. If vaccinating personnel providing essential services (15% of the 25-60-year-olds Italian workers), elderly persons, and 2-18 year-olds, the AR would also decrease among individuals 19-64 years of age. In particular, it would reduce by approximately two times the AR in unvaccinated 30-50-year-old adults. Excluding the elderly from vaccination would not affect the infected AR in the other age groups.

Discussion

The containment of an emergent pandemic strain of influenza is feasible, but it requires a combination of different interventions such as pre-pandemic vaccination, prophylactic use of antiviral drugs and social distancing measures. As other recent analysis show [1, 3], the outbreak could be mitigated through a combination of antiviral prophylaxis and reduction of social contacts. Vaccinating 2-18 year-olds would reduce by approximately two times the AR in unvaccinated adults, showing a clear herd immunity effect. These findings suggest that vaccinating children should be a higher priority than vaccinating elderly [20]. The role of restricting international travel remains controversial [2, 6, 7, 15, 23-24]. Our results show that assuming a $R_0 < 2$, the role of such restrictions is limited, but has a role if the R_0 were 2. The results of this study show that this individual based model is a convenient tool allowing the simulation of influenza spread and estimation of the impact of control measures with little computational effort.

Figure 1. Number of daily cases in the absence of interventions (solid lines) and by applying all the control measures (dashed lines) for $R_0=1.4$ (left), $R_0=1.7$ (middle) and $R_0=2$ (right). Circles represent the day of the first Italian case.



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A Simulation Model for Testing the Effects of Multiple Interventions During an Influenza Pandemic

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We used a stochastic simulation model to estimate the effectiveness of different intervention strategies during a 1957/1958-like influenza pandemic in a small urban community in the U.S. We tested both univariate and multivariate effects of age-based vaccination strategies, quarantine of ill individuals and their household contacts, and school closings. The outcomes modeled were illness rates, hospitalizations rates and death rates. In our model, one potentially effective combination strategy included vaccinating person aged 5-18 years old first, isolating ill individuals and quarantining their household contacts within 2 days of illness onset, and closing schools for at least 3 weeks.

Introduction

Influenza pandemics are associated with significant morbidity and mortality, (1;2) and they have the potential for significant economic burden. (3) The U.S. Centers for Disease Control and Prevention (CDC) has developed community-based guidance for interventions designed to slow the spread of a pandemic influenza virus. (4) This guidance considered results from several pandemic modeling studies. (5; 6) We previously published a simple stochastic simulation model which demonstrated significant beneficial effects of isolating ill individuals and quarantining their household contacts in reducing illness rates, hospitalization rates, and death rates associated with a 1957/1958-like pandemic influenza. (7) We also estimated small positive effects of short-term school closures of ≤ 21 days in length but loner school closures were not evaluated. Other studies have estimated significant benefits of longer-term school closings (5;6) and the recent CDC guidance suggested the use of school closings of up to 12 weeks during a severe pandemic. (4) A more recent study that examined the effects of school closures for a 1957/1958-like pandemic assumed lower illness rates among school-aged children relative to previous studies, and therefore estimated more modest effects of school closures on R_0 . (8) Previous modeling studies have also found significant benefits of vaccination of school-aged children given limited pandemic vaccine supply. (9) We modified the structure of our simulation model, and tested the effects of longer school closures (3 to 12 weeks in length), isolating ill individuals and quarantining their household contacts, age-based vaccination strategies and combinations of these three strategies.

Methods and Materials

The basic structure of our Susceptible-Exposed-Infected-Removed (SEIR) model has been described in detail. (7) We simulated an influenza outbreak in a small urban U.S. community of approximately 1000 households with 2500 household members. The simulation model used data from the A(H2N2) Asian influenza pandemic in 1957/1958, (10) and from studies on U.S. influenza-associated rates of excess morbidity and mortality. (1; 2; 11) Each person in the simulated community belonged to one of five age-dependent strata: preschool children (aged 0-4 years), school children (aged 5-18 years), adults (aged 19-64 years), seniors (aged 65+ years) living at home and seniors (aged 65+ years) living in a LTCF. In addition, each person belonged to one or more mixing groups, according to her/his stratum: households, daycare centers, schools, work places, long-term care facilities LTCF's and the community. Our SEIR model has several characteristics that differ from other simulation models for describing the transmission of influenza: (1) The probability of transmission depends on the total duration of all contacts between two individuals on a given day, rather than of the number of times they make a contact, (2) the transmission parameters do not depend on the population size, and (3) different contact parameters can be specified for week days and weekend days.

Interventions. We examined three different interventions: age-based vaccination strategies, school closings, and isolating ill individuals and quarantining their household contacts.

Age-Based Vaccination Strategies. For the age-based vaccination strategies, we tested the effects of vaccinating different age groups given a limited supply of vaccine. We tested the effects of vaccinating 10%, 20%, 30%, 40%, or 50% of the population. The age-based strategy vaccinated the target age group first and then uniformly vaccinated the other age groups with the remaining available vaccine. We assumed that the vaccine effectiveness for susceptibility (VE_s) was 30% and the vaccine effectiveness for infectiousness (VE_i) was 50%. (12) *School Closing.* We changed the model assumptions for school closings relative to the previously published model. In the new model, we closed all schools in the community when the prevalence of influenza illness among children in any one school exceeded a pre-determined threshold, set to 1%, 3%, 5%, or 10%. All schools remained closed for 3, 6, or 12 weeks. After the specified number of weeks, schools reopened, and closed again if the illness threshold was reached in any one school. When schools were closed, household and community number of contacts and duration of contacts were set at the higher weekend levels (i.e. approximately 2 times the weekday levels).

Isolating Ill Individuals and Quarantining Their Household Contacts. When isolating ill individuals and quarantining their household contacts was implemented, a given fraction of households were assumed to voluntarily comply with the guidelines. If a household complied, then ill persons and all the members of the same household were confined to their homes. We modeled the effect of confinement after either 1 or 2 days

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of illness. Fifty percent of infect adults and 75% of infected children were modeled as having severe symptoms. If symptoms were severe, then ill persons reduced their duration of contacts with other household members by 50%. When ill cases and other household members were confined, then an individual returned to school or to work a day after her/his illness ended (even if there were other ill people in the household). A person who did not become ill returned to school or work on the third day following the last day of illness of any household member (as the length of the latent/incubation period was assumed to be two days).

Effectiveness of Interventions. We first ran a set of 200 simulations with the baseline settings for all the parameters, without any interventions. The average rates for the three outcomes of interest -- overall illness rates, hospitalization rates and death rates -- were calculated for 200 simulations and used as baseline rates. For each intervention or combination of interventions, we ran a set of 200 simulations and used the averages of these simulations as estimates of the expected rates under this intervention. The effectiveness of each intervention was defined as: Effectiveness = [(Baseline rate) – (Rate with intervention)] / Baseline rate.

Results

Baseline Rates. For this study, the same baseline was used as in the previous manuscript. (7) The baseline rate for illnesses was 32.1%, the baseline rate for hospitalizations was 196.9 per 100,000 persons and the baseline rate for deaths was 63.4 per 100,000 persons.

Age-Based Vaccination Strategies. We examined the effects of age-based vaccination strategies in which 10%, 20%, 30%, 40%, and 50% of the population received vaccine. The results of age-based vaccination strategies, assuming vaccination rates of 20% and 50% of the population, highlight several general trends. (Table 1) If we assumed that 20% of the population could be vaccinated, the most effective strategy was to vaccinate the 5 – 18 year olds first. This intervention resulted in reductions in illness rates, hospitalization rates and death rates of among all age groups of 78%, 66%, and 65%, respectively. If 50% of the population could be vaccinated, reductions in outcomes were greater and there were relatively smaller differences in outcome rates across age groups except for the policy that would vaccinate the 19-64 year olds first. For vaccination of the other age groups first, the vaccination policy results in the reduction of illnesses, hospitalizations, and deaths by more than 90%. Uniform distribution of the vaccine across age groups results in better outcomes than preferentially vaccinating the 19-64 year olds. This difference in effectiveness is due to the large number of 19-64 year olds in the population, with the result that little or no vaccine would be available for use in the other age groups.

School Closings of 3, 6, and 12 weeks. The effects of closing schools for 3-, 6-, or 12-week periods were similar across school closing thresholds of 1%, 3%, and 5%. For example, when a 1% threshold was used for closing schools, illness rates were 8.2%, 7.6%, and 7.7% for 3-, 6-, and 12-week closings, respectively. In

terms of effectiveness, when we examined the effect of closing schools for 3 weeks based on a 1% influenza illness rate, the reduction in illnesses, hospitalizations, and deaths was 74%, 63%, and 60% respectively. (Table 1) When closing schools was based on a 3% threshold, the estimates of effectiveness were similar to the 1% threshold (i.e. reduction in illnesses, hospitalizations, and deaths of 67%, 55%, and 44%, respectively). When schools were closed based on a 10% illness threshold, the effectiveness was substantially lower than using the 1% threshold (i.e., reduction in illnesses, hospitalizations, and deaths of 34%, 20%, and 11% respectively).

Isolating of Ill Individuals and Quarantine of Household Contacts. In our models, confinement to home of all household members took place after one of the household members developed symptoms of influenza. We assumed a delay of 1 or 2 days between onset of symptoms (which coincided with the onset of infection) and the beginning of the confinement period. This delay and the proportion of households that complied with the confinement rules affected the effectiveness of the intervention. As expected, effectiveness increased with the compliance percentage and decreased with the length of delay in confinement. (Table 1) For example, given a delay of one day and 50% compliance, the effectiveness of these interventions on illness rates, hospitalization rates, and deaths rates was 65%, 64%, and 62% respectively. With 75% compliance, effectiveness exceeded 75%.

Table 1. Outcome rates and vaccine effectiveness for different strategies using SEIR model.

Strategy	Category	Outcome Rates			Effectiveness		
		Illnesses per 100 Person	Hosp Per 100,000 Persons	Deaths Per 100,000 Persons	Reduction in Illnesses	Reduction in Hosp	Reduction in Deaths
Baseline Model		32.1	196.9	63.4	0%	0%	0%
Vaccination of 20% of individuals *	Uniform	15.3	94.1	29.3	52%	52%	54%
	Age 0 - 4 Years	18.3	118	38.4	43%	40%	39%
	Age 5 - 18 Years	7.0	67.9	22.2	78%	66%	65%
	Age 19 - 64 Years	21.7	143.1	51.1	32%	27%	19%
	Age ≥ 65 Years	8.7	81.8	19.3	73%	58%	70%
Vaccination of 50% of individuals	Uniform	2.3	19.5	5.4	93%	90%	91%
	Age 0 - 4 Years	2.7	20.2	8.7	92%	90%	86%
	Age 5 - 18 Years	1.5	16.8	6.6	95%	91%	90%
	Age 19 - 64 Years	12.6	80.2	25.8	61%	59%	59%
	Age ≥ 65 Years	2.5	14.1	4.0	92%	93%	94%
School Closings 3 Weeks **	1% School Illness ***	8.2	73.7	25.5	74%	63%	60%
	3% School Illness	10.7	89.3	35.7	67%	55%	44%
	5% School Illness	13.6	113.8	42.4	58%	42%	33%
	10% School Illness	21.0	156.6	56.3	34%	20%	11%
Quarantine with 1 day delay after symptom onset	25% Compliance	22.4	140.4	47.8	30%	29%	25%
	50% Compliance	11.1	70.8	24.1	65%	64%	62%
	75% Compliance	3.9	26.4	8.3	88%	87%	87%
Quarantine with 2 day delay after symptom onset	25% Compliance	24.7	157.5	51.7	23%	20%	18%
	50% Compliance	14.2	92.7	30.5	56%	53%	52%
	75% Compliance	6.4	40.3	13.9	80%	80%	78%

*We vaccinated the preferred age group first and then uniformly vaccinated the other age groups with the remaining vaccine.

**Schools were close for 3 weeks when the given threshold was reached.

***Rate of illness among school children.

Combination Intervention Strategies. We examined several different combination strategies. To illustrate the effectiveness of combinations of intervention strategies, we present one of the models in which we vaccinated 25% of 5-18 year olds, closed schools when 5% illness rate was found in any one school, and assumed 25% compliance with household quarantine within 2 days of illness onset of any household member. (Table 2) When

we varied the length of school closures most of the benefit was obtained by closing schools for 3 weeks with little added benefit after 6 weeks. Relative to baseline outcome rates, closing schools for three weeks resulted in reductions in illness rates, hospitalization rates, and death rates of 83%, 72%, and 66%, respectively.

Table 2. Outcome rates and effectiveness for several combination strategies using SEIR model.

School Closing	Outcome Rates			Effectiveness		
	Illnesses per 100 Person	Hospitalizations Per 100,000 Persons	Deaths Per 100,000 Persons	Reduction in Illnesses	Reduction in Hospitalizations	Reduction in Deaths
0 Weeks	12.7	92.9	34.5	60%	53%	46%
3 Weeks	5.4	55.1	21.8	83%	72%	66%
6 Weeks	4.3	40.7	15	87%	79%	76%
12 Weeks	3.9	40.1	14.8	88%	80%	77%

*Model assumptions.

- 1) Vaccinated 25% of the 5-18 years olds
- 2) Closed all schools based on 5% illness rate in any one school
- 3) Assumed 25% compliance with isolation and quarantine within 2 days from illness onset

Discussion

We modeled a 1957/1958-like pandemic outbreak in a small urban community by using a stochastic SEIR simulation model and tested the effectiveness of age-based vaccination strategies, isolating ill individuals and quarantining their household contacts, and school closings. We found that a combination of strategies would likely be the most effective, consistent with the results of other modeling studies. (5; 6) For example, vaccinating persons aged 5-18 years old first, quarantining individuals and their household contacts within 2 days, and closing schools for at least 3 weeks was estimated to reduce morbidity and mortality rates by 66-83%. One difference in our model results compared to other model results was that school closings of 3-weeks in length were almost as effective as school closings of 12-weeks in length for illness thresholds below 5%. This was partially driven by the fact that we closed schools repeatedly if any one school exceeded the illness threshold. Our model has several limitations. First, we assumed illness rates based on one study from the 1957/1958 pandemic, (10) which found a very high illness rate among persons aged 5-18 years of age (i.e. 62% illness rate). Therefore, the benefits of an age-based strategy that targeted 5-18 year olds would be greater than a strategy that targeted any other age group given the assumed illness rates. A recent UK study suggested that the illness rates among 5 – 18 year olds may have been lower than 62% during the 1957/1958 pandemic. (8) Second, we assumed that when schools were closed that contact rates for children within the household and the community *increased* to their assumed weekend levels. During an actual pandemic, depending on the severity, children at home may *decrease* their contacts relative to the baseline levels that we used in our model. In future modeling studies, we plan to 1) examine the effects of different intervention strategies by using 1918- and 1968-like age-specific

illness rates, 2) examine the effects of alternative contact rates among school-aged children while schools are closed and 3) examine the effects of school closures triggered by community-wide, rather than school-based, influenza illness attack rates. We will also perform detailed cost-benefit analyses using a variety of alternative intervention strategies. CDC is also developing a software package based on our model that uses a simple, user-friendly interface so that local and state public health officials can model the estimated effects of alternative pandemic intervention strategies by using local data and assumptions.

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Proceedings Topic #12

Virus Host Interaction/Pathogenesis

**Oral
Presentations**

Amino Acid 226 In the Hemagglutinin of H9N2 Influenza Viruses Determines Cell Tropism and Replication in Human Airway Epithelial Cells

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H9N2 influenza viruses are endemic in poultry, particularly in Asia. Some of these viruses have transmitted to humans (1, 6, 9, 13). The seroprevalence of H9N2 viral antibodies in apparently healthy individuals was reported to be around 2% (13) or higher (2, 6). Similar to human H9N2 isolates, an increasing number of avian and swine isolates are shown to have Lysine (L) at amino acid position 226 of the HAs (3, 7, 8, 10, 12). The acquisition of L-226 in H9N2 viruses was thought to have occurred in the avian hosts and preceded their introduction into mammals (12). The biological importance of this molecular change, however, is poorly understood, and the features of the receptor-binding site that contribute to human infection have yet to be determined. Our studies showed that the presence of L-226 in the HAs enables H9N2 viruses to infect nonciliated epithelial cells during the first round of infection of a human airway epithelium (HAE) *in vitro* model, in a manner similar to human H1N1 and H3N2 virus infections (15). This human virus-like cell tropism is likely to allow the viruses to increase the ability of crossing to humans. More importantly, the Q to L substitution at amino acid position 226 in the HAs allows H9N2 viruses to replicate more efficiently (with 100 fold higher peak titers) in HAE cultures, indicating a possible increase of infection severity in humans. The mechanism for this increased growth ability remains unknown, although our group and others (11) have noticed that the nonciliated HAE cells seem to be more optimal for the replication and subsequent spread of influenza viruses with preference for SA α 2,6Gal receptors. Taken together, our findings provide insightful clues for explaining the presence of the L-226 signature in all but one of the H9N2 isolates from the documented clinical cases of H9N2 infection in humans (the exceptional isolate, A/Guangzhou/339/00, has M residue at position 226). Avian influenza viruses of some subtypes (H1N1, H3N8, H5N1, H5N3 and H7N1) have been reported to target not less than 80% of ciliated HAE cells (11, 14). However, the avian H9N2 isolates tested in our study did not show such tight restricted tropism for ciliated cells. In our studies, we observed that H9N2 viruses showed only modest differences in the proportion of ciliated vs. nonciliated cells infected. This is not likely due to the difference between our HAE cultures and those used by other groups, as similar results were obtained with the commercially ready-to-use HAE cultures. In addition, the mallard H1N1 and H7N3 isolates reproducibly targeted predominantly ciliated cells when tested with HAE

cultures produced in our laboratory. Consistent with the cell tropism in HAE cells, the tested H9N2 viruses displayed either exclusive SA α 2,6Gal or dual receptor specificity (bound both SA α 2,3Gal and SA α 2,6Gal), and none of them showed exclusive SA α 2,3Gal receptor specificity, in hemagglutination assays with resialylated CRBCs. The nonciliated HAE cell preference and SA α 2,6Gal receptor specificity, of the four recent H9N2 isolates tested, were obviously determined by L-226 in the HAs, as the mutation of the residue at HA position 226 resulted in the shift of the cell tropism and receptor specificity. However, it is noteworthy to mention that the three Q-226 containing isolates used in our studies have dual HAE cell tropism and dual receptor specificity rather than typical avian virus phenotype observed with other avian influenza viruses (ciliated HAE cell preference and exclusive SA α 2,3Gal receptor specificity). Residue glycine (G) at amino acid position 225 of HAs has been shown to be responsible for the dual receptor specificity of H1N1 viruses (5). We noticed that most avian H9N2 isolates have G and fewer have A or aspartic acid (D) at amino acid position 225 of HAs (10). It remains to be determined whether the dual cell tropism and dual receptor preference of some of the tested H9N2 viruses in the present study is due to the existence of G-225 in their HAs. All but one H9N2 viruses tested in our studies replicated to some extent in HAE cells. The exception, the early duck isolate A/duck/Hong Kong/448/78 (H9N2), did not show any replication in HAE cells even after 24 h PI. The failure of the virus to replicate in HAE cells was due to the restriction in its surface proteins, as a reassortant carrying the surface protein genes of A/guinea fowl/Hong Kong/WF10/99 (H9N2) and the internal component genes A/duck/Hong Kong/448/78 (H9N2) replicated readily in HAE cells. Considering this result and the observation that two additional early isolates (A/duck/Hong Kong/702/79 and A/quail/Hong Kong/A29845/88) have dual cell tropism, whereas the more recent isolates (A/chicken/Hong Kong/G9/97, A/chicken/Hong Kong/SF3/99, A/guinea fowl/Hong Kong/WF10/99 and A/duck/Hong Kong/Y280/97) showed cell tropism similar to that of human viruses, it seems reasonable to speculate that domestic poultry have provided an ideal environment for H9N2 viruses to evolve into strains more prone to infect humans. This concept is consistent with the previous observations that some poultry species, including chickens and quail, possess also SA receptors for human influenza viruses besides the typical avian virus-like receptors (4, 16). Since the late 1990s, the H9N2 viruses have been occasionally isolated from patients displaying flu-like illness (1, 6, 13). Fortunately, there is no evidence, so far, of human-to-human transmission, and the human isolates of H9N2 virus were of purely avian origin. Our results of growth kinetics indicated that avian H9N2 viruses, even those with L-226 in the HAs, grew to peak titers significantly lower (10-100 fold) compared to two prototype human H3N2 viruses. This is in agreement with the recent findings indicating that avian influenza viruses of the H5N3 subtype grew less efficiently than human H3N2 isolates in HAE cells (14). Our observations may help explain, albeit in part, the lack of transmission of H9N2

viruses among humans. However, considering their dual- or human virus-like tropism, H9N2 viruses are more likely to be transmitted to intermediate hosts (like pigs), which possess SA receptors for both avian and human influenza viruses, and thus increase the chance of reassortment with viruses of other subtypes. Nowadays, the opportunity for such reassortment continues to exist, as Peiris and colleagues have reported that H9N2 viruses are co-circulating with contemporary human H3N2 influenza viruses in pigs in Asia (12). Furthermore, such reassortment can also occur in humans in close contact with poultry species, in which H9N2 viruses are endemic. Our results are in favor of continuous surveillance studies of H9N2 viruses in Asia. The use of HAE cells on a routine basis could certainly be helpful in discriminating H9N2 influenza isolates that are more prone to infect humans. Acknowledgments: This work was supported by grants from NIH-NIAID (RO1-AI052155) and USDA (CSRESS NO 2005-35605-15388). We are indebted to Dr. Ruben Donis at the Centers for Disease Control and Prevention, Atlanta, GA for critically reading and editing the manuscript. We thank Dr. Laurel Glaser from the Mount Sinai School of Medicine and Dr. Liqun Zhang from University of North Carolina for helpful discussion in performing the studies. We would like to express our gratitude to Dr. Robert G. Webster and Scott Krauss for providing the viruses used in the study. We specially thank Dr. Haichen Song for help in the studies and in preparing the manuscript. We also thank Dr. Chinta M. Lamichhane for providing the monoclonal antibody used in the immunostaining.

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Detailed Pathogenesis of Swine Influenza Virus in the Natural Host

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Introduction

Swine influenza virus (SIV) is a major cause of acute respiratory disease in pigs [1]. Three SIV subtypes – H1N1, H1N2 and H3N2 – are enzootic in swine populations worldwide, and these subtypes do not seem to differ in virulence or pathogenicity. Typical outbreaks of swine “flu” are characterized by a rapid onset of high fever, depression, anorexia, laboured abdominal breathing and coughing. Weight loss or growth retardation may also occur, but mortality is usually low and the pigs recover within one week. Moreover, many SIV infections in the field have a very mild or subclinical course. Under experimental conditions typical SIV symptoms can only be reproduced by intratracheal inoculation of a high dose of virus. SIV has been shown to replicate in the nasal mucosa, tonsils, trachea, tracheobronchial lymph nodes and lungs of pigs [2]. In the lungs, viral antigen and/or viral RNA have been detected in epithelial cells of the bronchi, bronchioli and alveoli, and in alveolar macrophages [3]. Viremia or virus replication in extra-respiratory sites, in contrast, has only rarely been found [2, 4]. Still, the detailed pathogenesis of SIV remains unexplored. It is unknown, for example, whether the virus replicates better in the upper or lower respiratory tract and which are the major target cells. In addition, the possibility of virus replication outside the respiratory tract has rarely been examined. This study aims to investigate and elucidate these issues using intranasal inoculation, the natural route of SIV infection.

Materials and Methods

Seven 4-week-old conventional pigs were bought on a SIV negative farm and housed in Horsefall-type isolation units with positive-pressure ventilation. The individual pigs were inoculated intranasally with 10^7 50% egg infectious doses (EID₅₀) of the A/swine/Belgium/1/98 (H1N1) isolate in 3 ml of phosphate-buffered saline. The pigs were monitored clinically and one pig per day was euthanized from 1 to 7 days post inoculation (dPI). Nasal and oropharyngeal swabs were collected daily from 0 to 7 dPI or until euthanasia. Samples from the nasal mucosa (olfactory and respiratory part), nasopharynx, tonsils, trachea, four different parts of the lung (left apical and cardiac lobes, left diaphragmatic lobe, right apical and cardiac lobes, and right diaphragmatic lobe), retropharyngeal and tracheobronchial lymph nodes, spleen, ileum, colon, brain stem and conjunctiva were collected for virus titration and immunofluorescence (IF). Serum for virus titration was also collected at euthanasia. Virus titration was performed in Madin-Darby canine kidney (MDCK) cells. IF staining against influenza-A nucleoprotein was performed on all tissues. A minimum

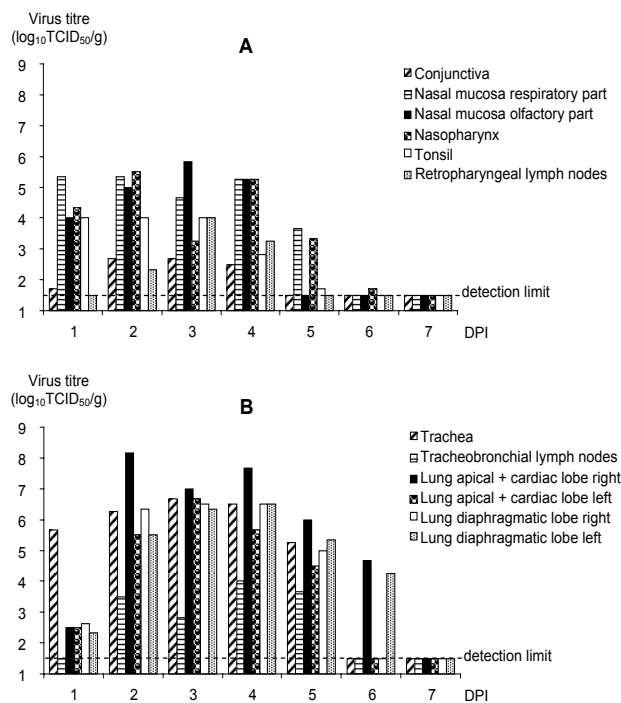
of four sections from each tissue were fixed in acetone and stained. A scoring system based on the relative percentage of positive cells in the epithelium was used to quantify the extent of IF in the upper respiratory tract samples, trachea, bronchi, bronchioli and alveoli.

Results

Clinical signs. Clinical signs, if present, were mild and lasted for only 1 day. Sneezing was occasionally observed in some pigs at 1 and 2 dPI. A transient fever (40.1–41.1°C) was observed in four of the seven pigs between 1 and 4 dPI, but there were no other general symptoms or prominent respiratory disease.

Virus titrations. SIV was isolated from nasal and oropharyngeal swabs of all pigs on days 1–5 PI except for oropharyngeal swabs in 2 pigs on day 5 PI. The mean virus titre was lower in the oropharyngeal swabs (1.8–5.3 log₁₀ TCID₅₀/100mg) than in the nasal swabs (4.3–6.2 log₁₀ TCID₅₀/100 mg). SIV was also isolated from all samples of the upper and lower respiratory tract and associated tissues. Figure 1 compares the virus titres in the upper and lower respiratory tract of the individual pigs euthanized between 1 and 7 dPI. Samples from the upper respiratory tract were positive from 1–4 (olfactory part of nasal mucosa and tonsils) or from 1–5 (respiratory part of nasal mucosa and nasopharynx) dPI. Virus titers in these samples were around 4–5 log₁₀ TCID₅₀/g tissue. The trachea and all four lung samples tested were positive from 1 throughout 5 dPI, whereas 2 out of 4 lung samples were positive in the pig killed at 6 dPI. Virus titers in the lungs and trachea usually exceeded 6.0 log₁₀ TCID₅₀/g between 2 and 4 dPI with a maximum of 8.2 log₁₀ TCID₅₀/g in apical and cardiac right lung lobes at 2 dPI. The titres were clearly higher than in the upper respiratory tract except on 1 dPI when titers in the lungs were around 2.5 log₁₀ TCID₅₀/g. The lymph nodes draining the upper and lower respiratory tract, were positive on days 2–4 (retropharyngeal) and 2–5 (tracheobronchial), though titers were still lower (2.3–4 log₁₀ TCID₅₀/g) than in the respiratory tract tissues. From all extra-respiratory tissues examined virus was only isolated from the brain stem and conjunctiva at 2–4 dPI, and virus titers were between 2.0–4.4 log₁₀ TCID₅₀/g and 2.5–2.7 log₁₀ TCID₅₀/g respectively. No infectious virus was isolated from the serum, spleen or the intestinal tract of any of the seven pigs.

Figure 1. Evolution of virus titres in the upper (A) and lower (B) respiratory tract and associated tissues after intranasal inoculation of pigs with an H1N1 swine influenza virus.



Immunofluorescence stainings. In the upper respiratory tract (URT) single viral antigen-positive cells were found in the nasal mucosa, nasopharynx, tonsils and lymph nodes. In the lymphoid tissues the cells often had a star-shape and were usually weakly positive. Fluorescing debris was found in the crypts of the tonsils between 2 and 4 dpi, but there were no clear viral antigen-positive cellular structures. The trachea revealed more positive cells than the URT. Virus was found from 2 to 5 dpi in single or groups of cells in the mucosa, and often in cellular debris in the tracheal lumen. In contrast to the URT the lungs showed massive numbers of positive cells between 2 and 6 dpi. Most positive cells were detected in the bronchioli. Frequently up to 100% of the epithelial lining was positive and a complete obstruction of the lumen with fluorescing debris, sloughed epithelial and inflammatory cells was observed. The bronchi and alveolar tissue also had many IF-positive cells, but less than the bronchioli. As an example, only 10-70 % of the bronchial epithelial cells were virus-positive. To the present moment antigen-positive cells were not detected in the conjunctiva, brain stem, intestinal tract samples or spleen of any of the 7 pigs. Identification of virus-positive cells remains to be performed.

Discussion

Though it is clear that mammalian influenza viruses cause an acute infection of the respiratory tract, the detailed pathogenesis of such viruses remains obscure. It is still unclear, for example, whether human influenza viruses replicate

mainly in ciliated or non-ciliated cells of the respiratory tract, or whether influenza in humans is mainly an upper or lower respiratory tract infection. Our data indicate that SIV has a stronger tropism for the lower respiratory tract of pigs, bronchioli in particular, than for the upper respiratory tract. We will continue to determine the exact cell tropism of SIV in the respiratory tract using cell-markers. The nature of the single weakly positive cells found in the lymph nodes and tonsils also remains to be determined. These cells may be antigen-presenting cells that phagocytose virus without allowing productive virus replication. The single extra respiratory tissues from which SIV could be isolated were the conjunctiva and the brain stem. Virus titers in the brain stem correlated with those in the olfactory part of the nasal mucosa, and we found no evidence for systemic spread of SIV. Both findings suggest that SIV may spread to the brain by olfactory nerves, as has been described in mice with the mouse-neuroadapted WSN/33 influenza virus [5] and reviewed by Kristensson [6]. In humans, H1N1 and H3N2 influenza virus infection has been occasionally associated with encephalitis and human influenza viruses have been isolated from the brain of fatal influenza cases during the 1971-1975 period [7]. A human H3N2 influenza virus as well as two highly pathogenic avian H5N1 viruses were also isolated from the brain of experimentally infected ferrets [8]. This suggests that not only avian influenza, but also common mammalian influenza viruses have the ability to invade the brain of humans and possibly of pigs. On the other hand viral antigen-positive cells have not yet been demonstrated in the brains of humans or pigs and it remains to be confirmed whether the virus has a true tropism for cells of the central nervous system of these species. We have previously suggested that pigs are valuable animal models for research on human influenza [9]. The present study will allow us to further compare the pathogenesis of influenza in pigs with that in other influenza models like mice and ferrets.

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Contribution of Viral and Host Genes to the High Pathogenicity of H5N1 Influenza Virus

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H5N1 influenza viruses transmitted from birds to humans in Asia, Europe and Africa cause high mortality. Viral and host genes involved in H5N1 pathogenesis must be identified to determine which factors may be therapeutic targets. We utilized reverse genetics to generate H5N1 reassortants combining genes of lethal A/Vietnam/1203/04 (VN1203), a fatal human case isolate, and non-lethal A/chicken/Vietnam/C58/04 (CH58). In vivo pathogenicity of these H5N1 viruses was tested using mice and ferrets. Exchange of the VN1203 polymerase genes with those of CH58 resulted in attenuation of virulence in vivo and reduced polymerase activity. Interestingly, substituting CH58 NS gene partially attenuated VN1203 in ferrets but not in mice. The role of host cytokine responses to these reassortant viruses was investigated using mice deficient in the hallmark inflammatory cytokines. Overall, our results suggest that the high virulence of avian H5N1 in mammalian species may be due to adaptive changes in viral polymerase complex genes.

Introduction

Currently, H5N1 viruses pose a threat to global health as they have been found to cause high rate of mortality in humans in Asia, Africa and Europe. The clinical symptoms include fever, viral pneumonia, encephalitis, and acute respiratory distress syndrome¹⁻⁷. H5N1 infections are also characterized by lymphopenia and high levels of inflammatory cytokines^{1,4,8,9}. There are major knowledge gaps in viral and host factors contributing to the high virulence of H5N1 in humans. In 2004, two H5N1 isolates from Vietnam became of great interest to our research. One virus was isolated from a fatal human case, A/Vietnam/1203/04 (VN1203) and the other was an avian isolate A/Chicken/Vietnam/C58/04 (CH58). Both of these H5N1 viruses are of the Z genotype, have the multibasic amino acid motif in hemmagglutinin (HA) and are lethal to chickens¹⁰. However, VN1203 was highly lethal to mammalian species, while CH58 was non-lethal^{10,11}. Sequence analysis of VN1203 and CH58 revealed only 31 amino acid differences¹⁰. Specifically, there are 3 amino acid differences in PB1 gene, 4 in PB2, 4 in PA, 5 in HA, 1 in NP, 5 in NA, 1 in M and 8 in NS. We hypothesized that the differences in virulence between VN1203 and CH58 may be due to tissue tropism as determined by surface glycoproteins HA and NA, evasion of host innate immunity by NS, or viral growth due to polymerase genes. It is not well understood whether adaptive changes in HA and neuraminidase (NA), NS1 or the polymerase

genes are important for interspecies transmission and virulence. Thus it is important to map the viral virulence factors in mammalian hosts which contribute to the pathogenicity of H5N1 viruses. Here we demonstrate the use of reverse genetics to generate recombinant H5N1 viruses of VN1203 and CH58 and test their virulence in mice. Our studies allowed us to identify the viral factors which contribute to the difference in high virulence of VN1203 as compared to CH58. In particular we examined the contribution of the viral surface glycoproteins HA and NA, NS gene, and polymerase genes. Mice were used in these experiments because they have been well established as a model that following H5N1 infection, have virus replication sites, cytokine production, and pathogenicity comparable to that in humans^{12,13}. We also investigated the contribution of host factors, specifically cytokines, to the pathogenicity of VN1203 H5N1 virus. High levels of pro-inflammatory cytokines, including TNF- α , IL-6, and CCL2, have been measured in human cells and mice infected with highly pathogenic H5N1 influenza virus^{7,9,12-15}. Yet, the importance of the high induction of pro-inflammatory cytokines, commonly referred to as the "cytokine storm", in the pathogenesis of H5N1 is not known. It is believed that the dysregulated cytokine production may be the main trigger of pathology and ultimately of death. Thus, we also studied whether inhibition of the host cytokine response is sufficient to protect against death caused by the highly virulent H5N1 VN1203. Mice genetically deficient in specific cytokines were used to examine the contribution of key inflammatory cytokines TNF- α , IL-6, or CCL2. Furthermore we studied whether cytokine suppression by treatment of mice with glucocorticoids protected mice from the high pathogenicity of H5N1.

Materials and Methods

Use of reverse genetics for generation of recombinant viruses. WHO collaborating laboratories provided A/Vietnam/1203/04 (H5N1) and A/chicken/Vietnam/C58/04 (H5N1) influenza viruses which were grown in chicken eggs. All work was conducted in biosafety level 3+ conditions. All eight viral genes were amplified by RT-PCR, and viral cDNAs were inserted into dual-promoter plasmid pHW2000¹⁶. Plasmid coding sequences were identical to PCR fragment sequences. Recombinant viruses were made by DNA transfection of MDCK/293 T cells. Transfection supernatant was injected into chicken eggs, and virus stock prepared, sequenced and titrated. The universal primer set for influenza A virus was used for RT-PCR of viral RNA¹⁷. Sequencing was performed by Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital.

Infection and Corticosterone Treatment of mice. Seven to ten week-old male mice were lightly anesthetized with isoflurane and inoculated intranasally with 10^3 EID₅₀ of virus in 50 μ l of PBS. C57Bl/6J, B6129SF2/J, B6129Tnfrsf1atm1Mak/J, B6129S6-Tnftm1Gkl/J, B6129S-Tnfrsf1atm1lmx, Tnfrsf1btm1lmx/J, B6129S2-Il6tm1Kopf/J, and B6129S4-Ccl2tm1Rol/J mice were purchased from Jackson Laboratories. Weight and survival were recorded. Corticosterone-treated mice had 30 μ g/ml of corticosterone in drinking water three days prior to infection,

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three days prior and throughout infection or three days post infection with virus. All animal studies were done in accordance with the St. Jude Children's Research Hospital animal care and use committee.

Minigenome assay. 293T cells were transfected with luciferase reporter plasmid and mix of PB2, PB1, PA, and NP plasmids of VN1203 and CH58 viruses. After 24 hours, cell extracts were made and luciferase levels were assayed. Experiments were done in triplicate.

Results

VN1203 is highly lethal while CH58 is non-lethal to mice. Using the eight-plasmid system reverse genetics system¹⁶, we generated two sets of plasmids encoding the genes of VN1203 and CH58. Transfection of cells with the eight plasmids resulted in recombinant viruses that were identical to their parental strains by sequence analysis. C57BL6 mice inoculated with 10^3 50% egg infectious dose (EID₅₀) of VN1203 had significant morbidity, as measured by weight loss, and all died. However, mice inoculated with the same dose of CH58 virus exhibited no disease symptoms.

Surface glycoproteins of CH58 do not attenuate the pathogenicity of VN1203 in mice. We tested whether the high pathogenicity of VN1203 in mice was caused by differences in tissue tropism due to the five amino acid changes in the HA and NA genes as compared to CH58. Thus we measured whether the viruses differed in the specificity of their HA for the receptors typical avian strains, α -(2,3)-linked sialic acid (SA), or for the typical human strains, α -(2,6)-linked SA. The results were that both VN1203 and CH58 had greater affinity toward synthetic α -(2,3)-linked SA than for the α -(2,6)-linked SA. We also produced a reassortant VN1203-CH58(HA,NA) virus that had the six internal genes of VN1203 and the HA and NA genes of CH58. Mice inoculated with 10^3 EID₅₀ of VN1203-CH58(HA,NA) virus had similar weight loss to mice infected with VN1203 and all died. Taken together, the surface glycoproteins of the VN1203 and CH58 do not alter the pathogenicity in mice.

NS gene does not contribute to differences in lethality between VN1203 and CH58 in mice. We also chose to investigate the contribution of the NS gene as it has been shown to regulate innate immune by inhibiting the host type I interferon defense system. Using the reverse genetics technology we generated single-gene reassortants that combined CH58 NS with the remaining seven VN1203 genes. All mice inoculated with VN1203-CH58(NS) had significant weight loss and died. Thus in mice, the viral NS gene does not contribute to the difference in lethality between the VN1203 and CH58 viruses.

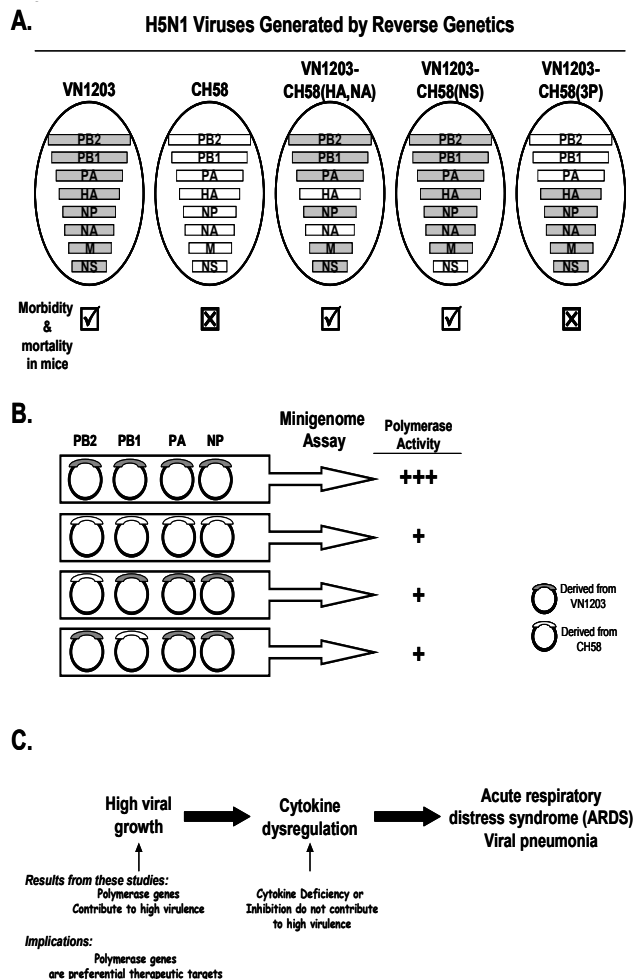
Polymerase genes contribute to lethality of VN1203 in mice. Between the highly lethal VN1203 and non-lethal CH58 viruses, there are eleven amino acid differences in the three polymerase genes. We generated a reassortant virus VN1203-CH58(3P), consisting of CH58 PB2, PB1, and PA genes and the remaining genes from VN1203. Remarkably, this virus was not lethal to mice. Mice inoculated with VN1203-CH58(3P) showed no signs of morbidity as measured by weight loss. We also

tested whether the three polymerase genes were sufficient to cause high lethality in mice by generating a reassortant virus encoding VN1203 polymerase genes and the remaining segments from CH58. All mice inoculated with this virus caused severe weight loss and 100% lethality. Therefore, the VN1203 polymerase genes are sufficient to transform the non-lethal CH58 virus into a lethal virus for mice. Mice inoculated with the single-gene VN1203-CH58(PB2) reassortant exhibited no weight loss, and an 88% survival rate. Furthermore, using reverse genetics we generated a virus of VN1203 with a point mutation in PB2 changing lysine (K) at position 627 to glutamic acid (E). Infection of mice with this virus with the E⁶²⁷ in PB2, like that of CH58, did not result in any morbidity or mortality. Thus, specifically the K⁶²⁷ of VN1203 PB2 contributes to lethality in mice. Similarly, mice inoculated with single-gene reassortant VN1203-CH58(PB1) resulted in 100% survival. Mice did lose weight following infection but recovered after 9 days. In summary, the results demonstrate that the PB2 and PB1 polymerase genes contribute to the high pathogenicity of VN1203 in mice. A schematic summarizing these results is shown in Figure 1A.

Polymerase activity of VN1203 is greater than CH58. The polymerase activity of VN1203 and CH58 were measured in a minigenome assay by transfecting 293T cells with the plasmids encoding the viral PB2, PB1, PA, and NP genes and a luciferase reporter plasmid which is regulated by the influenza non-coding regions¹⁸. The luciferase levels reflect the polymerase complex's transcription and replication activity. Our results demonstrated that the polymerase complex of VN1203 had 3.5-fold more polymerase activity than CH58 of the polymerase complex. Replacing the VN1203 PB2 or PB1 with that of CH58 significantly reduced the viral polymerase activity. These results are summarized in Figure 1B. Overall, the VN1203 polymerase complex had significantly higher activity than the CH58 polymerase complex.

Contribution of host cytokines to pathogenicity of VN1203 in mice. To examine the contribution of TNF- α , which is believed to be the main cause of lymphocyte-mediated lung injury, we inoculated mice genetically deficient in TNF- α or its receptors with VN1203 virus. The weight loss and rate of death were similar in wild-type mice and mice deficient in TNF- α , in TNFR1 receptor or in both TNFR1 and TNFR2 receptors. Thus, deficiency in TNF- α or its receptors does not protect mice from the morbidity and mortality following infection with H5N1. Mice deficient in the pro-inflammatory cytokine, IL-6 were also not protected from the high lethality of H5N1 virus infection. We also examined the contribution of the chemokine, CCL2 also referred to as monocyte chemoattractant protein-1 (MCP-1). Mice deficient in CCL2 were inoculated with VN1203 and experienced a weight loss and mortality similar to wild-type mice. Therefore, deficiency in TNF- α , IL-6, or CCL2 alone is not sufficient to protect mice against highly lethal H5N1 viruses. We tested the effect of corticosterone, the murine glucocorticoid, which is known to suppress cytokines. Mice were administered corticosterone prior to infection, throughout infection, or

Figure 1. Summary of Results. (A) Generation of reassortant viruses of VN1203 and CH58 using reverse genetics and the outcome on morbidity and mortality following infection of mice with these viruses. The genetic segments derived from VN1203 or CH58 are represented as grey or white respectively. Viruses that cause high morbidity and mortality are signified with a checked-symbol , while those viruses that do not are designated with the x-symbol . (B) Summary of polymerase activity measured for the indicated polymerase complexes as measured from minigenome assay. The polymerase genes derived from VN1203 or CH58 are represented as grey or white respectively. (C) Schematic of key events characteristic of highly pathogenic H5N1 viruses and potential therapeutic targets.



three days post infection with VN1203. Corticosterone-treated mice and untreated mice had similar rates of weight loss and death following infection with VN1203. Thus, glucocorticoid treatment does not reduce the lethality of infection with highly pathogenic H5N1 virus.

Discussion

In these studies, we use reverse genetics technology to map the viral factors that contribute to the high virulence of a H5N1 virus in mice. We demonstrate that the high pathogenicity of VN1203 is attenuated by the polymerase genes of the non-lethal CH58. Interestingly, the HA, NA, and NS genes of VN1203 do not contribute to the differences in lethality of VN1203 and CH58.

Furthermore, the results from the polymerase activity assay, established in these studies, suggest that antivirals targeting the polymerase proteins may be effective in protection against H5N1 virus infection. The polymerase activity assay may be used for high-throughput screening to test for potential antivirals. There is great interest in whether the dysregulated cytokine production following H5N1 infections is the primary cause of severe disease. Therefore the inhibition of the inflammatory cytokine response has been proposed as therapy for a person infected with H5N1 virus. Our results demonstrate that mice deficient in the inflammatory cytokines TNF- α , IL-6, or CCL2 had similar morbidity and mortality rates to wild-type mice following infection with VN1203. Treatment of mice with corticosterones also did not protect them against VN1203. Thus inhibition of the cytokine response to infection with virulent H5N1 is not sufficient to protect mice from the lethality of the virus. Figure 1C illustrates that highly virulent H5N1 viruses possess high viral growth efficiency, which results in cytokine dysregulation and ultimately ARDS, viral pneumonia and death. The findings from our studies propose that inhibition of viral replication may be more effective than inhibition of the cytokine response in ensuring host survival of H5N1.

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Proceedings Topic #13

Clinical Guidance and Policies

Oral Presentation

The Macroepidemiology of Influenza Vaccination in 73 Countries: An Update for 2004 and 2005

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Macroepidemiology of Influenza Vaccination (MIV) Study Group, France

Introduction

In the early 1990s, individual investigators began to gather country-specific information on the distribution of influenza vaccine and on vaccination recommendations and reimbursement policies. Over the next decade they gradually increased the number of countries included in their surveys and published their findings in several reports. In 2002, the Influenza Vaccine Supply International Task Force, and industry organization, was established and soon reported its findings on the global distribution of influenza vaccine for the years 2000-2003 [1]. However, country-specific data were not reported and many felt they were needed. For this reason, following the Options V meeting in Okinawa in 2004, an e-mail network of individual investigators was established. This group is known as the Macroepidemiology of Influenza Vaccination (MIV) Study Group, and in 2005 published its findings for 56 countries for the years 1997-2003 [2]. The MIV Study Group is entirely voluntary and receives no financial support from any source. This report updates the MIV Study Group survey for the years 2004 and 2005, extending its findings to 73 countries. It explores changes in influenza vaccination that have taken place since the re-emergence of H5N1 influenza in Southeast Asia in 2003 and explores the implications of these findings for pandemic vaccination.

Methods

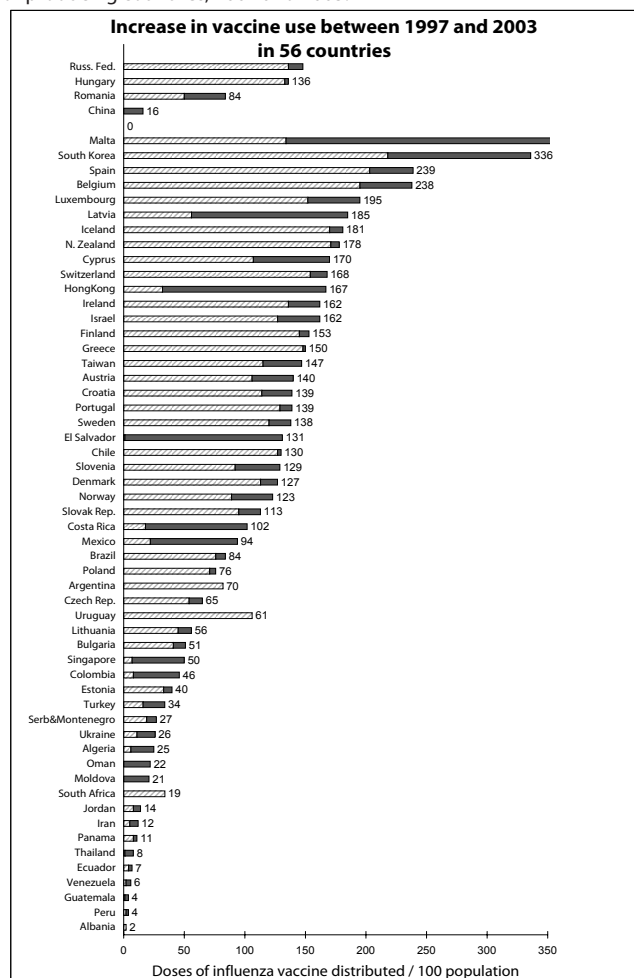
For almost all countries, individual MIV Study Group investigators were recruited from academic institutions or government health departments or laboratories. Using an annual e-mail questionnaire, each investigator reported information on the number of doses of influenza vaccine distributed and national recommendations for influenza vaccination and policies for vaccination reimbursement in his or her country [2]. For some countries, investigators had not been identified and information was obtained from vaccine companies. Influenza vaccine distribution was expressed as the number of doses distributed per 1000 total population using data reported in the US Bureau of the Census International Data Base [3].

Results

In 2005, the MIV Study Group survey covered the population of all countries in North America and Western Europe, all but 4% of the population of countries in Central and Eastern Europe and all but 12% of the population of countries in Central and

South America. Among the countries of Asia and Oceania (representing 57% of the world's population), only 43% of the people in these countries were included in the survey (35% in China alone). For the countries of the Middle East and Africa, only 18% of their people were covered by the survey. Overall, the 73 MIV Study Group countries surveyed included 51% of the world's 6.56 billion people. In 2005, the 73 MIV Study Group countries used 329 million doses of influenza vaccine, a 12.7% increase over the estimated 292 million doses used by all countries two years earlier [1, 2]. Similar to earlier years, large differences were noted between individual countries, although in most countries the levels of vaccine use in 2005 had increased somewhat compared with levels in 2002 (Figure). The leading country in 2005 was Malta, which increased its level of vaccine use from 134 doses/1000 to a remarkable 657 doses/1000 in 2005. Other leading countries in 2005 (doses/1000 population) were Canada (345), the Republic of Korea (336), Japan (303), the US (274) and Germany (255). In addition to Malta, several countries showed impressive increases over the 2002-2005 period: Japan (130), Republic of Korea (118), Hong Kong SAR (117) Latvia (102), El Salvador (91) Costa Rica (84) and Mexico (72). In several countries (Argentina, Italy, South Africa, the

Figure 1. Influenza vaccine distribution in vaccine producing and nonproducing countries, 2002 and 2005.



supply shortages (US). Vaccination recommendations showed little change between 2002 and 2005, although the age cut-off (US and Uruguay), vaccine use decreased from 2002 to 2005, sometimes due to levels decreased in several countries. By 2005, four countries (Kyrgyz Republic, Mexico, Republic of Korea, and the US) recommended influenza vaccination for all people 50 years of age and older, and seven countries (Austria, Canada, Chile, Mexico, the Republic of Korea, Taiwan and the US), had adopted policies for vaccinating all children 6-23 months in age. Some form of public reimbursement for vaccination was provided in 60% of countries, and these countries tended to have higher levels of vaccine use compared with countries with no public reimbursement. In general, levels of vaccine distribution were not correlated with the economic status of individual countries; often countries with similar levels of Gross National Income per capita (adjusted for purchasing power parity) had much different levels of vaccine distribution. In 2005, nine vaccine-producing countries (Australia, Canada, France, Germany, Italy, Japan, The Netherlands, the UK and the US; see Figure) used 59% of all doses of vaccine distributed throughout the world, but these countries had only 12% of the world's population. Three other countries (Hungary, Romania and the Russian Federation) had 2% of the world's population and used 7% of the world's vaccine. China's domestic production was small, and most of its vaccine was imported. Several countries that did not produce influenza vaccines (especially Malta, the Republic of Korea, Spain, and Belgium) had higher levels of vaccine distribution than several vaccine-producing countries (see Figure). Moreover, some of the most impressive increases in vaccine use in 2005 occurred in rapidly developing non vaccine-producing countries that previously had used little vaccine (Costa Rica, El Salvador, Latvia and Mexico). Limited information was available for several countries in Asia hard hit by H5N1 influenza. Nonetheless, most of the countries in this region that were included in the survey (Hong Kong, Japan, Republic of Korea, Singapore and Taiwan) increased their use of influenza vaccine considerably between 2002 and 2005, the only exception being Thailand. All but one of these countries (Japan) were non vaccine-producing countries.

Discussion

Influenza vaccination is steadily increasing throughout the world. The 329 million doses used in 2005 probably represented >95% of all doses used throughout the world; in 2003, the 56 countries included in the MIV Study Group survey also represented >95% of the total number of doses distributed worldwide [1, 2]. In general, influenza vaccine use continued to increase more rapidly in countries that don't produce it. Almost all doses of influenza vaccine distributed in non vaccine-producing countries were imported from five countries in Western Europe [2]. The disparity between vaccine-producing and non producing countries is likely to persist and could lead to difficulties for vaccine supply in the event of a pandemic [4, 5]. The UN System Coordinator has reported that almost 100 countries will want to be supplied with pandemic vaccines

[6], yet given what is known about formulating inactivated adjuvanted H5N1 vaccines, the world's vaccine-producing countries could not produce within six months enough doses to vaccinate their own populations, let alone people in non vaccine-producing countries [4]. It has been recognized for several years that vaccine-producing countries might prohibit the export of their vaccines to non-producing countries until their domestic needs have been met, and that this might lead to a global political as well as public health crisis [4]. A hint of what might be in store appeared in February 2007, when the Indonesian Minister of Health announced that her country would no longer send specimens of H5N1 viruses to the WHO influenza surveillance laboratories and would resume virus sharing only when it could be guaranteed access to a supply of affordable pandemic vaccine [5]. The World Health Assembly attempted to deal with this impasse in May 2007 when it passed a resolution calling on the Director General to increase the transfer of vaccine production technology to developing countries, establish an international stockpile of pandemic vaccines, devise a mechanism for financing pandemic vaccine purchases and renegotiate the terms of reference for virus sharing between countries and the WHO surveillance laboratories [7]. Whether these measures will solve the access problems for non vaccine-producing countries is difficult to say, but in all likelihood the potential for political crises, at least in the near term, will get worse before it gets better. The MIV Study Group survey provides country-specific "numerator" data on the use of seasonal influenza vaccines throughout the world. What are needed, however, are complementary "denominator" data on regional and global vaccine production, something that only the IVS International Task Force is in a position to provide [1]. In the meantime, the MIV Study Group will continue to expand its coverage to include a larger number of countries, especially the unrepresented countries in South and Southeast Asia, the Middle East and Africa. In this way it will contribute to the WHO effort to increase access to affordable pandemic vaccines in all countries [8].

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Trends in Influenza-Attributable Mortality in Four Countries: Implications for National Vaccination Programs

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A recent report found that influenza-attributable (excess) mortality did not decline during 1980-2000 in any US elderly age group despite an increase in influenza vaccination coverage from ~20% to ~65%. This result was unexpected. This study was designed to provide a more stringent test using data for 1968–2000 from Australia, Canada and France. In each country, vaccination directed to defined risk groups increased strongly over this period. Linear trends in excess mortality rates were modeled by age group separately for seasons dominated by A(H3N2) and [B & A(H1N1)] viruses. For 1968-2000, no slope estimate was statistically significant for either Canada or the US; however, five age groups in Australia and in France had statistically significant slopes. For 1980-2000, four age groups in Australia and two in France had significant slope estimates. The 95% confidence intervals for slope estimates for all age groups for 1980-2000 that could be compared with 1968-2000 overlapped. Longer-term trends for B and re-emergent H1 seasons in Canada (1950-2000) and the US (1953-2000) were significant. In all four countries, the large decreases observed were consistent with the expected effects of national vaccination programs. However, these declines appear to have been continuous over not only the period in which vaccination increased strongly but across the eras of circulation of three A-type viruses. Therefore, these declines were extraneous to the influenza virus. Excess mortality appears to have been an unfortunate choice as a measure of effect for national vaccination programs. At the present time, excess mortality rates have become very low in all age groups under age 70 in these countries.

Introduction

The three-year moving average of influenza-attributable (excess) mortality for persons age ≥ 65 years was chosen as a measure of effectiveness of the national influenza vaccination program of the United States (US)^{1, 2}. However, this measure exhibited erratic behavior throughout the 1990s, and excess mortality rates for no elderly age group exhibited a decline over the period 1980-2000 when vaccine coverage for elderly persons increased from ~15% to ~65%³. This was unexpected. To expand this inquiry, we examined data from three other countries, Australia, Canada, and France over the period of circulation of A(H3N2) viruses, 1968-2000.

Methods

A precedent study on US data found that it was necessary to separately analyze excess mortality by age group and for seasons dominated by A(H3N2) and [B & A(H1N1)] viruses³. For this study, we proposed to estimate age-specific excess all-cause and excess pneumonia & influenza (P&I) mortality for the entire period of circulation of A(H3N2) viruses, 1968-2000 and for the period 1980-2000, during which influenza vaccination increased strongly in all countries studied. It was known that excess mortality rates in Canada were similar to those in the US, and that overall levels of excess mortality were somewhat higher in both Australia and France⁴. Because we planned to analyze separately elderly age groups and to compare trends modeled for 1968-2000 with trends found for 1980-2000, we chose to estimate excess mortality using a method that provides estimates that are strictly additive by age group and for which the estimates for any season do not depend on which other seasons are included in the analytical sample. We used a modification of the digital filter method described previously⁵. The digital filter method uses the moving average of observed mortality (13 months centered on the index month), multiplied by a Hamming window to prevent filtering artifacts. Excess mortality is defined in this study as the sum over a defined influenza season of the difference between observed mortality and the Hamming moving average baseline. The influenza season was defined for each winter season and country as the set of months for which the difference between observed P&I mortality and the Hamming moving average baseline was contiguously positive for the age group ≥ 65 years. Excess all-cause mortality was estimated for all elderly age groups for all study countries and the US. Linear trends were estimated for each age group and country separately for seasons dominated by A(H3N2) and [B & A(H1N1)] viruses. Seasonal dominance was defined as accounting for $\geq 50\%$ of all isolates and/or named as such in an official publication^{6, 7, 8}. Slope estimates for an age group/country found to be statistically significant for 1980-2000 were compared to estimates of the slope for the same age group/country for 1968-2000, if these were also statistically significant. The intention was to compare any changes in slope to the history of vaccination coverage by age group and country.

Results

Vaccine Coverage. The data on vaccine distribution (doses per 1,000 population) suggests that the time course of vaccine coverage may have differed somewhat in these four countries, although all exhibited a general increase after 1980⁹. However, on an age-specific basis, the situation was much more uniform. Although data on age-specific distribution of vaccine was sparse for both Australia and Canada, point estimates suggest that coverage progressed quite similarly to the time course in the US, except that coverage for the age group 65-74 years was somewhat lower in Canada than in the other two countries^{10, 11}. In France, on the other hand, vaccine coverage progressed at quite different rates for different elderly age groups. In

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particular, the age group 65-69 years, remained at a relatively low level of coverage (between 30 and 40%) for the entire study period while older age groups had significantly higher coverage¹². Table 1. Summarizes the Results of Linear Modeling of Trends by Age Group, Virus Sub-Type and Country for Excess All-Cause Mortality.

Table 1. Statistically significant trends in excess all-cause mortality in Australia and France.

Country	Slope estimates for linear models of the trend in age-specific All-cause excess mortality					
	Australia			France		
Age group	H1&B 1968-2000	H1&B 1980-2000	H3 1980-2000	H1&B 1968-2000	H1&B 1980-2000	H3 1980-2000
45-49	NS	NS	-0.207	-0.074	NS	NS
50-54	NS	NS	-0.207	-0.340	NS	NS
55-59	-0.449	-0.195	NS	-0.157	-0.026	NS
60-64	-0.702	NS	NS	-0.289	NS	NS
65-69	-1.655	-1.187	-1.705	-0.450	-2.091	-1.312
		-2.123		-2.960		-2.870
70-74	-2.541	-1.509	-3.15	-0.749	NS	NS
		-3.572		-5.550		
75-79	-3.402	-1.460	NS	NS	-3.788	-1.126
		-5.343				-6.450
80-84	-7.912	-5.676	-8.572	-2.554	NS	NS
		-10.148		-14.590		
85+	-16.514	-8.967	NS	NS	-12.623	-5.500
		-24.062				-24.745

For the US and Canada for the period 1968-2000, no trend was statistically significant for any elderly age group. However, for Australia, five of six age groups had statistically significant slopes for 1968-2000; and slopes for three of these age groups were also significant for 1980-2000. For France, five of eight age groups had significant slopes for 1968-2000 and two of these were significant for 1980-2000. There were, then, five opportunities to compare slopes for the entire study period with those for the period in which vaccination increased strongly. In all cases, the slope estimates for the later period were indistinguishable from those for the longer period (95% confidence intervals mutually overlapped). In one age group for Australia, estimates were available for the period 1980-2000 for both A(H3N2) seasons and [B & A(H1N1)] seasons. These estimates too were indistinguishable (cf. Figure 1). Data were also available for all-cause mortality for a longer period for both Canada (1950-2000) and the US (1953-2000). We modeled linear trends for excess all-cause mortality for all age groups in both countries. All age groups (four of four) were significant for the US as were four of six age groups for Canada. The relationship of all significant trends with age was interesting (Figure 2). Trends for northern North America were highly similar to one another as were those for Australia and France. However, the two pairs were quite different. We noted that for all age groups under age 70 in all countries, the absolute value of excess mortality rates at the end of the study period was such that extrapolation of the estimated slope of decrease would produce near zero values in about one decade, the present time.

Figure 1. Linear trends for excess all-cause mortality rate for the age group 65-69 years in Australia for the two time periods studied. Trends were indistinguishable between time periods; and the magnitude of the decline was such that excess mortality rates would extrapolate to values close to zero at the present time.

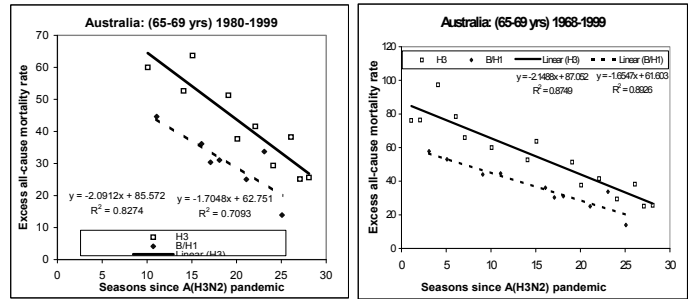
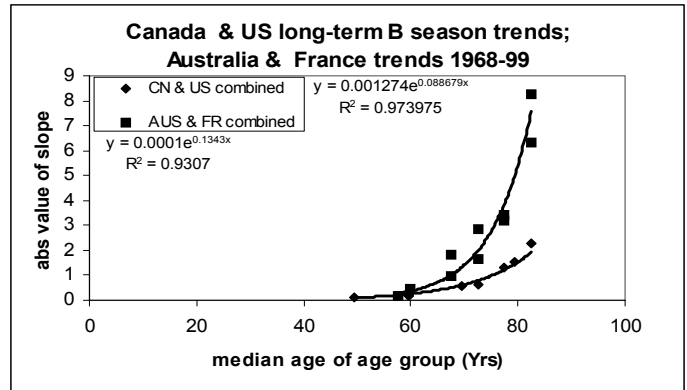


Figure 2. The age relationship for trends in excess all-cause mortality rates for Canada and the US for [B & re-emergent A(H1N1) seasons, 1950 and 1953 (respectively) to 2000; and for Australia and France for the time period 1968-2000.



Conclusion

There has been a strong decline in excess all-cause mortality in all studied countries throughout the study period, the era of circulation of A(H3N2) viruses. The rate of decline appears to have been lower in northern North America (nNA) than elsewhere; however excess mortality rates were also lower there at the start of the A(H3N2) era. The trend found in nNA extended back in time through the era of circulation of A(H2N2) viruses for about the last decade of circulation of A(H1N1) viruses. The net result is that for all persons under the age of 70, excess mortality rates in all studied countries are, at the present time, very low. The slopes of the observed decreases have a well-defined relationship with age, different in nNA than elsewhere. Because the observed trends are large and do not change detectably during the period during which influenza vaccine coverage ramped up strongly in all studied countries, these declines in excess mortality cannot be assigned to efforts at influenza control. Because the trends in nNA appear to be consistent across eras dominated by different A-type viruses, these trends appear to be extraneous to the influenza virus itself, i.e., due to advances in public health and/or medicine.

Against such a strongly declining background, it is very difficult to detect further increases (more strongly negative) in the rate of decline in excess all-cause mortality. It is, therefore, unfortunate, that this measure was chosen to gauge the effectiveness of national vaccination programs. This is the first observation that influenza has essentially ceased to be mortally virulent in man under age 70 years in economically developed countries. Influenza-attributable mortality is also strongly declining in persons of greater age, but rates there are still appreciable. Influenza-attributable mortality in middle-aged to elderly persons is very closely exponentially distributed with age. Somewhat paradoxically, since the number of persons of increasing age continues to increase strongly in all developed countries, and since the efficacy of influenza vaccine likely decreases with increasing age, the total number of deaths due to influenza is likely to remain somewhat the same for the next several decades, unless higher efficacy vaccines are developed or substantial herd immunity arises.

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Proceedings Topic #14

Preclinical Vaccines and Other Intervention Strategies

Oral Presentations

Induction of Protective Immunity in Mice Against Antigenically Distinct Influenza Virus H5N1 Strains With Recombinant MVA-Based Vaccine

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Since 2003, the number of human cases of infections with highly pathogenic avian influenza viruses of the H5N1-subtype is still increasing and therefore the development of safe and effective vaccines is considered a priority. However, the global production capacity of conventional vaccines is limited and insufficient for a worldwide vaccination campaign. In the present study an alternative H5N1 vaccine candidate, based on the replication deficient modified vaccinia virus Ankara (MVA) was evaluated. C57BL/6J mice were immunized twice with MVA expressing the HA-gene from influenza virus A/Hongkong/156/97 (MVA-HA-HK/97) or A/Vietnam/1194/04 (MVA-HA-VN/04). Subsequently, recombinant MVA-induced protective immunity was assessed after challenge infection with three antigenically distinct strains of H5N1 influenza viruses: A/Hong Kong/156/97, A/Vietnam/1194/04 and A/Indonesia/5/05. Our data suggest that recombinant MVA expressing the HA of influenza virus A/Vietnam/1194/04 is a promising alternative vaccine candidate that could be used for the induction of protective immunity against various H5N1 influenza strains.

Introduction

Since the first human cases of H5N1-infections in 1997, influenza viruses of this subtype caused outbreaks of avian influenza worldwide associated with an accumulating number of bird-to-human transmissions. As of 25 June 2007, 315 human cases were recorded of which 191 proved to be fatal [1]. The availability of safe and effective vaccines is considered of great importance to limit the impact of a future pandemic on the human population. However, the development of such vaccines and their production is not straightforward: at present the combined vaccine production capacity of all manufacturers is not sufficient for timely provision for a worldwide vaccination campaign. There is a clear need for alternative vaccine delivery systems and production technologies that could help to overcome this problem. Furthermore, different antigenically distinct clades of H5N1 viruses have been identified [2] and an ideal vaccine would also induce cross-protective immunity against these antigenic variants. In the present study we evaluated a candidate vaccine based on a replication deficient poxvirus vaccine strain as a vector system: modified vaccinia virus Ankara (MVA). MVA has been tested originally in >120,000

individuals and proved to be a particularly safe candidate vaccine against human smallpox [3]. More recently, recombinant MVA expressing foreign genes proved successful in evoking immune responses and providing protection against diseases caused by viruses, bacteria, parasites or tumors from which the antigens were derived [4,5]. MVA vector vaccines are safe, deliver heterologous antigens efficiently and can be produced at a large scale under the requirements of Good Manufacturing Practice (GMP) [4-7]. Other properties are: capacity to induce well-balanced B and T cell responses, extreme host-range restriction, possibility of long term storage (stockpiling) and easy production at BSL-1 conditions in chicken embryo fibroblasts (CEF) and baby hamster kidney cells [4,7-11]. Two different recombinant MVA viruses expressing the HA-genes of H5N1 influenza viruses A/Hongkong/156/97 (A/HK/156/97) or A/Vietnam/1194/04 (A/VN/1194/04) were evaluated in a mouse model to assess their potential to induce protective immunity against three different H5N1 viruses [12].

Material and Methods

Vaccine preparation. The HA-genes of influenza H5N1 viruses A/HK/156/97 and A/VN/1194/04 were amplified by RT-PCR and cloned into the MVA expression plasmid pIII_dHR-PsynII to generate the MVA vector plasmids pIII_d-HA-HK/97 and pIII_d-HA-VN/04, which direct insertion of the HA-genes into the site of deletion III within the MVA genome [9] resulting in recombinant viruses MVA-HA-HK/97 and MVA-HA-VN/04 which express the respective HA genes under control of the vaccinia virus-specific promoter PsynII [13]. The production of HA protein by MVA vector viruses was confirmed by Western blot analysis (see also figure 1) and found to be comparable. For vaccination purposes the viruses were purified and used at a dose of 10⁸ PFU in 100 µl PBS. Whole-inactivated NIBRG-14 virus was used as positive control at 2 µg HA/50 µl mixed with the adjuvant Stimune[®] (Cedi-Diagnostics, Lelystad, the Netherlands). Negative control mice were inoculated with PBS.

Influenza viruses. Influenza viruses A/HK/156/97, A/VN/1194/04 and A/Indonesia/5/05 (A/IND/5/05) were inoculated in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluid was harvested after 3 days. Infectious virus titers were determined in Madin Darby Canine Kidney (MDCK) cells as described previously [14]. **Mice.** Female specified pathogen-free 6-8 weeks old C57BL/6J mice were used. Animals were divided in five groups of 18 mice and immunized with PBS, MVA-HA-HK/97, MVA-HA-VN/04, wtMVA, or Stimune[®]-adjuvanted NIBRG-14. Immunizations were performed intra-muscularly, 50 µl in the left hind leg and 50 µl in the right. Four weeks later, blood samples were collected and animals were immunized again as described above. After another four weeks, again blood samples were collected and each of the five vaccine groups was divided into three sub-groups of six animals each. The sub-groups of each vaccine group were inoculated with 10³ TCID₅₀ of influenza virus A/HK/156/97, A/VN/1194/04 or A/IND/5/05 in 50 µl PBS by the intranasal route. Animals were weighed every day until day 4 after infection and then euthanized and

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specific organs were taken out. Intra-muscular immunizations, intranasal infections, blood sampling and euthanasia were carried out under anesthesia with inhalative isoflurane. During the 5 days of infection with the H5N1 influenza virus, animals were placed in filter-top cages in bio-safety level 3 containment facilities.

Virus titers in organ tissues. Organs were snap frozen and stored at -70°C . Organs were homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in transport medium and quintuplicate ten-fold serial dilutions of these samples were used for inoculation of MDCK cells to determine virus titers. **Serology.** Antibodies specific for influenza viruses A/HK/156/97, A/VN/1194/04 or A/IND/5/05 were detected using a standard hemagglutination inhibition (HI) assay and a micro virus neutralization (VN) assay.

Histopathology. Formalin-inflated lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at $4\mu\text{m}$ and stained with hematoxylin and eosin for histological evaluation. Sequential slides were stained using an immunoperoxidase method with a monoclonal antibody directed against the nucleoprotein of influenza A virus [15]. **Statistical analysis.** Data for viral titers and antibody titers were analyzed using the two-sided Student's t test and differences were considered significant at $P < 0.05$.

Results

Serology. Upon a single vaccination with MVA-HA-HK/97 mice developed antibody responses against the homologous virus strain with geometric mean titers (GMTs) of 1629 and 239 measured in HI- and VN-assays, respectively. These antibodies however, did not cross-react with the influenza virus strains A/VN/1194/04 or A/IND/5/05. Four weeks after the booster vaccination the homologous antibody GMTs in the HI- and VN-assays were 1370 and 744, respectively. Again no cross-reaction was observed with the other H5N1 strains. After the first vaccination with MVA-HA-VN/04 none of the mice developed HI antibodies against the homologous strain and only one animal developed VN antibodies. After a second dose all animals responded and the GMT increased to 20 and 64 as measured by HI- and VN-assays, respectively. These antibodies cross-reacted with the H5N1 strain A/HK/156/97 and to a limited extent with the strain A/IND/5/05. The adjuvanted NIBRG-14 vaccine preparation, which was included in the experiments as a positive control induced robust antibody responses against the homologous A/VN/1194/04, which cross-reacted with the strains A/HK/156/97 and A/IND/5/05 both in the HI- and VN-assays.

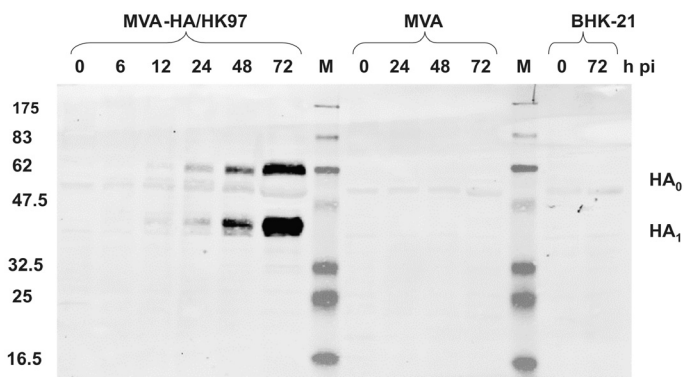
Clinical signs. Vaccination with MVA-HA-HK/97 or MVA-HA-VN/04 prevented the development of clinical signs seen in mice immunized with PBS or wtMVA like hunched posture, rapid breathing, ruffled fur and decreased muscle strength after infection with influenza virus A/HK/156/97 or A/VN/1194/04. MVA-HA-VN/04 vaccination also prevented the development of clinical signs caused by infection with influenza virus A/IND/5/05. The observed protection against clinical signs correla-

Table 1. Clinical and virological outcome 4 days post infection of immunized mice.

Vaccine	A/Hongkong/156/97			A/Vietnam/1194/04			A/Indonesia/5/05		
	Weight loss ¹	Lung virus titers ²	IHC ³	Weight loss	Lung virus titers	IHC	Weight loss	Lung virus titers	IHC
wtMVA	11.5	8.4	+++	10.4	6.7	+++	16.9	8.5	+++
MVA-HA-HK/97	1.9	1.6*	-	2.4	4.9	+	12.0	6.8	+++
MVA-HA-VN/04	3.2	4.2	-	2.1	1.6*	-	1.1	2.9	+/-
NIBRG-14	2.9	1.4*	-	1.2	1.5*	-	-1.1	1.6*	-
PBS	16.2	7.9	+++	16.9	7.8	+++	18.6	8.9	+++

¹The percentage of weight loss is indicated relative to the body weight before infection of the mice. ²Indicated are the average virus titers in the lungs expressed as 10Log, * all mice tested below the detection limit of the assay. ³Indicated are the frequencies of infected cells in lung tissue by arbitrary units, -: no infected cells detectable, +/-: rare, +: isolated infected-cells present, +++: abundant (especially in the bronchiolar epithelium)

Figure 1. HA protein synthesis in BHK-21 cells infected with MVA-HA-HK/97 at different time points post infection (h pi). The figure shows a Western blot analysis using an H5-specific rabbit antiserum. Uninfected cells or cells infected with non-recombinant MVA served as negative controls.



ted with reduced loss of bodyweight after infection (Table 1). In PBS and wtMVA immunized mice an average loss of bodyweight of 16.2% and 11.5% was observed post infection with influenza virus A/HK/156/97 (Table 1) or 16.9% and 10.4% post infection with influenza virus A/VN/1194/04, respectively. This was largely prevented by vaccination with MVA-HA-HK/97 or MVA-HA-VN/04 (Table 1). Also infection with influenza virus A/IND/5/05 caused severe loss of bodyweight in control mice, which was significantly reduced by vaccination with MVA-HA-VN/04 but not by vaccination with MVA-HA-HK/97.

Virus replication in lungs. After infection, average lung virus titers of $10^{7.9}$, $10^{7.8}$ and $10^{8.9}$ TCID₅₀/gram tissue were observed for PBS control mice infected with influenza viruses A/HK/156/97, A/VN/1194/04 or A/IND/5/05 respectively. Mice vaccinated with wtMVA were not protected and similar average virus titers were found in the lungs of infected mice (Table 1). Vaccination with MVA-HA-HK/97 prevented replication of influenza virus A/HK/156/97 in the lungs completely whereas with MVA-HA-VN/04 vaccination a reduction of virus replication in the lungs was observed. Vaccination with MVA-HA-VN/04 prevented replication of virus A/VN/1194/04 in the lungs completely, whereas vaccination with MVA-HA-HK/97 only partially reduced virus replication. This reduction was statistically significant compared to PBS-inoculated mice $P <$

0.05). Vaccination with MVA-HA-VN/04 also partially prevented replication of influenza virus A/IND/5/05 in the lungs. Vaccination with MVA-HA-HK/97 did not prevent replication of influenza virus A/IND/5/05 and all six mice tested positive (Table 1). Vaccination with the inactivated whole-virus NIBRG-14 adjuvanted with Stimune® prevented replication of all three H5N1 strains tested. In general, the lung virus titers correlated with the number of infected cells in lung tissue detected by immuno-histochemistry (IHC) (Table 1).

Discussion

In the light of the pandemic threat caused by influenza H5N1 viruses, the availability of sufficient doses of safe and effective vaccines is considered a priority [1,2]. In the present study we have evaluated recombinant MVA expressing the HA genes of two different influenza H5N1 viruses for the induction of protective immunity against three antigenically distinct influenza H5N1 viruses in a mouse model. Vaccination with MVA expressing the HA of influenza H5N1 viruses induced virus-specific antibody responses, which correlated with protection against homologous and heterologous challenge infection. The co-circulation of antigenically different influenza virus strains complicates the development of effective vaccines considerably. The use of three antigenically distinct viruses allowed the assessment of cross-protective immunity induced by vaccination. Vaccination with MVA-HA-HK/97 induced strong antibody responses, even after a single immunization, however these antibodies were not cross-reactive in HI- and VN-assays with A/VN/1194/04 or A/IND/5/05. Vaccination with MVA-HA-VN/04 induced good virus-specific antibody responses after two immunizations, which cross-reacted with A/HK/156/97 and to a lesser extent with A/IND/5/05. The NIBRG-14 vaccine preparation was included in the experiments as a positive control and in combination with the Stimune® adjuvant induced strong (cross-reactive) antibody responses. The HI- and VN-antibody titers measured against the three H5N1 strains correlated with protection against challenge infection as assessed by observing clinical signs of disease and weight loss, lung virus titers and histo-pathologic changes and detection of virus-infected cells *in situ*. In addition, partial protection was also seen in the absence of detectable cross-reacting antibodies, e.g. against infection with A/VN/1194/04 in MVA-HA-HK/97-vaccinated mice. We conclude that MVA-based H5N1 vaccines are promising vaccine candidates with favorable properties regarding safety, effectiveness, the potential of rapid large-scale production and the potential of long term storage (stock-piling), which may be important in the face of an emerging pandemic.

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Lipo peptide Vaccines Illustrate the Potential Role of Subtype-Crossreactive T Cell Responses in the Control of Seasonal and Pandemic Influenza

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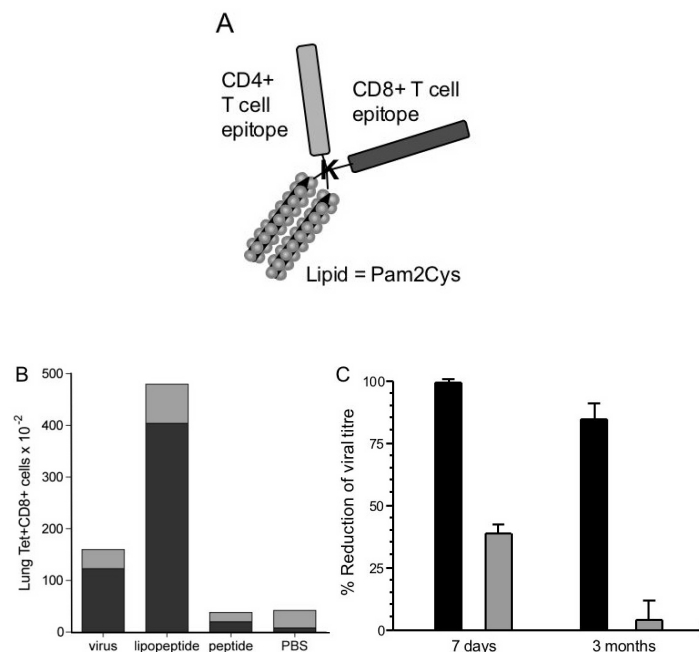
The best form of protection against influenza is high-titred virus-neutralising antibody specific for the challenge strain. However, this is not always possible to achieve by vaccination due to the need for predicting the emerging virus, whether it be a seasonal drift variant or the next pandemic virus, for incorporation into the vaccine. Our view is that providing additional protection in the form of heterosubtypic immunity, i.e. immunity active against all viruses of type A influenza regardless of subtype or strain, will give additional security in situations where protective antibody responses are not achieved. The main mediator of heterosubtypic immunity is the CD8⁺ cytotoxic T cell (1). Targets for CD8⁺ T cells, unlike those for antibody responses, are located on the internal conserved proteins of the virus, hence the breadth of these effector cells. CD8⁺ T cells do not prevent infection because their recognition structure on epithelial cells, MHC class I molecules bearing viral peptide, are only generated following infection of the cell. On recognition of this MHC-peptide complex, lysis of infected cells occurs and the viral load in the lungs is substantially reduced. Secretion of the antiviral cytokine IFN γ by the stimulated T cells may also aid in reducing viral load. One could envisage that induction of a potent CD8⁺ T cell response might decrease the load of virus in the lungs sufficiently to protect against severe disease and even death though not infection. This might be important in situations where antibody levels are suboptimal, such as in the elderly where antibody-inducing vaccine efficacy is relatively low. Co-priming of a CD8⁺ T cell response might also reduce the levels of non-responsiveness in situations of conventional vaccine mismatch or in the elderly experiencing "original antigenic sin". Studies of the efficacy of such responses in man are very rare but McMichael et al. (2) have demonstrated that, in the absence of specific antibody, high levels of cytotoxic T cells can reduce the amount and period of viral shedding. If vaccines that boost heterosubtypic CD8⁺ T cell responses in addition to inducing antibody to the circulating strain were in common use, it might be expected that at the start of a pandemic, although the antibody would be inappropriate, the T cell responses could potentially diminish the morbidity and mortality typical of a newly emerging viral subtype. Induction of CD8⁺ T cells by influenza and other viruses is a process that is dependent on the dendritic cell

(DC). Virus is capable of abortively infecting immature DC and triggering their activation to become mature licensed antigen-presenting cells. This process does not require help from CD4⁺ T cells but co-induction of this subset of T cells is essential for priming CD8⁺ T cells that will form a robust memory population. Mimicking this process by vaccination can be accomplished using lipo peptide technology. Lipo peptides have shown their strength in recent years by providing immunogens for the safe and efficient induction of CD8⁺ T cells (reviewed in 3). The lipo peptides we have designed are totally synthetic and have a CD4⁺ and a CD8⁺ T cell epitope linked via a lysine residue to which lipid is attached in a branched configuration. We have previously shown that branched immunogens are better able to be presented by DC to T cells in vitro and are also more stable in non-inactivated serum (4). Branched lipo peptides can be more soluble than the equivalent linear version, which may have advantages for quality control and even delivery (5). Our choice of lipid was determined by the efficiency of different lipo peptides to mature DC as measured by the upregulation of cell surface MHC class II and the CD86 costimulatory molecule as well as other functional parameters such as DC cytokine secretion (6). We found that lipo peptides based on the lipid moiety S-[2,3-bis(palmitoyloxy)propyl]cysteine or Pam2Cys, which corresponds to the lipid component of macrophage-activating lipo peptide 2 (MALP-2) from *Mycoplasma fermentans* (7), were the most active (6). We have also shown (6, 8) that the mechanism of DC maturation involves engagement of the lipid with Toll-like receptor 2; signalling through this receptor triggers NF κ B-dependent readout of those genes that initiate the maturation process. A Pam2Cys-containing lipo peptide based on T cell epitopes from influenza virus (Fig. 1A), including the immunodominant BALB/c CD8⁺ epitope TYQRTRALV from the viral NP, does not induce any antibody and therefore allowed us to assess the contribution of T cells independently of the humoral response. Our strategy involved a *single dose* of the lipo peptide delivered intranasally to mice in the absence of additional adjuvant. Four weeks after a relatively low dose (9 nmol) of lipo peptide, we could not detect any CD8⁺ T cells in the lungs of vaccinated mice that bound tetrameric class I molecules bearing the CD8⁺ T cell epitope present in the vaccine (Tet⁺CD8⁺ cells). However, when these mice were challenged with influenza virus, considerable numbers of these cells could be found in the lungs 5 days later. This lung Tet⁺CD8⁺ T cell population resulted from expansion and/or recruitment of vaccine-induced CD8⁺ T cells and were not found in response to the challenge virus itself in unprimed mice (6). The same pattern of results was obtained when specific IFN γ -producing CD8⁺ T cells were measured. When a higher dose of lipo peptide (45 nmol) was used to vaccinate mice, a lung resident population of Tet⁺CD8⁺ T cells could be directly detected even without viral challenge (9). When mice were infected with virus 3 months after vaccination, these lung resident CD8⁺ T cells were rapidly activated as measured by downregulation of CD62L (Figure 1B). The number and activation status of the lipo peptide-induced lung resident

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Tet⁺CD8⁺ T cells were of the same order of magnitude, and in fact somewhat greater, than those induced by virus infection itself. The equivalent non-lipidated peptide also induced CD8⁺ T cells but these were far fewer in number (Figure 1B), were under-represented in the lung versus the spleen (not shown), and only about half the population became activated in response to viral challenge (Figure 1B).

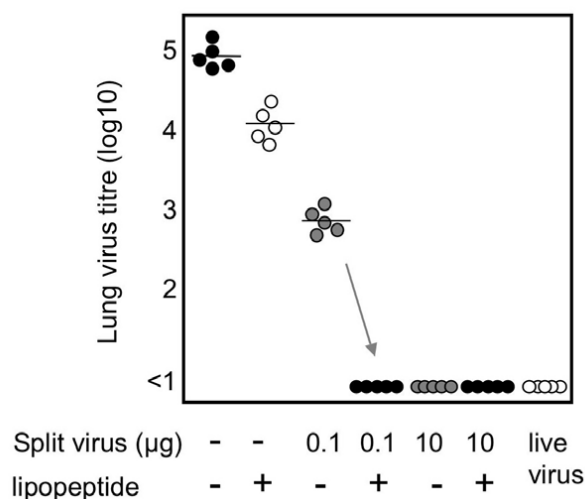
Figure 1. Lipopeptide-induced immune responses. A. Schematic representation of the structure of the synthetic lipopeptide. B. Mice were either infected with A/Mem/1/71 influenza virus or inoculated intranasally with a single dose (45 nmole) of lipopeptide or the equivalent non-lipidated peptide or with PBS. Three months later mice were challenged intranasally with 10^{4.5} pfu A/Mem/1/71 virus and 5 days later lung cells were stained with MHC class I tetramer, anti-CD8 and anti-CD62L. Shown are the number of Tet⁺CD8⁺ T cells. The dark grey represents the fraction of cells activated in response to infection (CD62L^{lo}). C. Mice (n=5) were vaccinated with lipopeptide (black bars) or non-lipidated peptide (grey bars), challenged with A/Memphis/1/71 virus 7 days or 3 months following vaccination, and the titre of virus in the lungs 5 days later determined by plaque assay.



These lipopeptide-induced T cells were capable of causing significant reduction of pulmonary viral loads (Figure 1C). If mice were challenged with A/Memphis/1/71 virus 7 days after a single dose of lipopeptide, when the activated primary effector population was present, virtually all virus (>99%) was gone from the lungs on day 5. Effector T cells induced by vaccination with the non-lipidated peptide were also able to reduce the viral load, although to a lesser extent (approx. 40%). This non-lipidated peptide-induced clearing response waned rapidly with time such that when mice were challenged with virus at 3 months post vaccination they were unprotected. In contrast, the significant memory CD8⁺ T cell population induced by the

lipopeptide vaccine was able to provide an 85% reduction in lung viral loads at 3 months. We have examined mice as late as 9 months after single dose vaccination and can still demonstrate significant protection of the lung in lipopeptide but not non-lipidated peptide-primed mice (9). In a separate study, using a related dipalmitylated branched lipopeptide, we examined the ability of lipopeptide to augment the clearance of virus in mice vaccinated with a *suboptimal* dose of inactivated detergent-split A/Memphis/1/71 virus vaccine (Figure 2). This lipopeptide is not as effective as the Pam2Cys-based lipopeptide and 25 nmol administered subcutaneously resulted in only a log decrease in viral titre 5 days after challenge when compared to nonvaccinated mice. Nevertheless, when combined with a suboptimal dose (0.1 µg) of split virus, which alone gave a two log reduction in viral titre, a synergistic effect was observed which resulted in complete viral clearance. When an optimal dose of split virus (10 µg) was used, which alone provides complete protection, co-administration of the lipopeptide did not compromise the protective effect of the antibody.

Figure 2. Addition of lipopeptide to split virus vaccine improves viral clearance. BALB/c mice were vaccinated with pal2-lipopeptide (25nmole) subcutaneously and either 0.1 or 10 µg A/Memphis/71 HA in the form of inactivated split virions, or combination of lipopeptide and split virus as indicated. After 4 weeks the mice were challenged with 10^{4.5} pfu A/Memphis/1/71 virus and 5 days later the titre of infectious virus in the lungs was determined by plaque formation in MDCK cells. Symbols represent individual mice.



Lastly, we have examined the ability of the Pam2Cys lipopeptide to protect against death by virulent mouse-adapted A/PR8/34 virus. Non vaccinated mice infected with a lethal dose of this virus have to be culled at the humane endpoint starting on day 6 post infection and are all dead by day 12. In contrast, a single intranasal dose of lipopeptide was able to completely protect against death from this virus (data not shown). We conclude from these studies that CD8⁺ T cell-inducing lipopeptides that target the conserved internal components of the virus

induce enhanced pulmonary clearance of virus and can protect against death from a highly virulent strain. Such lipopeptides show the potential to increase the efficacy of the current split virus vaccine when the antibody-inducing component is suboptimal e.g. in situations of vaccine mismatch or general non-responsiveness as in the elderly. An advantage of these vaccines is that they do not rely on knowledge of the emerging viral strain. If administered routinely with split virus vaccine to maintain CD8⁺ T cells at effective levels, then the effects of a pandemic could be significantly reduced. Such a vaccine strategy might also lessen the demand for antivirals in the period prior to development of specific pandemic vaccines and would achieve the same result of decreasing severity and duration of infection without the need for continuous administration as is the case with anti-virals. Our studies clearly illustrate the potential of designing vaccines that, unlike current vaccines, can exploit both arms of the immune response.

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Proceedings Topic #1

Disease Surveillance and Impact

Poster Presentations

Economic Impact of the 2005/2006 Influenza Epidemic in France

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¹GROG National Coordination Centre-OPEN ROME, Paris, France; ²Add GROG Association; ³Collective Name of GROG Network

Influenza is a frequent seasonal and epidemic viral disease, leading many patients to a medical contact, with a potentially important complication rate (morbidity and mortality) in non-vaccinated people over 64 years or with chronic disease. To quantify the average cost of an influenza case and the economic impact of influenza epidemics is not an easy task: the clinical influenza pattern can be very different from one patient to another and precise influenza diagnostic is rarely done, leading to crude quantitative impact estimate; the direct cost (consultations, treatment, hospitalisation...) varies, according to the patient's age, his health status and the epidemic's intensity and severity; the indirect costs (daily allowance, production loss...) are very difficult to quantify. French economists agree to say that the direct cost of an influenza epidemic varies from 300 to 800 million of Euros, according to the different seasons. The French national influenza surveillance network (GROG) is an early warning system, based on the data from community health care professionals who weekly describe their activity and take rhinopharyngeal swabs in patients presenting with influenza like illnesses. Each swab is accompanied by a standardised form describing the clinical symptoms of the patient. At the end of the surveillance season, seasonal influenza incidence is estimated through these weekly GROG data (acute respiratory infection incidence, number of medical contacts, virological results...). Objective: To evaluate the direct cost of the 2005/2006 influenza epidemic through the GROG surveillance data.

Methodology

Retrospective study of all the 2005/2006 confirmed influenza cases seen in the GROG network. The analysis of these data leads to estimate the direct cost of an influenza case, according to the age of the patient and the intensity of symptoms. Study physicians: GPs or paediatricians involved in the GROG surveillance network and having had at least one influenza positive swab during the 2005/2006 season. Study patients: patient swabbed by a study physician for an influenza like illness and having had an influenza positive swab (classical virology or rapid test). All the cases are identified by an anonymous number.

Data Collection

at the end of the surveillance season, an additional form is sent to the study physicians for each influenza positive patient. The filled forms, registering the therapeutic attitude (number of consultations, prescriptions, sick leaves...) is sent back to the GROG coordination (Open Rome, Paris, France) by

post or fax. Direct cost estimate: cost of medications, medical and paramedical practitioners fees (visits, consultations, laboratory...), in outpatients or at the hospital. Levels of clinical intensity: Low influenza case: influenza case where patients have needed only one medical consultation, without sick leave prescription nor hospitalisation (nor death). High influenza case: influenza case where patients have needed a medical home visit and/or a sick leave prescription, without a second medical contact nor an hospitalisation (nor death). Complicated influenza case: influenza case where patients have needed more than one medical contact (consultations and/or home visits).

Analysis

All the collected forms have been keyed in a specific database using official drugs coding (CIP code) to perform an automatic calculation of the prescription cost. Statistical analysis is performed with Excel. This results can be compared with data of the 2001/2002 season obtained using the same methodology. Results: 964 forms have been sent to the 297 study physicians; 558 forms (58%), filled by 162 (55%) physicians, 123 GPs et 39 paediatricians, have been received back. Two of the 558 forms have been excluded as the prescription items were not filled. The analysis is based on 556 flu cases. During the 2005/2006 surveillance season, influenza B (and some A/H1N1) viruses circulated on a moderate epidemic way. The GROG network estimates that, in France, during the 2005/2006 epidemic, 2.554.000 people have had an outpatient care contact (GP or paediatrician) for a flu case. As usually when influenza B circulates, this epidemic caused mainly low cases, in children. On average, each flu case had 1.2 medical contact (consultation or visit). Almost 85% of the flu cases needed only one medical contact. No hospitalisation has been necessary. 554 patients (99.6%) had a drug prescription, with an average of 2.9 drugs (1st medical contact) and 1.5 drugs (2nd medical contact) prescribed. Specific influenza antiviral drugs were prescribed for 29 cases (5.2%). Each influenza case led to an average direct cost (Table 1) of 37 to 51 Euros, according to the patient's age (drugs: 13.70 € on average; physicians fees: 24.90 € on average). 8 of the 556 flu cases (1.4%), all under 10 years old, needed a paramedical contact (physiotherapy) ; 30 cases (5.6%) needed complementary examination (biology, X-Ray...).

Table 1. Average direct cost (Euros) of influenza cases according to the patient's age France, 2005-2006 season. Source: GROG/Open Rome.

Age	Influenza clinical intensity					
	Low		High		Complicated	
	2001/2002	2005/2006	2001/2002	2005/2006	2001/2002	2005/2006
0-14 years	9	10	10	11	18	19
15-64 years	11	15	13	15	20	30
>64 ans years	26	21	13	-	18	28
Total	10	11	12	13	21	23

The average number of medication prescribed for flu and the corresponding cost increase with patient's age and intensity of symptoms is shown in Table 2.

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Table 2. Average prescription cost (Euros) according to the patient's age and the intensity of flu symptoms France, 2001-2002 Et 2005-2006 seasons. Source: GROG/Open Rome.

	Average cost (Euros)
Children (0-14 years)	
Practitioners fees	24,95
Drugs	11,42
Total	36,37
Adults (15-64 years)	
Practitioners fees	24,73
Drugs	18,23
Total	42,96
Adults over 64 years	
Practitioners fees	27,50
Drugs	23,85
Total	51,35

Compared with 2001/002, and despite a recent upgrading, the average direct cost of a flu case shows a moderate increase, especially on the drug item (drugs: 12.50 € on average; physicians fees : 25.30 € on average in 2001/2002). These data lead to estimate the direct cost of the 2005/2006 influenza epidemic to 103 million Euros. Cost of sick leaves (daily allowance, production loss...) must be added to this direct cost: 4.8 days for sick leaves on average for 70% of working adults with flu; 3 days for sick leaves to care about a sick child in ¼ of the children flu cases.

Discussion and Conclusion

Epidemiological and early warning influenza surveillance systems can be useful tools to get an access to reliable medico-economic data. In France, each winter, influenza epidemics lead to million of medical contacts for flu cases. On an epidemiological point of view, the 2005/2006 season can be described as a moderate flu season (influenza B); the influenza attack rate has been very low in people over 64 years. No hospitalisation for flu has been described through the 556 study cases, suggesting an hospitalisation rate under 2‰. However our data lead to estimate that influenza stays an expensive disease. In 2005/2006, each flu case with an outpatient care contact have led to expenses of: drugs: average of 10 to 30 €/patient, according to the patient's age and the intensity of flu symptoms, physicians fees: average of 25 to 28 €/patient, leading to an average direct cost from 37 to 51 €/flu case. At last, the total direct cost of the 2005/2006 epidemic wave is estimated to 103 million of Euros (2/3 for physicians fees, 1/3 for drugs). Cost of sick leaves (daily allowance, production loss...)

must be added to this direct cost. Compared to the 2001/2002 season, there was, in 2005/2006, a moderate increase of the average prescription cost and a stable physicians fees cost (despite a recent upgrading).

Acknowledgements

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Impact of Influenza Epidemics Over 7 Winter Seasons

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Seasonal influenza is a reality almost every winter, with an important social and economical impact. The objective of this study is to contribute to the evaluation of the impact that seasonal influenza represents each winter, presenting the morbidity and virological data analysed by the National Influenza Centre over seven influenza winter seasons (1999/2000 to 2005/2006). Methods: Clinical and epidemiological data from Influenza-like illness (ILI) cases were reported to, and analysed by, the National Influenza Centre and the National Observatory of Health. The intensity and duration of the epidemic periods were described based on the weekly incidence rates for ILI. Also, the excess of population with ILI was found as a function of the baseline for the incidence of ILI. Nasopharyngeal swabs were collected for virological characterisation of influenza viruses in circulation, by typing and genetic and antigenic analysis. Results: The majority of notifications originated from individuals with 15-44 years, of the female gender, mainly from the north of Portugal. A statistically high association with a confirmed influenza case was found for high fever, cough and contact with another ILI case. Influenza A(H3) viruses were dominant during the 1999/2000, 2001/2002, 2003/2004 and 2004/2005 winter seasons. Influenza B viruses were mainly detected during 2000/2001 and 2002/2003. During the 2005/2006 season, both influenza B and A(H1) viruses were detected. With the exception for the 2003/2004 and 2005/2006 winters, the majority of influenza strains analysed was similar to those included in the influenza vaccine for the respective season. On average, the epidemic periods associated with the circulation of influenza type A(H3) viruses were longer and the incidence rates recorded were higher (peak incidence reached within 3 weeks). The highest increase in the excess of population with ILI, 0.8%, was recorded for the 2001/2002 winter, associated with the circulation of influenza A(H3) viruses. Comments: This study aims for a better knowledge of the impact of influenza epidemics in Portugal, contributing to the improvement of clinical and virological monitoring systems for influenza and for a better planning and, if necessary, the implementation of preventive and/or therapeutic measures in future influenza epidemics.

Introduction

The probability of emergence on an influenza pandemic has been one of the major public health concerns worldwide. Still, one can only speculate when this pandemic will occur and how severe it will be. Seasonal influenza, however, is a reality almost

every winter, with an important social and economical impact. The objective of this study was to contribute to the evaluation of the impact that seasonal influenza represents each winter, presenting the morbidity and virological data analysed by the National Influenza Centre over seven influenza winter seasons (1999/2000 to 2005/2006).

Methods

Cases of influenza-like illness (ILI) in this study were notified to the National Influenza Centre by the Network of Sentinel Medical Practitioners and the Network of Emergency Units, in the context of the virological component of the National Influenza Surveillance Programme. The distribution by age group, gender and region, and the signs and/or symptoms present were analysed. Nasopharyngeal swabs were collected for virological characterisation of influenza viruses circulating in each winter season. Within the epidemiological component of the Programme, ILI cases were also notified to the National Observatory Health. The intensity and duration of the epidemic periods and the excess of population with ILI were described based on the weekly incidence rates for ILI and as a function of a defined baseline.

Results

Demographic data. During the seven influenza winter seasons studied, 56 Sentinel Medical Practitioners have participated, on average, per season, in the virological component of the National Influenza Surveillance Programme. The number of collaborating Emergency Units has been increasing since the network was established in 1990, reaching 35 Units in 2005/2006, and includes, at least, one Health Centre and one Hospital (usually a District Hospital) per District. The two networks combined collected and sent to the National Influenza Centre 4724 nasopharyngeal swabs from patients with a clinical diagnosis of ILI, during the seven winters. The majority of ILI cases reported, 51% (2409), was observed in individuals in the age group of 15-44 years. 18.4% were children between 0-14 years and 28.2% were adults over 45 years. In terms of gender, 56.6% (2676) of cases were observed in women and 42.9% (2026) in men. This distribution was consistent for all seasons. In terms of geographic origin, the majority of cases (25.8%) were reported at the Northern region of the country, followed closely by the Central (21.7%) and the Lisboa e Vale do Tejo Region (22.6%). The southern regions of Alentejo and Algarve reported 27.3% of cases and the Islands of Madeira and Açores accounted for 1.5% of the ILI cases reported.

Virological analysis. Influenza infection was laboratory confirmed on 2217 (46.9%) ILI cases and the percentage of positive results varied between 30.3% in 2005/2006 and 60.6% in 2003/2004 (Figure 1a). Either Influenza type A(H3) or B circulated predominantly in one season following an alternating pattern (type A(H3): 1999/2000, 2001/2002, 2003/2004 e 2004/2005; type B: 2000/2001, 2002/2003), with the exception for the 2005/2006 winter during which both types B and A(H1) were found in circulation. Genetic analysis of

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the HA1 subunit of the haemagglutinin revealed that, with the exception for the 2003/2004 and 2005/2006 winters, influenza viruses circulating in Portugal were similar to the vaccine strains for the respective winter. During 2003/2004, influenza viruses type A(H3) studied were similar to A/Fujian, while the vaccine included the strain A/Panama. Influenza viruses type B found during 2005/2006 were similar to B/HongKong of the B/Victoria lineage, while the vaccine was formulated with B/Shanghai of the B/Yamagata lineage. Antigenic analysis (data not presented) corroborated these findings. Of the signs/symptoms which are usually associated with a higher risk and probability of having influenza, fever (odd ratio, OD: 3.966), cough (OD: 3.573), contact with another patient (OD: 1.632) and chills (OD: 1.425) were the ones with a statistically significant association ($p < 0.05$) with a confirmed influenza laboratory diagnosis.

Epidemiology. Incidence rates for ILI were calculated by the National Observatory of Health on a weekly basis (Figure 1b) with the information collected through the Network of Sentinel Medical Practitioners which covers over 3% of the Portuguese population (max. population under observation: 324716 inhabitants). An average of 2000 ILI cases per season were notified during the seven winter seasons. The intensity and duration of the epidemics were very heterogeneous during the period of this study. The epidemic period lasted, on average, between 8-9 weeks in winters associated with the predominant circulation of influenza type A(H3), with the highest values of the incidence rates being reached within 3-4 weeks. In winters associated with the circulation of influenza type B, the epidemic periods were shorter (1-3 weeks) and of lower intensity. The impact of influenza epidemics, measured in terms of the excess of population with ILI relative to baseline levels, shows that the epidemics associated with the circulation of influenza A(H3) were the most severe, with an average increase of 0.6% (ranging from 0.38% in 1999/2000 to 0.85% in 2004/2005) of the population with ILI. The average percentages of excess population with ILI by age group (Figure 2a) were similar. However, the excess was above the average for the age groups 0-4 and 5-14 years during 2001/2002 and 2003/2004, and for the age groups 15-64 and over 65 years during 2001/2002 and 2004/2005.

When influenza B was circulating, the excess of population with ILI was lower, 0.04% on average (ranging from 0.00% in 2005/2006 to 0.06% in 2000/2001 and 2002/2003). During these seasons, the age group 5-14 years recorded the highest values of excess population with ILI (Figure 2b). Comments: Influenza activity was higher during winters associated with the circulation of influenza A(H3), with longer epidemic periods, higher in duration and intensity. In fact, 2 of the 3 highest values of the incidence rate recorded since 1990 were observed during the last 5 winters. During these seasons, all age groups recorded high values of excess population with ILI. The lowest incidence rates of the last 16 years were recorded during influenza B seasons, with the age group 5-14 years

registering the higher values of excess population. These differences may be explained by higher mutations rates on influenza type A. Genetic analysis revealed that, although the strains circulating were generally similar to the vaccine strains, differences were observed on amino acids located near and on antigenic sites of the haemagglutinin. The incidence rates and estimation of excess population with ILI presented are known to be underestimated for several reasons, and data should be interpreted with care. It has been estimated (data not published) that for each patient with ILI consulting a Sentinel Medical Practitioner, 5 do not seek medical care. Also, the population in this study is not representative of the Portuguese population. Still, the association between the type of influenza virus in circulation and the incidence of ILI continues to be monitored.

Figure 1. Virological analysis of ILI cases and ILI incidence rates.

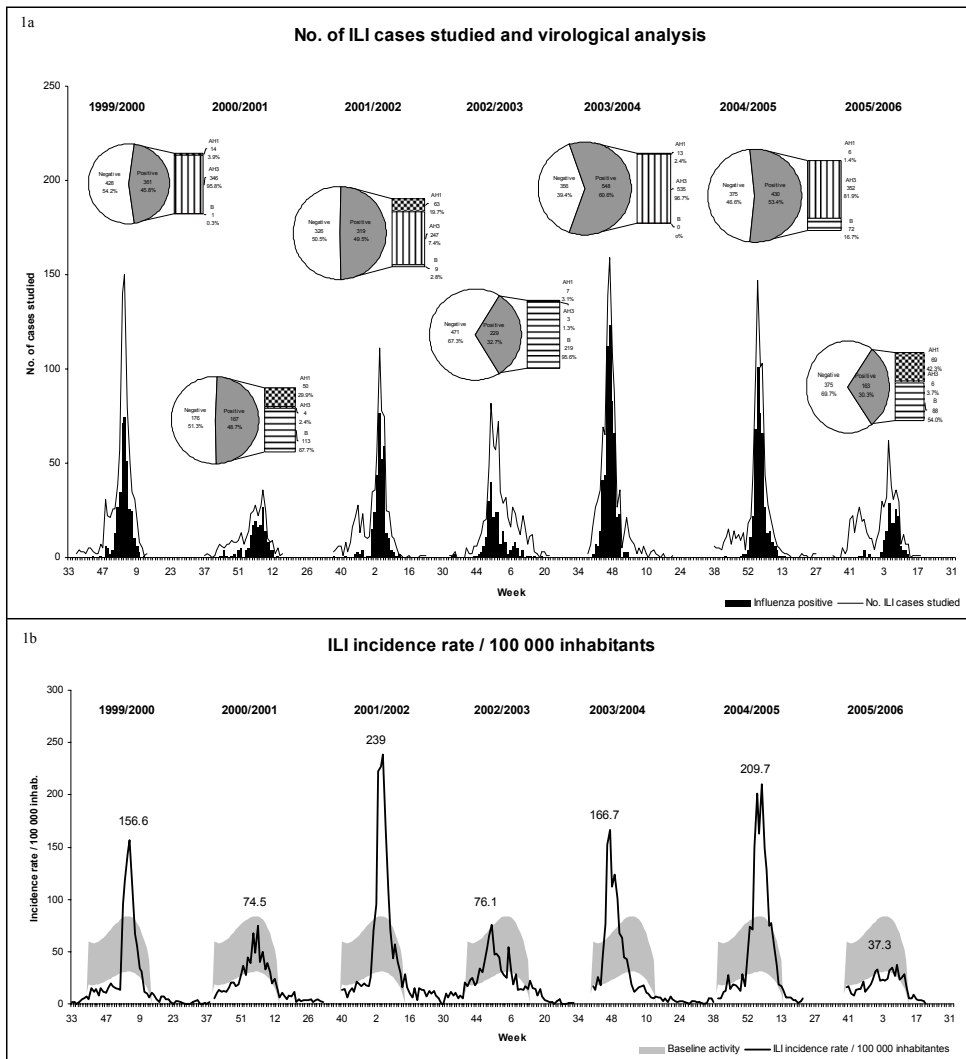
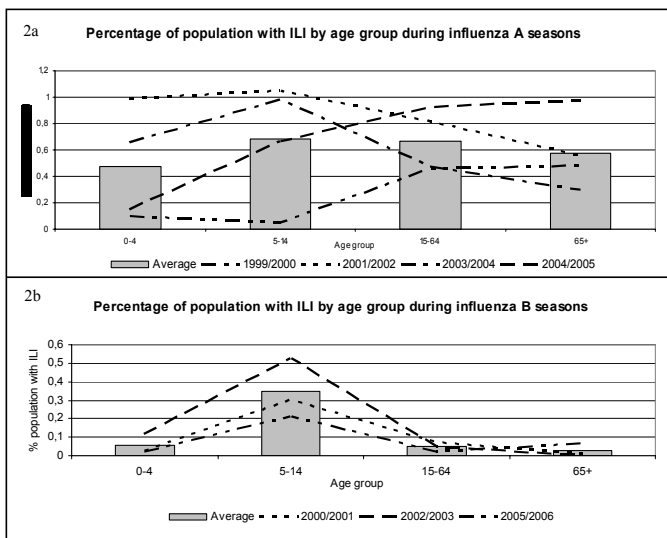


Figure 2. Average percentages of excess population with ILI by age group during influenza A and B seasons.



Surveillance of Influenza and Respiratory Illness Over 40 Years Using the Weekly Returns Service

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Aim: To provide information on a general practice-based sentinel surveillance scheme that has monitored the incidence of influenza-like illness (ILI) and other respiratory diseases in England and Wales for 40 years. **Introduction:** The Weekly Returns Service (WRS) of the Royal College of General Practitioners (RCGP) is a clinical information system based on a sentinel network of general practitioners who record diagnostic data from every consultation. Since 1966, the WRS has collected data on a selected number of diseases; since 1998 all data have been entered into electronic patient records and tabular extracts are taken on a twice weekly basis allowing analysis of every disease/condition reported. **Results:** The WRS is best known for its reporting on ILI and has monitored events such as the 1968/69 pandemic, the 1977 pandemic and the last major epidemic in the UK (1989/90). The main clinical impact in the UK of the Hong Kong influenza pandemic was felt during the winter of 1969/70, when the WRS recorded the highest rates of ILI. Incidence was highest in the age groups 15-44 and 45-64 years. In the subsequent ten winters, incidence remained elevated but has since gradually decreased and is now less than a fifth of the 1969 rate. In collaboration with the UK Health Protection Agency, the RCGP co-ordinates an influenza swabbing scheme. Patients presenting with ILI are swabbed using a nose/throat swab, which is tested for influenza A (H3 and H1), B and RSV. Clinical and virological data from the same population are integrated. **Conclusions.** The WRS provides a reliable surveillance service that is based upon a sentinel network of doctors who record clinical data to very high standards of quality. Integration of microbiological testing increases the potential of the system to interpret clinical data.

Introduction

Sentinel surveillance provides the ability to observe infectious and communicable diseases and provide warning of unusual activity, which may ultimately result in a threat to public health. In recent years, sentinel surveillance schemes have also played an increasingly important role against the threat of bioterrorist attacks [1, 2]. In the UK, national surveillance of infectious and communicable diseases consists of general practitioner (GP) community-based schemes and syndromic surveillance. The Royal College of General Practitioners Weekly Returns Service (WRS) [3], QResearch [4], QFLU [5] and GPRD [6] are all GP surveillance systems; NHS Direct is a telephone health helpline providing the only syndromic surveillance scheme in the UK [7]. All the GP schemes, excepting GPRD, are used for

contemporaneous surveillance, producing weekly morbidity reports with the potential to “switch” to daily reporting in the event of a national emergency e.g. an influenza pandemic. The WRS is the longest running system, established at the Birmingham Research Unit in 1964. Post-1998 the system has been completely electronic allowing the capture of all diagnostic consultation data from the sentinel network. The WRS is considered the gold standard for sentinel surveillance in the UK, especially for influenza surveillance; many other similar European surveillance schemes have based their infrastructure on that of the WRS. In this paper we shall describe the nature of the data collected by the WRS in respect to respiratory illness, in particular influenza-like illness (ILI), and how we utilise these data to provide an insight into the differing impact and etiology of common respiratory diseases that circulate the community each winter.

Methods

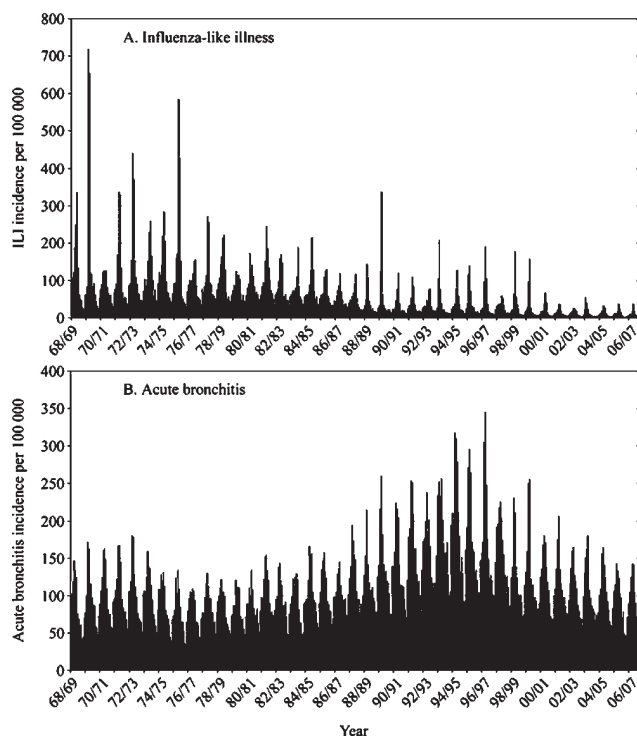
The current WRS network (2007) comprises 103 practices monitoring a population of over 920,000 and is now five times the population monitored in 1967, enabling us to provide robust data at a regional level. The WRS is broadly representative of the UK national population in respect of socio-demographic spread [8]; this has been further improved in recent years following an expansion of the network in 2005 [9]. GPs record each clinical diagnosis in Read code, which is assembled into the disease categories and rubrics of the International Classification of Disease Version 9 (ICD9) for analysis. New episodes of illness are distinguished from ongoing consultations to provide a more accurate reflection of weekly disease incidence. Data are obtained by gender in eight age groups; <1, 1-4, 5-14, 15-24, 25-44, 45-64, 65-74 and 75+ years. Data can be analysed at national (England and Wales), regional (three “super-regions”, North, Central, South; and 10 Health Authority Regions) and general practice level. For this paper, diagnostic data were examined for ILI (ICD9 487), acute bronchitis/bronchiolitis (ICD9 466, 490), acute otitis media (ICD9 381.0, 382.0, 382.9) and common cold (460, 465). We also obtained virological reports for respiratory syncytial virus (RSV) from the Health Protection Agency (HPA).

Results

The WRS has monitored the incidence of ILI in the community for over 40 years and provides the only source of UK clinical incidence data from the Hong Kong influenza pandemic of 1968/69. The major clinical impact of this pandemic in England and Wales was recorded during the following winter, 1969/70, when incidence rates of ILI peaked during the first week of 1970 at an all-age incidence rate of 1252 per 100,000 (Figure 1A). This remains the highest rate of ILI recorded by the WRS, although there were major epidemics during the winters 1972/73, 1975/76 and 1989/90. Following the 1969/70 pandemic, rates of ILI remained high for about 10 years, but have since shown a steady decline. The last year of significant activity in the UK was the millennium winter (1999/00), when

ILI was almost exclusively confined to the 45-64 and 65+ years age groups; concurrently, the WRS recorded high rates of acute bronchitis in the elderly, the result of which was a health service that crumbled under the pressure. In contrast to ILI, the 40-year time series of another commonly diagnosed respiratory illness, acute bronchitis, shows a very different trend (Figure 1B). During the mid-1990's there was a surge in the incidence of acute bronchitis, this coincided with similar increases in asthma attacks, although the reasons for these increases are unclear [10]. Since this peak, rates of acute bronchitis have also been steadily falling, a trend that has been seen across all upper and lower respiratory tract infections diagnosed in general practice in recent years [11].

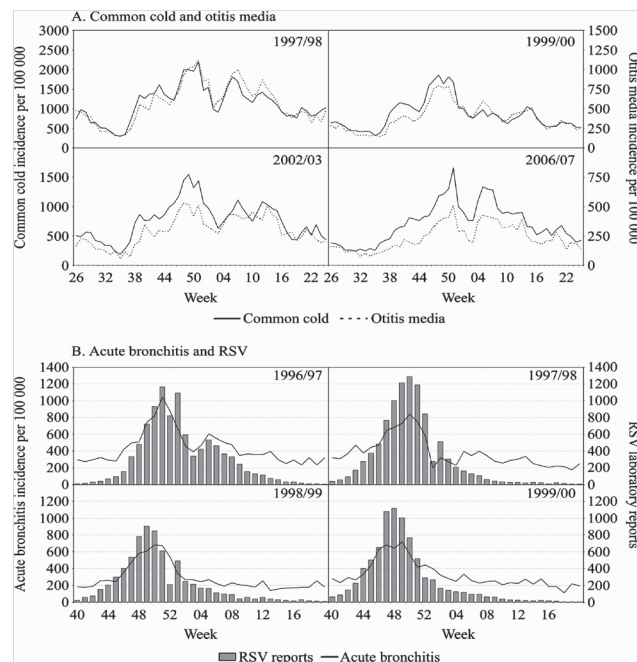
Figure 1. The historical incidence of A) Influenza-like illness and B) Acute bronchitis recorded by the weekly returns service over the years 1968/69 to 2006/07. Clinical incidence rates are displayed as all-age mean weekly rates in 4-weekly periods I.E. 13 bars per year.



Age specific analysis of incidence data can provide an insight into shifting burden of disease in different age groups. During the 1969/70 pandemic, the burden was mainly found within the working age group and less within the young and old, similar to the 1918/19 pandemic. An analysis of age specific incidence rates of ILI in seasons where influenza A H3N2, H1N1 subtypes, and influenza B predominated reveals that H3N2 viruses have the ability to infect all age groups (e.g. 1969/70, 15-44 years; 1989/90, 0-4 years; 1999/00, 65+ years). In those years where H1N1 viruses were the predominant subtype, it is mainly the younger age groups affected (0-4 years and 5-14 years) and in influenza B years the burden of clinical disease mainly lies in school children aged 5-14 years (data

not presented). Comparative analysis between sets of clinical data, and analysis of clinical and laboratory data can provide a better understanding of the etiology of certain respiratory diseases. Otitis media and the common cold are two commonly diagnosed respiratory illnesses, especially in young children. Figure 2A demonstrates how a comparison of WRS incidence data for these two conditions shows a remarkable correlation over a 52 week period; this is shown consistently over four separate years. This type of data can potentially provide direction for patient management; figure 2A suggests that the occurrence of otitis media is strongly associated with the viral pathogens that are known to cause the common cold [12], thereby providing evidence that the antibiotic treatment of young children with acute otitis media is unnecessary in the majority of cases. Similarly, by comparing clinical data for acute bronchitis in young children aged 0-4 years with laboratory data for RSV isolations (for which over 95% are isolated from young children) we can demonstrate a close association, providing strong evidence that RSV is the predominant viral pathogen associated with clinical cases of acute bronchitis in young children (Figure 2B).

Figure 2. The use of WRS data to determine the etiology of different respiratory illnesses. A) Incidence of Otitis media and common cold in young children (0-4 Years) over four years; B) Incidence of Acute bronchitis in young children (0-4 Years) and laboratory reports of Respiratory Syncytial Virus (RSV) 1996/97-1999/00.



The WRS, in collaboration with the HPA, co-ordinates an influenza swabbing scheme each winter, which has collected data for over ten winter seasons [13, 14]. The main advantage of this project is that the swabs are taken from patients who have consulted with an ILI or other acute respiratory infection,

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thereby combining clinical and virological data. Combined nose and throat swabs are tested by multiplex RT-PCR for a number of pathogens including influenza A (H3N2 and H1N1), influenza B and RSV (types A and B). The samples have also been tested retrospectively to assess the community burden of newly discovered respiratory pathogens e.g. human metapneumovirus [15]. The collection of these samples provides valuable antigenic data; the first influenza viruses to appear at the start of a winter epidemic are usually isolated from community-based samples, such as those provided by the WRS and therefore provide early data on the antigenicity of the dominant circulating strains, which are important for determining the strain composition for the influenza vaccine for the following season.

Conclusions

The WRS has a 40-year history of disease surveillance and provides a unique opportunity to study long-term trends. One of the major strengths of the system is the quality of data capture by the participating GPs. Data are captured for each individual consultation i.e. even for two or more episodes during the same consultation, which ensures that all data are recorded and utilised within the system. GPs record each episode as either a first, new, or ongoing episode. This allows for the discrimination between genuine new episodes of disease, and continuing consultations for more persistent and chronic conditions; the weekly surveillance reports only count first and new episodes of disease. Historically the WRS is renowned for its surveillance, and epidemiological research, on influenza and the clinical outcomes associated with this viral infection. Incidence rates of ILI recorded by the WRS are monitored by the UK Department of Health and National Institute for Clinical Evidence (NICE) to provide evidence of significant influenza activity in the community to command the signal for the widespread use of neuraminidase inhibitor drugs by GPs during the winter season [16]. The WRS also monitors the seasonal uptake of influenza vaccine in all age groups, but in particular the elderly, which can identify problems with uptake associated with manufacturing supply and/or distribution problems. The peak clinical activity of ILI was recorded during the 1969/70 pandemic, after which there was a decade of heightened activity, and since when the incidence of ILI has followed a gradual downward trend. We have previously hypothesised that the continual drift that the current influenza A H3 virus has undergone over the last 40 years might have compromised the transmission potential of the virus, indicating that the natural cycle of the H3 subtype might be coming to an end, making way for a novel subtype to succeed [17]. Should this happen, the WRS surveillance system can provide the means to monitor the clinical impact of the emergence of a novel strain in the community, thus providing data that will be vital in the planning required to minimise the public health impact of such an event. Acknowledgements: We are grateful to the Weekly Returns Service general practitioners and their staff for providing the clinical incidence data. Dr Elliot is jointly funded by the Royal College of General Practitioners and the Health Protection Agency.

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Variability of Estimators for Excess Mortality

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Introduction

For nearly one hundred years, excess mortality has been used as the primary measure of epidemic or pandemic severity. The early use of excess mortality as a tool for the epidemiological study of influenza dates to Collins, Langmuir, and Serfling. More recent work has appeared by Simonsen, Viboud, Thompson, Reichert, and others. Excess mortality during an influenza season is defined as “the difference between the number of deaths observed and the expected baseline in the absence of influenza” [1]. Implicit in this definition is a statistical model to determine the “expected” seasonal baseline. Thus estimating excess mortality is a two-step process: 1) estimate seasonal baseline; 2) calculate observed excess above baseline. Typical approaches to determining seasonal baseline include regression models [1-3], non-parametric smoothing [4], or alternative methods e.g. using viral surveillance data [5]. Characteristics of several of these methods are summarized in a recent review article [6]. Historically, the most common approach to this problem has been to model seasonal baseline with a periodic regression model (reflecting early work [2], this is often called Serfling’s method). Such models use standard linear regression techniques, and include as independent predictors a secular trend (typically linear or quadratic in time) as well as a combination of sine and cosine terms to approximate the annual rise and fall characteristic of seasonal influenza. In order to tolerate residual variability, a threshold above baseline is established, below which observations will not contribute to the calculation of excess. This threshold is typically 1.96 residual standard deviations above baseline, but practices vary and there are no firm guidelines based on statistical principles. Excess mortality is then estimated by summing positive residuals above the resulting model fit plus threshold. While calculating a point estimate for excess mortality is relatively straightforward, calculating valid standard errors or confidence intervals around this estimate is more challenging. Since standard errors are not easily calculated with usual regression techniques, estimates of excess mortality are often simply reported without confidence bounds. Strong arguments have been made for the importance of including such bounds around estimates, especially when reporting health statistics [7]. In general, ignoring sources of uncertainty can lead to invalid statistical inference and potentially incorrect conclusions. Moreover, reporting estimates without bounds may cause unnecessary confusion and controversy when the imprecise nature of the reported figures is not made explicit [8,9]. While precise quantification of uncertainty is probably not crucial for most applications, it is nonetheless important to understand the principles and

mechanics behind this quantification. In this paper, we begin the study of variability in OLS estimators of excess mortality and describe what we believe to be the first findings on this topic.

Materials and Methods

We specified a stochastic model to simulate time series data with dynamic characteristics similar to those of typical influenza surveillance data. Our prototypical data set was the influenza mortality data available from CDC (i.e. either the weekly 122 Cities Mortality data or monthly mortality data from NCHS). The underlying simulation model was essentially identical to Serfling’s model. The process consisted of a linear trend, a seasonal baseline represented by a pair of harmonic terms, and a lag-one autoregressive structure for the residuals:

$$y_t = \alpha_0 + \alpha_1 t + \alpha_2 \sin(2\pi t / 52) + \alpha_3 \cos(2\pi t / 52) + X_t + \varepsilon_t$$

where:

y_t = observed count (or rate) of deaths at week t

ε_t = error term, with $\varepsilon_t = r\varepsilon_{t-1} + z_t$, $z_t \sim N(0, \sigma^2)$

α_i = constant coefficients

X_t = excess mortality (possibly zero)

We assumed a 52-week annual period, slightly shorter than the true periodicity which follows the (roughly) 365-day solar cycle. Summing the estimates of X_t over a specified period, e.g. a selected influenza epidemic, gives an aggregate estimator of excess mortality, which we will write $X = \sum X_t$. To quantify variability, we generated 10,000 independent realizations of the stochastic process above, and calculated regression-based estimates of excess mortality for each simulation. The empirical standard error (ESE) was computed as the width of the central 95% of the resulting distribution, i.e. $ESE = Q_{97.5} - Q_{2.5}$ where $Q_{97.5}$ and $Q_{2.5}$ refer to the empirical quantiles of the computed estimates at 97.5% and 2.5%, respectively. Several factors relating to methodology and data were identified *a priori* as potential influences on the accuracy and precision of the estimator. One set of parameters was selected as point of reference, and the influence of each factor on variability was expressed as a Standard Error Inflation Factor (SEIF) with respect to the reference case. The individual factors considered were:

- Length and magnitude of outbreak
- Number of observations used to estimate baseline
- Residual standard deviation (σ)
- Lag-one autocorrelation of residuals (ρ)
- Temporal aggregation

Results

Data were simulated using the following parameters for the underlying process: ($\alpha_0 = 0$, $\alpha_1 = 0.1$, $\alpha_2 = \alpha_3 = 100$). Excess mortality X_t was modeled with a simple step function of magnitude 300 for 20 consecutive time periods. A subset of results are displayed (Figure 1) and summarized (Table 1). Base simulations with reference parameters (i.e. denominator when

Options for the Control of Influenza VI

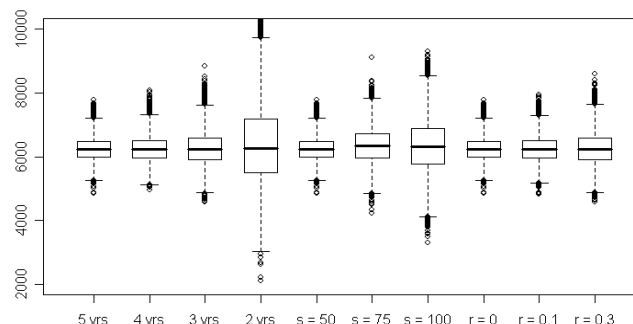
calculating SEIF) used five years of data to estimate baseline, with residual parameters ($\sigma = 50$, $\rho = 0.0$). These simulations are denoted with bold type in Table 1. Graphical depictions of selected results are displayed in Figure 1.

Table 1. Summary of selected results. For purposes of calculating SEIF, referent (base) parameter settings are indicated in bold.

Parameter		ESE	SEIF
Years of data	5	1434	1.00
	4	1613	1.12
	3	2044	1.43
	2	5195	3.62
Residual sd (σ)	25	719	0.50
	50	1434	1.00
	75	2186	1.52
	100	3185	2.22
Autocorrelation (ρ)	0.0	1434	1.00
	0.1	1580	1.10
	0.2	1756	1.22
	0.3	1999	1.39

None of duration, magnitude, nor timing of excess mortality X_t had a substantial impact on the precision of the estimates (results not shown). Estimates were generally unbiased, although underestimation was common when the magnitude of excess was small relative to the residual standard deviation σ . To evaluate the influence of temporal aggregation, the time scale was collapsed by a factor of four to simulate change in temporal scale from weekly to monthly data. This resulted in slight underestimation and a modest increase (10%) in variability. Estimates from the aggregated data tended to be especially sensitive to small changes in regression methodology. In practice, temporal aggregation is often achieved using calendar units (i.e. calendar months) instead of consistent time periods (such as four-week periods). We have not yet investigated these complexities, which may lead to additional uncertainty in estimates. In the absence of autocorrelation, OLS regression standard errors generally will increase linearly with s , and decrease according to the inverse square root of the number of independent observations. Variability of estimates for excess mortality follow a similar pattern. However, including autocorrelation in the generating process ($\rho \neq 0$, bottom four rows of Table 1) had an additional effect on variability.

Figure 1. Results of 10,000 simulations with different parameters. The y-axis represents estimated excess mortality. The letters "s" and "r" denote residual standard deviation σ and lag-one autocorrelation ρ respectively.



Discussion

Our results demonstrate that although an OLS regression model can not distinguish between data with uncorrelated errors and data with serial autocorrelation, the variability of estimates from such models is dependent upon this autocorrelation. As a practical consequence, OLS regression (i.e. Serfling) is insufficient to quantify uncertainty in the estimated excess mortality. Instead, computing valid confidence intervals for excess mortality requires simultaneous estimation of baseline as well as the autoregressive characteristics of the residuals. Estimates of these parameters are available using alternative statistical methods (e.g. periodic autoregressive (PAR) models or Hidden Markov models (HMMs) [10-12]). It is important to note that the variability exhibited by Serfling-type OLS estimators is not simply a consequence of the methodology. Rather, it is a result of stochastic uncertainty that is present in any statistical model, and thus will also affect other existing methods. In this paper we have reported our initial findings and discussed some of the practical methodological issues that will arise when quantifying uncertainty in estimates of excess mortality. To make this study of practical use to epidemiological research using excess mortality as a measure of influenza activity or severity, future work will focus on data-derived calculation of valid confidence intervals and will investigate similar issues with other available methodologies.

Acknowledgements

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Influenza Surveillance in the Eastern Mediterranean Region, Africa, Eastern Europe and the Central Asian Republics

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NAMRU-3 continues to expand influenza surveillance within the Eastern Mediterranean Region and other regions as an integral part of the World Health Organization (WHO) global influenza surveillance program. Trilateral agreements between NAMRU-3, WHO-EMRO, and host countries have been established to support regional surveillance. Additional countries from Africa, Eastern Europe, and Central Asia have entered into bilateral agreements with NAMRU-3. Isolation and characterization of circulating virus strains from these regions contributes to the overall decision for the annual vaccine formulation. Participating laboratories were assessed and provided necessary equipment, reagents, and training. Throat swabs collected from patients with influenza-like illness were processed for virus isolation with subtyping conducted using hemagglutination inhibition kits provided by a WHO Collaborating Center for Reference and Research on Influenza (Centers for Disease Control (CDC), Atlanta, Georgia). Positive specimens or putative isolates from collaborating sites were forwarded to NAMRU-3 for additional testing and/or confirmation with representative isolates being forwarded to the CDC. From July 2005 to June 2006, a total of 4,524 specimens were collected from 12 countries. A total of 399 (10%) influenza viruses were isolated from 3,982 samples collected in Egypt. Seventy-three isolates (18.3%) were confirmed as influenza type A and 326 (81.7%) as type B. Sixty-nine of the 73 influenza A isolates were H1N1 (A/NEW CALEDONIA/20/99-LIKE) and four were H3N2. Influenza B isolates were B/OHIO/01/2005-LIKE. From other countries, influenza was isolated from 65 (12%) of 541 specimens from Kazakhstan, Uzbekistan, Kyrgyzstan and Ukraine. Of these, 57 were influenza B, five were influenza A H1N1, and the remaining three were influenza A H3N2. Between July 2006 and January 2007, an additional 1,984 specimens were processed resulting in 42 (2.4%) influenza isolates from Jordan, Kyrgyzstan, Oman, and Syria. Of these isolates, 35 were influenza B, two were influenza A H1N1, and five are pending subtyping. Development of influenza surveillance networks is crucial for identifying circulating influenza strains to incorporate in the seasonal vaccine. Capacity building in countries with the development of National Influenza Centers to expand diagnostic capabilities strengthens these efforts. Training to enhance the quality of sample collection and processing is required during this process.

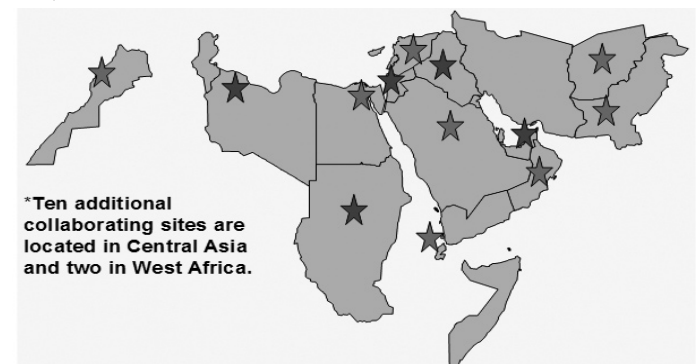
Introduction

The epidemiology of influenza A in humans follows three general patterns: (1) local sporadic outbreaks; (2) regional or nationwide epidemics; and (3) worldwide pandemics. Local and regional outbreaks are due to limited antigenic change. Pandemics result from antigenic shift where the antigenic structure of the virus is considerably changed. This allows the virus to replicate in an immunologically naive population with devastating results. Pandemic strains have included H1N1 in 1918-19, H2N2 in 1957-58 and H3N2 in 1968-69 (Figure 1). Currently, influenza A (H3N2) and A (H1N1) are circulating worldwide. The DoD Global Emerging Infectious Disease Surveillance Program provided resources for human seasonal influenza surveillance and work is conducted in conjunction with each participating country's Ministry of Health (MOH) and in the Middle East with the World Health Organization (WHO) Eastern Mediterranean Regional Office (EMRO). The US Naval Medical Research Unit No. 3 (NAMRU-3) continues to expand influenza surveillance within the Eastern Mediterranean Region (EMR) and other regions as an integral part of the WHO global influenza surveillance program. Trilateral agreements between NAMRU-3, WHO-EMRO, and host countries have been established to support regional surveillance. Additional countries from Africa, Eastern Europe, and Central Asia have entered into bilateral agreements with NAMRU-3. Primary objectives were: To isolate and characterize circulating influenza virus strains. To establish an influenza surveillance network within the EMR and provide virological and epidemiological information to the member countries and the WHO Global Influenza Program. Results are supplied to the WHO Task Force on Influenza Vaccines for evaluation and potential incorporation into the seasonal vaccine. Secondary objectives were: To develop capacity for in-country surveillance of influenza and other respiratory diseases. To identify viral respiratory pathogens other than influenza viruses causing morbidity. To develop the capacity for influenza culturing and identification within the Central Laboratories of the participating countries.

Materials and Methods

Study sites: See Figure 1.

Figure 1. Current (★) and prospective (★) seasonal surveillance sites within EMR.



Poster Presentations: Disease Surveillance and Impact

Study population. Patients presenting to outpatient clinics of collaborating hospitals or poly-clinics in each country, meeting the WHO influenza case definition.

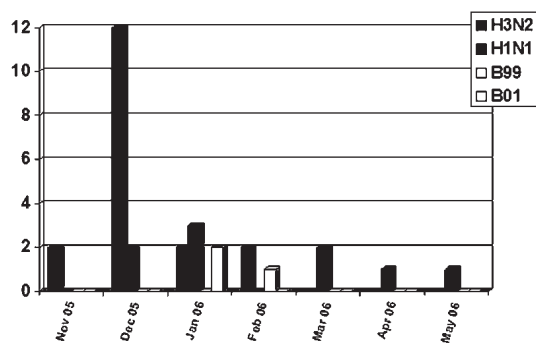
Sample collection. Throat swab samples were placed in virus transport medium and stored immediately in liquid nitrogen immediately after collection.

Virus isolation/identification. Cell lines used: MDCK (mainly for influenza viruses), H292 and LLC-MK2 (mainly for parainfluenza virus, enteroviruses, and adenoviruses). Virus isolation was conducted in a BSL-2 facility. After observation of a cytopathic effect on MDCK cells viral isolates were identified by hemagglutination test and subtyped using hemagglutination inhibition kits provided by the WHO Collaborating Center for Reference and Research on Influenza (CDC, Atlanta, Georgia). Viruses isolated on H292 and LLCMK-2 were identified by immunofluorescent antibody test (IFAT) using kits from LIGHT DIAGNOSTICS, CA, USA.

Results

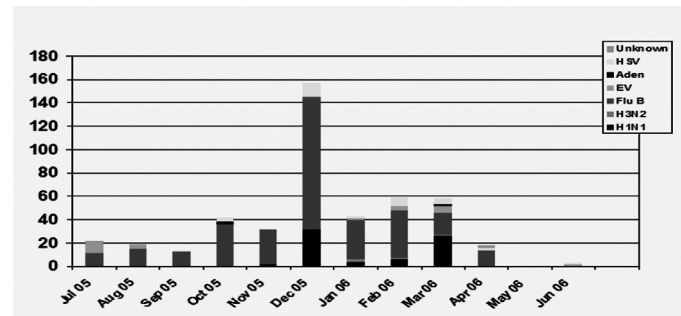
Training was conducted at NAMRU-3 for influenza virus isolation and identification and in collaborating countries during 2005 to 2007. One hundred and twenty eight technicians and professionals from 20 countries (Egypt, Libya, Sudan, Kenya, Nigeria, Ghana, Jordan, Syria, Saudi Arabia, Iraq, Kuwait, Oman, Yemen, Afghanistan, Uzbekistan, Kazakhstan, Azerbaijan, Georgia, Bulgaria and Ukraine) were successfully trained. National Influenza Centers have been established in some countries (Egypt, Syria, Jordan, Oman, Uzbekistan, Kazakhstan, Azerbaijan and Ukraine). Isolated influenza viruses were characterized at NAMRU-3 and representative isolates were sent to CDC for final recommendations for the next season's influenza vaccine. From July 2005 to June 2006, a total of 4,524 specimens were collected from 12 countries. A total of 399 (10%) influenza viruses were isolated from 3,982 specimens collected in Egypt. Seventy-three isolates (18.3%) were confirmed as influenza A and 326 (81.7%) as type B. Sixty-nine of the influenza A isolates were H1N1 (A/NEW CALEDONIA/20/99-LIKE) and the remaining 4 were H3N2. Influenza B isolates were all B/OHIO/01/2005-LIKE. Influenza A were detected from Nov 05 to March 06, while influenza B was detected all year around, peaking during winter season (Figure 2).

Figure 2. Distribution of influenza and other respiratory viruses in 2005-2006 in Egypt.



In addition to influenza viruses, 28 enteroviruses, 3 adenoviruses and 33 herpes simplex viruses were isolated and identified by IFAT. A total of 30 (8.3%) influenza viruses were isolated from 363 samples collected in Oman. Twenty seven were influenza A (18 H3N2 and 9 H1N1) and 3 were type B (2 B01 and one B99). Twenty-seven of 30 (90%) isolates were detected during Dec 05 to Feb 06 (Figure 3). Influenza viruses were isolated from 65 (12%) of 541 specimens from Kazakhstan, Uzbekistan, Kyrgyzstan and Ukraine. Of these, 57 were influenza B, five were influenza A H1N1, and the remaining three were influenza A H3N2. For the current season, July 2006 and January 2007, an additional 1,984 specimens were processed, resulting in 42 (2.4%) influenza isolates from Jordan, Kyrgyzstan, Oman, and Syria. Of these isolates, 35 were influenza B, two (only from Oman) were influenza A H1N1, and five are pending subtyping.

Figure 3. Distribution of influenza and other respiratory viruses in 2005-2006 in Oman.



Conclusions

During the 2005/2006 season, influenza B viruses predominated in all collaborating countries, except Oman where 94.5% of isolated influenza were type A (mainly H3N2). The WHO reported during the same season that the predominant influenza virus worldwide was type A (mainly subtype H3N2). Egypt participated continuously over the past year and has the most comprehensive data amongst our collaborating countries. Both influenza A (mainly H1N1) and B viruses co-circulated in Egypt with influenza B viruses predominating. Interestingly, in the previous season (2004/2005), influenza B predominated but all influenza A were H3N2 indicating significant influenza A subtype variations between seasons. During the current season (July 2006 to January 2007), influenza B was still the predominant isolate (83.3%) among specimens from Jordan, Kyrgyzstan, Oman and Syria. Development and expansion of influenza surveillance networks is crucial for identifying circulating influenza strains for incorporation into the seasonal vaccine. Capacity building in countries with the development of National Influenza Centers to expand diagnostic capabilities strengthens these efforts. Training to enhance the quality of sample collection and processing is required during this process.

Options for the Control of Influenza VI

Acknowledgements

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FluWatch: 11 Years of Influenza Surveillance in Canada

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Background: Since 1996 Canada has had a co-ordinated national influenza surveillance program, *FluWatch*, which monitors the occurrence and spread of influenza activity. *FluWatch* consists of a network of sentinel laboratories and primary-care practices, provincial and territorial health ministries, paediatric hospitals and the National Microbiology Laboratory. **Methods:** Eleven seasons of *FluWatch* surveillance data were reviewed to identify similarities, differences and trends in influenza activity in Canada over the years. Indicators and key attributes of *FluWatch* were also compared to other international influenza surveillance systems to assess other strengths and limitations of the program. **Results:** Seven out of 11 seasons were predominated by influenza A virus circulation during which the over 65 and under 5 age groups were most affected. Five of the 11 seasons saw onset dates peak between early to mid-February. Influenza activity started in the west of Canada and moved eastward in 8 of 11 seasons. Surveillance indicators show that 8 of 11 seasons were relatively mild. Highlights of the *FluWatch* program include year-round surveillance of key activity indicators and high per capita influenza testing and strain characterizations rates compared to other international systems/networks. **Conclusion:** Overall, *FluWatch* is a comprehensive system for timely year-round surveillance of seasonal influenza activity and is comparable to other international influenza surveillance systems.

Introduction

Canada's national influenza surveillance system, *FluWatch*, was implemented in 1996 to enhance the existing influenza surveillance by collecting consistent and timely national data. Prior to *FluWatch*, interpretation of influenza data at the national level was complicated due to provincial/territorial variations in measurement and reporting of influenza activity and was less timely (e.g. due to delays in reporting of surveillance data). Today, *FluWatch* is a collaborative effort between the Public Health Agency of Canada (PHAC), Provincial and Territorial Ministries of Health, sentinel public health and hospital-based laboratories, sentinel primary care provider reporting programs, and the Immunization and Monitoring Program Active (IMPACT) paediatric hospital surveillance network. Over 11 years, *FluWatch* has expanded and improved through strengthening of its individual components, addition of new indicators, implementation of mechanisms for more timely collection of data and information dissemination, and strengthening of relationships between collaborating

FluWatch partners. *FluWatch* objectives include: 1) early detection of influenza activity in Canada; 2) provision of timely and up-to-date information on influenza activity in Canada and abroad to professionals as well as the public; 3) monitoring of circulating strains of influenza virus, including new sub-types and antiviral resistance; and 4) contribution of virological surveillance information to the World Health Organization (WHO) to assist with decision-making to determine the vaccine components for the subsequent season. *FluWatch* indicators include: (a) number and proportion positive of laboratory detections for influenza and other common respiratory viruses; (b) representative influenza strain and antiviral resistance monitoring; (c) rate of influenza-like illness (ILI) consultations per 1000 all cause patient visits; (d) regional influenza activity level assessments; (e) influenza-associated hospitalizations and deaths in children; and (f) international influenza activity.

Methods

Eleven years of *FluWatch* surveillance data (1996/97 – 2006/07 seasons) were reviewed to identify similarities, differences and trends in influenza in Canada over the years. (Note that results for the 2006/07 season are preliminary and include data up to 28 April, 2007). Key indicators and attributes of the *FluWatch* program were compared to other international influenza surveillance systems/networks (the Centers for Disease Control and Prevention (CDC) in the United States and the European Influenza Surveillance Scheme (EISS)) to assess strengths and limitations of the program.

Results

Laboratory Identifications. Seven of 11 seasons were predominated by influenza A virus circulation, one was predominated by influenza B, and three were mixed (influenza A/B) seasons (Table 1). In influenza A predominant seasons, the over 65 and under 5 age groups were most affected whereas in mixed or predominantly influenza B seasons most cases were reported in children. Percent peak in laboratory detections averaged 22% per season (range 13–33%). On average, 10% of all influenza tests per season were positive (range 9–14%). Per capita influenza testing rates in Canada have increased approximately three-fold over 11 years. Five of the 11 seasons saw onset dates peak between early to mid-February (range: mid-December to mid-March).

Antiviral Resistance. The proportion of influenza A isolates resistant to amantadine increased significantly in the 2005/06 season (76.5%) and declined in 2006/07 (25.6%).

ILI Consultation Rates. The weekly peak in ILI consultation rates ranged from 31–149 per 1,000 patient visits during the influenza season compared to an average rate of 8 per 1,000 in the off season (Table 1). The four highest peak season rates were observed in periods where influenza A predominated, while the lowest rate (31 per 1,000) was observed in the predominantly influenza B season (2000/01).

Influenza Activity Levels and Outbreaks. Influenza activity started in the west of Canada and moved eastward in eight of the 11

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seasons (Table 1). In the other three seasons, influenza activity started in the provinces of Ontario and Quebec. Between 46–762 outbreaks in long-term care facilities were reported per season since 2002-2003 (mean=303), with the highest number reported in 2004-2005 (A/Fujian/411/02(H3N2) season). *Influenza-associated Hospitalizations and Deaths in Children*. Since the 2004/05 season, between 327-391 (mean = 364) laboratory-confirmed paediatric hospitalizations and between two to five deaths (mean = three) were reported in Canada per season. The majority of the hospitalizations were due to influenza A infection (range: 62%-87%). Most hospitalised cases were reported in those less than two years of age, except in the 2005-2006 mixed A/B season where most cases were reported in children above this age range. Overall Severity: Surveillance data indicate that eight of the 11 seasons were relatively mild, except for the 1998/99 (A/Sydney/3/97(H3N2)) and 2003/04–2004/05 (A/Fujian/411/02(H3N2)) seasons which were moderate in severity.

International Comparisons. Per capita influenza laboratory testing rates are high in Canada, compared to other influenza surveillance programs (Table 2). Sentinel practitioner coverage for monitoring ILI represent the population well. In Canada, influenza activity level assessments are reported by surveillance region, which often represents an assessment of activity for a smaller geographical area than would assessments at the state or country level. FluWatch reports on influenza surveillance year-round, unlike other influenza systems.

Discussion

Overall, surveillance data indicate that influenza A predominant seasons appear to have a greater impact on the elderly and the very young and that milder seasons tend to be associated with predominance of influenza B, A(H1N1) or mixed A/B circulation. Although it was observed that for the majority of the seasons under surveillance influenza activity began in Western Canada and later appeared in the East, the tendency for the west to east spread is not well understood. It is also difficult to predict timing of peak activity for influenza seasons as peaks have ranged from mid-December to mid-March as observed over 11 seasons. The increase in influenza testing observed over the years is likely due to increased testing by clinicians (especially in response to emerging infectious disease alerts as seen post-SARS and currently with avian influenza outbreaks occurring in Asia) and an increase in the number of participating sentinel laboratories. In addition, the increase in the number of long-term care facility outbreaks reported is due in part to improved reporting standards and methods over the years. PHAC issued a recommendation to stop the use of amantadine for treatment of influenza in January 2006 (midway through the 2005/06 season) and may have assisted in the reduction of the proportion of amantadine resistant isolates observed in the 2006/07 season. Overall, Canada has a comprehensive system for timely surveillance of seasonal influenza activity, which is comparable to other international influenza surveillance systems/networks. Highlights of the *FluWatch* program include

year-round surveillance of key indicators and high per capita influenza testing and strain characterizations rates compared to other international systems/networks. Influenza surveillance in Canada has improved substantially over the past 11 seasons, both in terms of the quality of data reported and the addition of new indicators. However the system still lacks real-time indicators of severity in the adult population, including hospitalizations and mortality data. Future directions include practical options for: development of real-time collection systems for adult severity indicators; community-based monitoring of morbidity and societal disruption (e.g. workplace absenteeism); use of web-based reporting and dissemination tools; and increasing system flexibility/sustainability for rapid response to public health emergencies.

Table 1. Summary of FluWatch data, 1996/97-2006/07 seasons.

Influenza Season	1996/97	1997/98	1998/99	1999/00	2000/01	2001/02	2002/03	2003/04	2004/05	2005/06	2006/07*	11 Season Summary
Total Cases	1930	3802	4203	5907	3935	6258	2953	7708	10000	6590	4934	5298 (range 1,930-10,000)
Influenza A	64%	99%	86%	98.5%	32%	87%	55%	98.5%	86%	61%	87%	7/11 influenza A seasons
Influenza B	36%	<1%	14%	1.5%	68%	12%	45%	0.5%	14%	39%	13%	3/11 mixed influenza A/B seasons
Predominant Strains	A/Muhan/395/95(H3N2) B/Beijing/184/93	A/Sydney/3/97(H3N2) B/Beijing/184/93	A/Sydney/3/97(H3N2) B/Beijing/184/93	A/Sydney/3/97(H3N2)	A/New Caledonia/20/99(H1N1) B/Yamanashi/166/98	A/Panama/2007/99(H3N2) A/New Caledonia/20/99(H1N1)	A/Panama/2007/99(H3N2) B/Hong Kong/330/01	A/Fujian/41/02(H3N2)	A/Fujian/41/02(H3N2) A/California/07/04(H3N2) B/Shanghai/361/02	A/California/07/04(H3N2) A/Missouri/67/05(H3N2) B/Malaysia/2506/04	A/Missouri/67/05(H3N2) A/New Caledonia/20/99(H1N1)	seasons with predominance of A/Sydney/3/97(H3N2) and A/Fujian/41/02(H3N2) were found to be most severe**
per capita influenza testing rates (per 100,000 pop'n)	91	145	180	195	178	185	192	291	314	271	238	207 (range 91-314)
% peak in lab detections	17%	26%	18%	25%	17%	26%	13%	33%	25%	20%	21%	22% (range 13-33%)
average % positive influenza detections over the season	9%	12%	10%	14%	8%	12%	6%	12%	13%	9%	9%	10% (range 6-14%)
peak timing of case symptom onset dates	mid-Dec	mid-Feb	Feb	early Jan	early Feb	Feb	early Mar	mid-Dec	early Feb	mid-Mar	early Mar	5/11 in early to mid-February (range mid-December-mid-March)
age distribution of cases by type	< 5 yrs	> 65 yrs	> 65 yrs	> 65 yrs	< 5 yrs	< 5 yrs	< 15 yrs	< 5 & > 65 yrs	> 65 yrs	< 15 yrs	< 5 & > 65 yrs	< 5 & > 65 affected in predominantly A seasons; children affected in mixed influenza A/B or predominantly B seasons
peak in ILI consultation rates (per 1000 patient visits)	123	91	52	149	31	58	40	80	49	49	50	70 (range 31-149)

* Results as of 28 April, 2007.
** based on number of cases, activity levels, outbreaks and ILI consultation rates.

Table 2. International comparisons between *FluWatch*, CDC and EISS.

Surveillance Indicator	FluWatch	CDC	EISS
Per capita influenza laboratory testing per 100,000 population (avg. 00/01-06/07 seasons)	234 (range 152 - 317)	43 (range 31 – 56)	3.3 (range 3.0 – 4.3)
Proportion of influenza isolates with strain identification (avg. 02/03-06/07 seasons)	10.2% (range 7.4 – 15.7%) Avg N=875	4% (range 2.9 – 5.4%) Avg N=795	30.6% (range 20.2-63.9%) Avg N=2486
Sentinel practitioners per capita monitoring ILI (2005-2006 season)	~1/30,000	~1/250,000	~1/100,000* *varies by country
Influenza activity level assessment	P/T regional level	State level	Country or region level
Real-time severity indicator	mortality & hospitalizations (paediatrics only)	mortality (P&I) & hospitalizations (paediatrics only)	--
Reporting period	year-round	seasonal	mostly seasonal

The Impact of Influenza Among Families With Children With Influenza Infection

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Introduction

Influenza is responsible for significant economic and societal costs¹. The work loss of influenza infection on affected households is difficult to estimate and quantify. Parents of children with confirmed influenza who presented to emergency secondary care were invited to complete a questionnaire to evaluate the impact of influenza on their family.

Methods

During winter 2006-07, we screened children presenting with febrile illness to Leicester Children's Hospital emergency unit with a rapid antigen test for influenza A and B (Roche Diagnostics). The aim was to identify influenza-infected children who could be approached and considered for enrolment into an oseltamivir treatment study. Subjects offered oseltamivir were restricted to those whose symptom onset at presentation was less than 48 hrs duration and who were >1 – 12 years of age. Parents were requested to complete a questionnaire on day 7-10 following presentation and diagnosis of their child. We collected information on the number and age of household contacts, duration and timing of influenza-like illness in any household member during the previous 2 weeks, number of medical consultations generated, any treatments received (prescription or over-the-counter use) and absenteeism from work or school due to personal ill health and/or the need to provide child care.

Results

Thirty-five questionnaires were distributed to parents and 29 (83%) were returned. All households returning questionnaires contained one child who had a positive influenza rapid antigen test at hospital within 48 hrs of onset of symptoms and received oseltamivir as part of a clinical trial. Subsequent culture of nasal specimens confirmed influenza A (H3N2) in all children enrolled and whose parents completed the questionnaire. All questionnaires were fully completed between day 8 and 10 after the child diagnosis and commencement of oseltamivir treatment. Of the 29 households, 21 (72%) described influenza-like illness in at least one occupant other than the known influenza-positive child. Of these affected households, 6 (29%) described influenza-like illness in another occupant before onset of symptoms in the known positive child, suggesting that the child who presented to hospital should not always be considered as the index case within the household.

Influenza-like illness in adult household contacts. The 29 households surveyed contained 59 adult contacts (range 1-3

per house, mode 2), aged 19-48 (mean 36 years). Forty-eight (81%) of the household adults attended work: 30 (51%) in fulltime and 18 (31%) in part-time employment. Five (8.5%) adults had recognised indications for influenza vaccination (these were 3 asthma, 1 diabetes mellitus, 1 chronic renal failure) but only 2 of these subjects (40%) were seasonally vaccinated against influenza. Neither of the vaccinated adults reported influenza-like illness during the questionnaire period. Of 59 household adults, 29 (49%) described febrile respiratory illness during the questionnaire period. Nineteen (66%) reported symptoms in the week after the positive influenza diagnosis in their child, but 10 (34%) reported onset of symptoms before their child. Twenty-two (76%) adults reported use of non-prescription antipyretic or analgesic medications. Nine (31%) of the 29 adults with influenza-like illness sought medical advice: 5 attended their primary care practitioner (GP), 2 attended NHS walk-in centres, and 2 attended a hospital emergency department. The medical consultations resulted in antibiotic prescriptions in 6 (21%) cases. No antiviral therapy was prescribed. The range of time to return to normal activities (defined as normal daily work or home routine) and their baseline level of health following the ILI was reported as 1-14 days, (mean 7.4 days, mode and median 7 days).

Absenteeism in adult household contacts. Twenty-five (52%) of 48 working adult household contacts reported absenteeism from work due to personal ill health or a need to provide care for their child with a range of 1-14 days (mean 4.5 days, median and mode 3 days) resulting in a total loss of 136 work days. Work absenteeism due to personal ill health was reported by 14 adults for a range of 1-14 days (mean 4.5 days, median 4 days, mode 3 days) with a total loss of 63 work days. Work absenteeism due to child-care requirements was reported by 16 adults for a range of 1-14 days (mean 4.6 days, median 3 days, mode 2 days) with a total loss of 73 work days. Five adults reported absenteeism for a combination of personal ill health and need for child care. Overall, the mean work absenteeism was 2.8 days for each working adult household contact.

Influenza-like illness in children household contacts. The 29 households contained 63 children (range 1-5 per house, mode 2), aged between 1 month and 14 yrs (mean 6.2 yrs, mode 2 yrs). Each of the 29 households contained 1 child with culture-confirmed influenza H3N2 who received oseltamivir treatment in a clinical study. Forty-eight children (76%) attended either nursery or school. None of the children in the households had ever received seasonal influenza vaccine. Of the 34 remaining siblings, 18 (53%) experienced influenza-like illness during the questionnaire period of which 10 (55%) reported symptoms concurrently or after the positive influenza case was diagnosed. The 45 children with symptomatic influenza-like illness in the households surveyed generated 60 medical attendances giving 1.33 medical consultations per child per influenza-like illness. There were more medical consultations at hospital emergency department than in primary care (31 versus 29 attendances). Forty-one (91%) and 14 (31%) of 45 symptomatic children received antipyretics and antibiotic prescriptions respectively.

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No antiviral therapy was routinely prescribed outside the clinical trial.

Absenteeism in children. Thirty-two (67%) of 48 household children contacts who attended school or nursery reported absenteeism with a range of 1-10 days (mean 4.6 days, mode 5 days) with a total loss of 113 school or nursery days.

Discussion

These 29 households containing at least 1 laboratory confirmed paediatric influenza A H3N2 case generated a total of 69 medical consultations, 20 antibiotic prescriptions, 138 lost adult workdays and 113 lost school or nursery days. Although a proportion of household influenza-like illness among adult and sibling contacts may be due to infection with concurrent non-influenza viruses, it is clear that influenza has a significant impact on working families and healthcare providers. This survey was conducted in 2006-07 during which all local isolates were influenza A H3N2 which may exhibit greatest impact on household adult contacts. Among school children, absenteeism is a useful measure of influenza activity. School absenteeism has been shown to correlate to the epidemic curve and may rise up to one third higher than its base levels during influenza activity. Up to 37% of all enrolled school children suffered absence during the 1976-77 epidemics². The high incidence of influenza in children is not only an important healthcare problem, but also impacts upon family economics by requiring substantial loss of work by at least one of the parents. The transmission of influenza within age groups and populations varies with each epidemic and depends on immunity to the prevalent strain. For influenza A H3N2, preexisting immunity is often limited because of frequent antigenic changes giving potential to have the greatest clinical impact, whereas influenza A H1N1 and B are more antigenically stable with less variability. Typically, influenza attack rates are highest in school age children with lower rates in adults, and households with children have the highest occurrence of influenza³. Although it is generally considered that children introduce influenza into households, the presenting child should not be assumed to be the index case, as up to one third of households describe influenza-like illness in other occupants before the onset of symptoms in the diagnosed child. We identified that more medical consultations for influenza-like illness among children occurred in hospital emergency departments than in primary care settings. In the UK, one of the traditional measures of seasonal influenza activity in the community has been general practice consultation rates for influenza-like illness collected by the UK Royal College of General Practitioners network⁴. Whilst sentinel practices should receive and submit information regarding emergency department attendances for their patients, it may be that changes in the provision of out-of-hours care and increasing attendances to hospital emergency departments underestimate the burden of influenza. As all the children in this survey with confirmed influenza A H3N2 were treated with oseltamivir within 48 hrs of symptom onset, it would be expected that their duration of illness and infectivity

would be shorter than in untreated children⁵ suggesting that the impact of influenza on most households is likely to be greater. Whilst oseltamivir has been demonstrated to prevent secondary infection in households when used as post exposure prophylaxis⁶, its use in the UK is limited despite its potential for reducing the economic burden and societal disruption attributed to influenza among households.

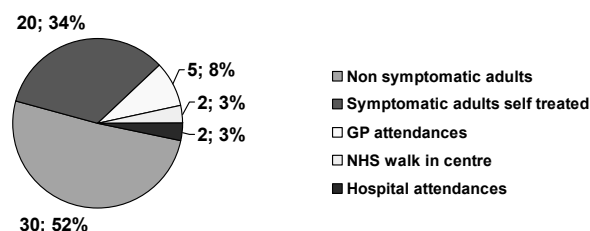
Sources of Funding

The oseltamivir treatment studies were supported by Hoffman-La Roche. Conflict of interest: IS has received funding from pharmaceutical industry for research support, speakers fees and travel to international meetings from companies including GSK and Roche who manufacture neuraminidase inhibitors. PB and JS are employees of Hoffman-La Roche who manufacture oseltamivir.

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Figure 1. Influenza-Like illness in adult household contacts (N=59) of confirmed influenza-positive children.



Synchronous Oscillation of Influenza Virus Activity and Influenza-Like Illness in a Subtropical City

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Clinical surveillance of influenza-like illness (ILI) and virological surveillance for influenza virus activity have been widely adopted as critical measures to monitor influenza outbreaks. Due to non-stationary seasonality of influenza virus activity, the correlation between ILI and influenza virus activity in the tropics is less well defined, compared with the temperate regions. We used wavelet analysis to model the oscillation coherence between influenza virus activity (revealed by virological surveillance) and consultation rates of ILI in both General Out-patient Clinic (GOPC) and General Practitioner (GP) settings, during the study period of January, 1998 to May, 2006. We found significant ($p < 0.05$) coherence with influenza virus activity for the annual periodic mode during the periods 1998-1999 and 2002-2006, for ILI in GP and also in GOPC setting. Oscillation of influenza virus activity was estimated to lag behind ILI in GP and ILI in GOPC by 4 weeks and 2 weeks, respectively. Our results provide further evidence to support that ILI surveillance could be a reliable predictor for influenza outbreaks in the tropic.

Introduction

Clinical surveillance for influenza-like illness (ILI) and virological surveillance for influenza virus activity have been widely adopted as critical measures to monitor influenza epidemics. Unfortunately, it is difficult to separate influenza infections from other acute respiratory diseases solely based on symptoms, making it unreliable to detect early-stage influenza outbreaks based on increased consultation rates of influenza-like illness. Although virological surveillance is regarded as a more accurate indicator for influenza virus activity than clinical surveillance, it could not provide a timely warning to influenza outbreak due to time delay and limited budget. As a result most virological surveillance is conducted to detect antigenic drift/shift of influenza viruses for the purpose of developing vaccines [1]. Many studies have examined the sensitivity and specificity of diagnosis criteria for ILI to virologically confirmed influenza infections [2,3]. However, few studies investigated synchrony between ILI consultation rates and influenza virus activity in the tropics and subtropics. This study aimed to model the oscillation relationship between ILI consultation rates and influenza virus activity revealed by virological surveillance. Unlike the temperate regions where influenza

epidemics occur in relatively fixed periods (mostly winter time) [4], the subtropical/tropical regions are featured with influenza circulating throughout a year and with relatively unpredictable epidemics [5]. Taking account of unstable influenza seasonality, in this study we used wavelet analysis which is suitable for modeling the non-stationary time series [6], to examine association between ILI consultation rates and the virus activity in terms of timing and magnitude of their oscillations.

Methods

We obtained the weekly consultation rates of ILI, both in GP and GOPC settings, and weekly virological surveillance data from January 1998 to May 2006. We used the wavelet analysis to model seasonal oscillation of ILI consultation rates and influenza virus activity (represented by weekly proportions of specimens positive for influenza A/H3N2, A/H1N1 and B from the virological surveillance data). The wavelet analysis decomposes a nonstationary time series into different oscillation modes over time. The wavelet coherence was adopted to examine the association between ILI consultation rates and the virus activity in terms of timing and magnitude of their oscillations. High coherence suggests the capability of one time series to predict another one. We also used the phase analysis to quantify the delay (lag) period between ILI consultation rates in both GP and GOPC settings and the virus activity. A detailed description for wavelet analysis and wavelet coherence can be found in Grinsted (2004) [7].

Results

Influenza virus activity showed an obvious semiannual pattern in 1998-2000 and 2003, with one sharp spike in the winter and another peak in the late spring/early summer; while in 2001-2002 and 2004-2006, only an annual seasonal pattern with one broad peak from winter to summer was observed. Similar seasonal patterns were also observed in ILI consultation rates, in both GP and GOPC settings. Wavelet coherence analysis showed that ILI consultation rates in GP were in coherence with influenza virus activity at the annual cycle, and their coherence was significant ($p < 0.05$) throughout the study period, although their coherence at the semiannual cycle was found significant only in the years with semiannual seasonality (1998-2000 and 2003). High coherence between ILI consultation rates in GOPC and influenza virus activity was observed at both the annual and semiannual cycles, with a pattern similar to coherence between ILI in GP and virus activity. Analysis for the phase difference between ILI consultation rates and influenza virus activity at the annual cycle showed that overall, the oscillation of ILI consultation rates in either GP or GOPC settings was in synchrony with the oscillation of influenza virus activity. ILI consultation rates in GP setting led influenza virus activity by an average of 4.3 weeks (ranged from -0.3 to 6.0 weeks), while those in GOPC led by an average of 1.6 week (ranged from -2.5 to 4.3 weeks). The lag between influenza virus activity and ILI consultation rates was relatively longer in the years with annual cycle (4.9 weeks for GP and 2.7 weeks for GOPC) than

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the semiannual-cycle years (3.7 and 0.3 weeks for GP and GOPC, respectively) (Table 1).

Table 1. Average phase difference (range) in weeks between ILI consultation rates and influenza virus activity at the 48-56-week periodic mode, for years with semiannual cycle (1998-2000 and 2003) and years with annual cycle (2001-2002, 2004-2006).

	Semiannual-cycle years		Annual-cycle years		Overall	
GP	3.7	(-0.3,6.0)	4.9	(2.7,6.0)	4.3	(-0.3,6.0)
GOPC	0.3	(-2.5,4.1)	2.7	(0.8,4.3)	1.6	(-2.5,4.3)

Discussion

Our findings suggest that ILI consultation rates in both GOPC and GP settings increase consistently ahead of the increase of influenza virus activity. As in influenza surveillance it is extremely important to issue a warning before influenza epidemics actually occur in the community, this result provides further evidence to support that clinical sentinel surveillance could provide reliable and timely signals for outbreaks of influenza infections. Another research topic of interest for influenza surveillance is probably how to build a real-time model in order to determine an appropriate threshold in posting an alarm to the public. In line with our results, a recent study in Hong Kong, which used the same clinical surveillance data as this study, demonstrated that the short-term models based on ILI consultation rates in GP setting could provide timely alerts within the two weeks of influenza epidemics, by calculating the specificity and sensitivity of the short-term models with virological surveillance data as the gold standard [8]. We also found that ILI consultation rates in GP increased even several weeks before increase of ILI in GOPC, suggesting that clinical surveillance in GP setting, compared with that in GOPC setting, could probably provide a timelier call for control measures to influenza spread. Using wavelet analysis and phase analysis, we were able to estimate the time variation of lag between oscillation of virus activity and ILI consultation rates. On average increases of influenza virus activity was estimated to lag behind increases of ILI consultation rates in GP approximately by 4 weeks, and behind ILI consultation rates in GOPC by 2 weeks. The estimated phase difference between ILI consultation rates in GP setting and virological surveillance was rather constant during the study period. ILI consultation rates in GOPC appeared out of phase with virus activity at the beginning and end of the study period, very likely due to the edge effects. Interestingly, we also found that the lag between virus activity and ILI consultation rates was longer in the years dominated with annual cycles. We postulate that in the years with annual influenza seasonality, the dominant influenza strains tends to have low virulence and these viruses need a longer time to fully spread in the community. Besides its application in modeling oscillation of clinical and virological surveillance data, wavelet analysis could be used to develop a prediction model for the influenza outbreaks with adjustment for the mildly changing coherence.

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Towards Ontology-Driven Influenza Surveillance From Web Rumours

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Surveillance of infectious disease rumours on the Web remains a potentially valuable source of information to public health workers. We argue that computer systems that perform this task require high-quality knowledge sources including a taxonomy of structured concepts, variant terms, including laymen's language, with equivalence relations across languages. The objective of the BioCaster ontology is to help fulfill this role for major languages in the Asia-Pacific region. In this paper we present a summary of the first version of the ontology and briefly describe features which make it useful for influenza surveillance.

Introduction

The recent H5N1 avian influenza epidemic has highlighted the need for improved surveillance to minimize the effect of future pandemics. With timeliness, coverage and the high cost of traditional information infrastructure a concern in the Asia-Pacific region, the ability to exploit unsubstantiated Web news is emerging as a new modality for early detection of disease outbreaks. Several systems have already been deployed including GPHIN [1] and MiTaP [2]. However substantial challenges remain including the massive size of the Web, its multilingual nature and the uncontrolled proliferation of terms. We argue that without knowledge intensive methods search times for Web news will overwhelm scarce expert resources. To support the development of intelligent systems we present a multilingual ontology focused on the needs of infectious disease surveillance with vocabulary in six Asia-Pacific languages (English, Chinese, Japanese, Korean, Thai and Vietnamese) and briefly discuss its application to influenza surveillance. The BioCaster Ontology (BCO) serves the needs of the infectious disease surveillance community by bridging the gap between the uncontrolled use of terminology, including laymen's terms, in online news and the need for a computable semantics in surveillance systems. The ontology therefore seeks to serve the dual purpose of enabling advanced search on newswires by experts using their own vocabulary and also automated understanding and alerting of events reported in online news. Our domain of interest is basically a subset of biomedicine that is focused on mediating the integration of textual content in various languages. Textual content in biomedicine, especially in news reports, exhibits considerable variability which needs to be systematized. A plethora of major nomenclatures and classification systems already exist that we

can draw on including SNOMED CT [3], the Unified Medical Language System [4] and ICD10 [5] as well as lexical ontologies such as EuroWordNet [6] each with varying degrees of rigor, coverage and accessibility. Most of these are mono-lingual domain ontologies with a scope far broader and deeper than the application ontology we have in mind for BCO. Few such resources though exist for Asia-Pacific languages, exemplifying the need for high quality cross-language resources to support biomedical applications.

Materials and Methods

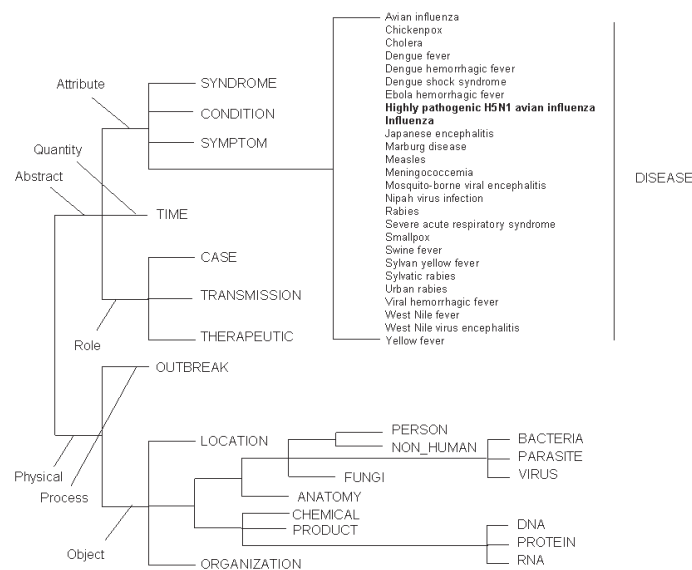
Discussions with epidemiologists and analysis of WHO consultation reports (e.g. [7]) revealed several scenarios for information surveillance including: the moment of transition from animal-to-human transmission and sustained human-to-human transmission, the spread of a virulent pathogen across international borders and the deliberate release of a virulent pathogen into the population. For the top level of our ontology various extant ontologies were considered and SUMO (the IEEE Suggested Upper Merged Ontology) [7] was chosen. This essentially covers non-lexicalized domain independent classes such as attribute, role or process and should enable interoperability across other ontologies. We then surveyed a collection of 1000 news articles and identified eighteen target entity classes of terms including virus, bacteria, disease, syndrome, symptom and host. The classes were chosen to reflect the granularity of entities that could be found in news articles. In practice the survey of the inventory for entity types is often not trivial because ad-hoc concept classes incorporate a mixture of substance and role viewpoints for example the class of people and cases. We were helped in our analysis by adapting the formal method suggested by Guarino and Welty [8] and detailed in [9]. Key relations were also identified between target classes such as host-pathogen, disease-symptom and pathogen-transmission. A list of high priority pathogens was then formed from a survey of notifiable diseases in the region by a geneticist supported by an epidemiologist and a computational linguist. Pathogens included the H5N1 subtype of influenza A virus. Following this groundwork, workflow to fill in the domain terms then generally followed the procedure set out in the EuroWordNet project [6]. An ontology fragment was first identified that was based on the first 27 pathogens in our high priority list. We then harvested terms from news articles using named entity recognition [10] before validating and defining them. Among 28140 English documents processed between April 2006 and early July 2006, we harvested 1695 unique entities for diseases, 399 for symptoms, 37825 for locations, and 262 for anatomy. In order to bootstrap the ontology development we looked for term pairs in known associative relations using mutual information [2] as the measure. Once the English term set was established for the fragment we proceeded to describe term equivalence across the six languages. This work was undertaken by linguists fluent in each of the languages.

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Results and Discussion

In the first release version of the BCO we have described terminology in six languages for 27 pathogens and the diseases they cause, 108 symptoms, 10 routes of transmission, and 6 syndromes of those diseases. In total the synonyms for each language consist of 479 English terms, 274 Japanese terms, 296 Korean terms, 283 Chinese terms, 361 Thai terms and 265 Vietnamese terms. Links were made to external ontologies and nomenclature such as ICD10, LOINC, MedDRA, MeSH and SNOMED CD. The resulting ontology has given us a compact structuring focused on one domain and application. As can be seen from the structural framework given in Fig. 1 the class of diseases includes influenza and its daughter Highly pathogenic H5N1 avian influenza. Within the ontology the two are related by a narrower term relation and each has a set of synonymous terms in the six languages. A single term was also selected as the preferred term for each concept. This is useful for systems that need to unify output so that users do not need to be concerned with variations. In our work so far we have presented a taxonomy of objects which aims to meet the need of a computable semantics for disease outbreak surveillance from news. In future work we expect to keep expanding the ontology and term banks year by year. We are now focusing on the design of a hierarchy of events related to disease outbreaks. The major challenges here though include considerations of event granularity, event inclusion (e.g. a single case and a group case) and temporality. (BCO is freely available to browse and download at <http://biocaster.nii.ac.jp>).

Figure 1. Structural overview of the BioCaster Ontology. Entity classes are shown capitalized.



Acknowledgements

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Seasonal Burden of Influenza-Like Illness (ILI) in a Community-Based Health Care Facility in Urban Subtropical Brazil

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Background

Influenzavirus (Flu) epidemics are associated with high morbidity and mortality, mainly in high-risk groups. Children younger than five may experience particularly heavy burden of Flu-related illness and a major proportion of the total medical visits and hospitalizations associated with seasonal influenza occur in children. In addition, children are considered to play a pivotal role as sources of household spreading of virus during outbreaks. This is reflected by the increase in work absenteeism reported by adults who have children in their households. Importantly, an overall increase in medical visits has been reported during Flu season, when there is also an increase in the frequency of acute otitis media (AOM) and related prescription of antibiotics. Detailed information on the actual impact of Flu in the different age groups has not been systematically collected by prospective surveillance in Brazil. Therefore, we attempted to assess the proportion of medical visits related to influenza-like illness (ILI) during eighteen months in a community-based primary health care facility in the city of Ribeirao Preto, state of Sao Paulo, southeast Brazil.

Methods

All medical visits, ILI-related or not, recorded from May 2006 through June 2007 were reviewed. Each and every week samples collected from patients with ILI were submitted to the virology laboratory for Flu detection by RT-PCR. The data recorded on medical visits and general attendance to the health care facility was analyzed in relation to Flu activity. Circulation of Flu was defined as the period of time between the first and last time that the virus was documented in clinical samples by the virology laboratory. ILI was defined as any respiratory symptoms associated with fever. The monthly frequencies AOM and pneumonia occurring in the same health care facility in the same period were also recorded.

Results

From January/2006 to June/2007 a total of 256,016 medical visits to that health care facility were recorded, and 43,254 (16.8%) of them were classified as ILI. The monthly number of total medical visits ranged from 7,363 to 10,641, and that of ILI-related medical visits ranged from 641 to 2037, for average 1159 ILI-related visits per month. Laboratory data determined that Flu season was May through July, both in 2006 and 2007. During Flu season the number of overall medical visits per month increased in all age groups, mainly in children younger

than 5 years of age (average plus 2 standard deviation - SD) and in those between 5 and 14 years of age (average plus 1 SD). ILI-related visits also increased remarkably during Flu activity, especially among adults who showed an overall increase in ILI-related visits of more than 1 SD and total medical visits increased by up to 2 SD above average (Figure 1). The lowest rates of total and ILI-related medical visits happened in people over 60. In May of 2006, the first month of influenza circulation, the number of ILI related visits was higher in children 0 to 14 years of age (1,913 visits) as compared to that among people 15 to 59 years of age (692 visits). In the next month (June of 2006), the number of ILI related visits increased for persons 15 to 59 years of age for a total of 1,027 visits and decreased to a total of 1,698 in children 0 to 14 years of age. In July of 2006, the third month of the epidemic, the total ILI related visits decreased to 767 and 522 visits in patients 0 to 14 and 15 to 59 years of age, respectively. This pattern was also observed in 2007, yet less pronounced than in 2006. Children 0 to 4 years of age had high rates of ILI-related visits around the year, yet with an increase during Flu activity. On the other hand the highest monthly numbers of medical visits occurred in individuals 25 to 59 years of age (Figure 1). Monthly numbers of AOM related visits ranged from 77 to 336 episodes per month, with peak during the months of influenza circulation (Figure 2). Visits for AOM occurred more in children less than 4 years of age (1924 episodes in the period January-December 2006). Pneumonia occurred in all ages, for a total of 2723 episodes. However, it was more frequent in children 0 to 4 years of age: 791 episodes from January to October 2006, ranging from 22 to 153 per month. The peak occurrence of pneumonia occurred during months of Flu activity. In the period from January to June of 2007, the total number of pneumonia episodes was 1708, being higher in people 20 to 59 years of age (503 episodes) and in children 0 to 4 years of age (441 episodes).

Conclusions

Flu seasonal outbreak in Ribeirao Preto was associated with an increase in the numbers of medical visits, both in general and ILI-related. This increase happens mainly in children younger than 14 years. Lower numbers of ILI-related visits among the elderly may be due to influenza vaccination targeted to this age group. Influenza circulation was also temporally associated with increased numbers of AOM and pneumonia episodes in children less than 4 years of age. The pattern of age-related occurrence of pneumonia was different in epidemics of 2 consecutive years, and in 2007 it was more frequent in adults aged 25 to 59 years. Vaccination of children younger than 2 years, as well as school-aged children, will very likely reduce the numbers of ILI-related and total medical visits in the region.

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Figure 1. Monthly frequencies of ILI-related medical visits distributed by patient age ranges, from January 2006 through June 2007.

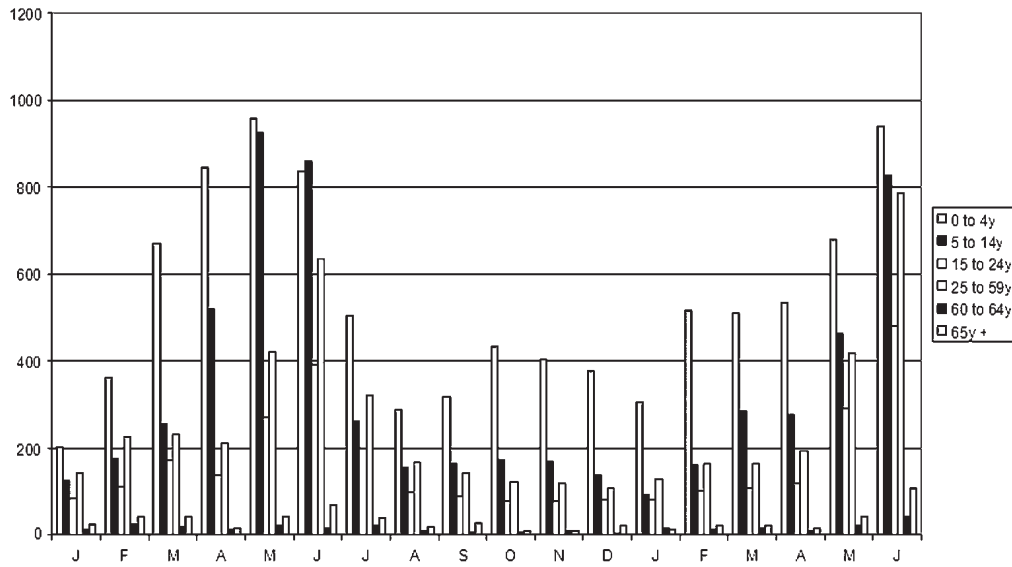
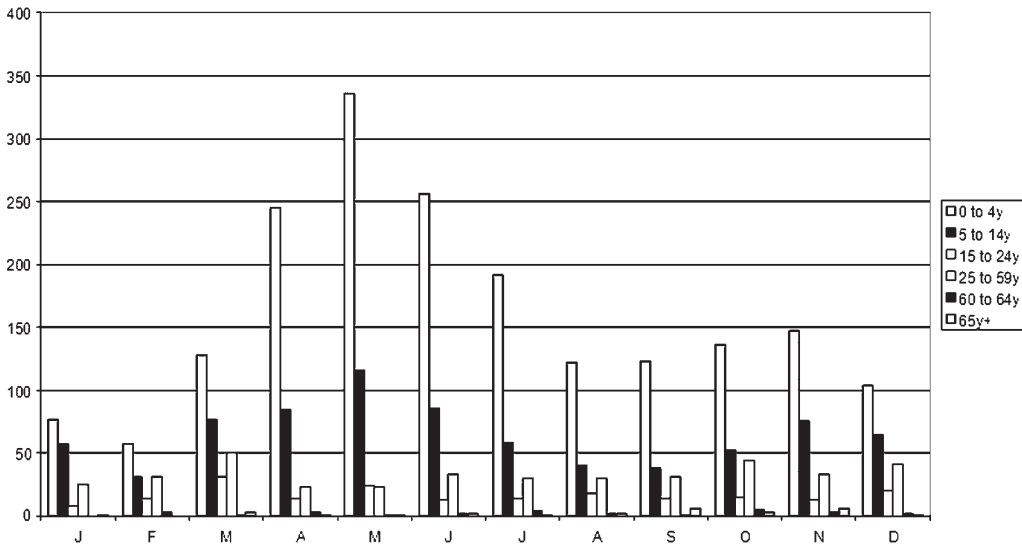


Figure 2. Monthly frequencies of OMA distributed by patient age ranges, from January through December 2006.



Associations Between the Timing of Influenza Seasons in Europe and Weather: A Pilot Study Using European Surveillance Data

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Introduction

Epidemic influenza occurs almost exclusively in winter and very early spring in temperate zones such as Europe. Although it is clear there are climatic influences for such seasonal variations in the timing of influenza occurrence, the actual factors behind them are not known. In Japan simple correlation of influenza reports and temperature and humidity suggested the number of medically diagnosed influenza or influenza-like illnesses in children fell with the number of warm days as well with the number of days with high (>60%) relative humidity¹. More recently there has been a suggestion using time series modelling of influenza reports in England of a tendency for weeks with warmer weather and high windspeed to protect against the flu season occurring then (Wilkinson et al, submitted). Over the last few years its been noted in European Influenza Surveillance System (EISS) data that the peak of influenza activity often starts first in the west and then moves east² (Paget J et al. Options for the Control of Influenza Conference 2007). We conducted a pilot study using new data from European country-scale weekly surveillance information to see whether the occurrence of influenza is influenced by weather. The modelling approach used incorporates elements of the "time series regression" traditional found fruitful in air pollution and weather-health studies³.

Methods

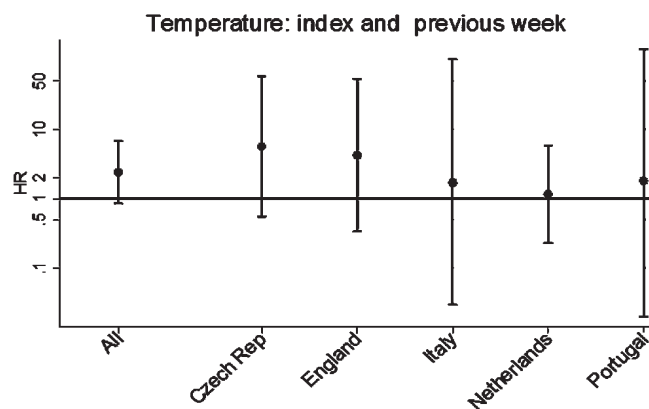
EISS has been collating and integrating clinical and virological data from a number of European countries since 1996. The number of countries have increased from 9 to 32 over this time. The countries included were those where several seasons were available and weather data were easy to hand. The methods followed the approach of Wilkinson (submitted) in seeking to identify weather conditions predictive of a peak, after discounting the average seasonal pattern. Peak weeks were defined for each winter season in each country for each year as those where the maximum of virologically confirmed cases (sentinel and non sentinel laboratory reports sent to EISS) were more than four times the median rate. If the maximum was below this no peak was considered to have occurred, and that season was excluded from analyses. To analyse if peaks are predicted by weather and to estimate the strength of any such association the occurrence of peaks (y-variable) of preceding temperature, humidity and windspeed in the

preceding two weeks (x-variables) were analyzed using Cox's Proportional Hazards model. To allow for the possibility of slower effect of weather we repeated the analysis using the preceding four rather than two weeks of weather. The model was fitted using all country data combined (but stratifying by country) and separately for each country. The association between preceding weather conditions and the occurrence of a peak was summarised as the hazard ratio associated with the average (across countries) of derived 10th-90th centile increase in the weather variable in question, calculated after adjusting for season (by stratifying for week in the season). This scale choice affects the size of the hazard ratios, but not their statistical significance.

Results

Information on weekly virologically confirmed cases was available for approximately October-March from six countries, starting in 1996-97 and ending in 2006. Weather data was available for five of these. In total reasonably complete data was available for 38 winter seasons. Two country-years had no peaks leaving 36 seasons for analysis.

Table 1. Hazard ratio of a peak week (of influenza laboratory reports) associated with a change from the 10th to the 90th centile of the distribution of the weather variable.



*Theoretical average of the range between 10th and 90th centile within each country and week stratum.

Before adjustment for normal seasonal patterns, preceding cold weather and low humidity were significantly associated with peaks. However, because temperature strongly follows a regular seasonal pattern, the association with temperature changes radically on adjustment for season, to a suggestive association of HIGH temperature in the previous two weeks with the occurrence of peaks (HR 2.41 95% CI 0.85,6.80). The hazard ratios for humidity changed less on adjusting for season; they lost statistical significance, though the association with humidity in the last four weeks was borderline (0.34 (95%

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0.11,1.08). Adjusted and unadjusted analyses showed high windspeed associated with peaks, but these associations never approached statistical significance.

Figure 1.

Determinant	10th%ile	90th%ile	unit*	HR unadj(95% CI)	HR adjust(95%CI)	p-value
<i>Av week 0-1 week prior</i>						
Temp (°F)	33.0	56.2	10.2	0.65(0.43,0.97)	2.41 (0.85,6.80)	0.09
Rel humidity (%)	76.0	91.0	13.5	0.45(0.25,0.82)	0.51 (0.20,1.35)	0.16
Wind speed (Knots)	2.3	8.9	4.4	1.38(0.77,2.47)	1.94 (0.75,5.01)	0.16
<i>Av week 0-3 weeks prior</i>						
Temp (°F)	34.8	57.2	7.8	0.58(0.42,0.81)	1.32 (0.52,3.32)	0.55
Rel humidity (%)	76.2	90.2	11.3	0.53(0.30,0.95)	0.34 (0.11,1.08)	0.06
Windspeed (Knots)	2.3	8.6	3.5	1.32(0.78,2.22)	1.21 (0.48,3.00)	0.68

Country-specific hazard ratios were too imprecisely estimated to allow meaningful interpretation (Figure). Discussion: This preliminary analysis suggests that it is feasible to examine the timing of the peaks of seasonal influenza in relation to weather. By concentrating on the timing of annual peaks we avoided having to allow for intra-year variations in the numbers of susceptible (ie non-immune) persons in the population, which could distort direct modelling of weekly occurrence data. Apart from avoiding complex modelling, this also avoids questionable assumptions on the proportion of total cases included in data series – on which data are sparse. The findings themselves are limited by low power. Despite this they, rather surprisingly, do not support an association with preceding cold weather but there is an intriguing suggestion high relative humidity may be a protective factor consistent with the study in Japan. This approach is novel, using data not previously used for this purpose. It extends the analysis in the UK by examining variations over a wider geographical and climatic conditions. Inclusion of more countries as well as methods to deal with heterogeneity of weather at the sub-country level should yield greater statistical precision.

Acknowledgements

The authors would like to acknowledge the following persons for providing the national influenza surveillance data: Czech Republic: Jan Kyncl and Martina Havlickova (National Institute of Health, Prague) England: John Watson and Maria Zambon (Health Protection Agency, London) and Douglas Fleming (RCGP Birmingham Research Unit, Birmingham) Italy: Fabrizio Pregliasco (University of Milan, Milan), Isabella Donatelli (Istituto Superiore di Sanita, Rome) and Filippo Ansaldi (University of Genova, Genova) The Netherlands: Ge Donker (NIVEL, Utrecht), Berry Wilbrink and Marianne van der Sande (RIVM, Bilthoven) and Jan de Jong (Erasmus University, Rotterdam) Portugal: Isabel Marinho Falcao, Jose Marinho Falcao and Helena Rebelo de Andrade (Instituto Nacional de Saude)

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Influenza Virus Surveillance Efforts by the US Department of Defense (DoD): A Unique "System of Systems"

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The US DoD-GEIS Influenza Surveillance System has three major components consisting of: 1) sentinel surveillance among military and dependent personnel in the US and overseas; 2) population-based surveillance among US military recruits undergoing initial entry training, shipboard personnel deployed to geographically-dispersed regions of the world, and patient groups of Mexican-Americans along the border with Mexico; and, 3) international surveillance of local (host nation) populations in five geographically dispersed regions outside the United States. The US Air Force ran the only DoD influenza surveillance program (known as "Project Gargle") from 1976 into the late 1990's. The present global influenza surveillance system was initiated in earnest in 1996-1997 and has grown tremendously in the past year.

Overview of DoD-GEIS Network

Our surveillance system is laboratory-based, consisting of a rather large network of military and civilian health facilities, research laboratories and field sites accruing specimens from over 275 participating sites in 56 countries around the world (Figure 1). Depending on the geographic location of collection, nasopharyngeal wash or swab specimens and associated epidemiologic information are sent either to the US Air Force's reference laboratory in Brooks City Base, Texas or to the US Navy's Respiratory Disease laboratory in San Diego, California. Influenza isolate subtyping and genetic sequencing data are also provided to the US Centers for Disease Control and Prevention (CDC) and represents a significant contribution to the World Health Organization (WHO)-led efforts in influenza vaccine development. Enhancements to the DoD's surveillance and response capabilities have taken place within the past year. Additional field and laboratory-based surge capacity is being expanded in 2007-08. The strategic location of high-level, biosafety level 3 (BSL-3) laboratories around the world (a total of 8 anticipated by end of 2008) provides the DoD with a unique capability which is leveraged by our global partners, especially in avian influenza enzootic regions. This unique "System of Systems" approach plays a key role in facilitating real-time influenza strain monitoring as well as laboratory training and pandemic influenza (PI) preparedness and response activities throughout the world. The contributions by DoD-GEIS have had a large impact on the selection of new vaccine strains for Northern and Southern Hemisphere

human seasonal influenza vaccines and will also continue to contribute to the future development of PI vaccines. Air Force Institute for Operational Health Activities: The Air Force's laboratory-based respiratory disease surveillance, managed by the Air Force Institute for Operational Health (AFIOH) in Brooks City-Base, Texas, began as a program known as "Project Gargle" back in 1976. With the advent of DoD-GEIS as a coordinating entity as well as the formation of the US Navy's (NHRC's) respiratory disease laboratory, AFIOH's surveillance efforts were complemented in the late 1990's and it subsequently became a WHO Collaborating Laboratory in 2003 (Figure 2). For specimen procurement, the patient must meet the Influenza-like Illness (ILI) case definition: fever (oral temperature) of $\geq 38^{\circ}\text{C}$; and cough or sore throat of <72 hours duration. A completed Influenza Surveillance questionnaire that accompanies the sample greatly adds to data analysis and interpretation. AFIOH's contribution to the DoD influenza surveillance program has increased significantly from 1997, when a total of 1,662 respiratory specimens from 19 sentinel sites were processed, to Fiscal Year 2006 (FY06) when a total of 4,303 specimens were collected from 65 sentinel sites, 63 non-sentinel sites, and 3 of the 5 overseas DoD laboratories (Kenya, Thailand, and Peru). Also in FY06-07, the US Army Center for Health Promotion and Preventive Medicine (CHPPM)-West at Fort Lewis, Washington, began obtaining specimens from rural Ministry of Health-based surveillance clinics in four countries of Central America, whereas the US Navy's reference lab in Lima, Peru expanded their sampling to 3 countries in Central America and 7 in South America. In addition, the US Army's Landstuhl Regional Medical Center in Germany was designated the forward reference laboratory in Europe to detect ILI throughout the region. Specimens positive for influenza are subsequently forwarded to AFIOH for further characterization. Recent annual respiratory sample submission workload has reached upwards of 5,000 specimens. In addition to Influenza A and B, specimens are also tested for other respiratory diseases. Of the total specimens processed, roughly 15% have been positive for Influenza A and 5% have been positive for Influenza B virus. All specimens positive for influenza are typed and a portion (all overseas isolates and isolates of interest from remaining sites) are subtyped. Select specimens are also sent to CDC in Atlanta, Georgia. Since 1998, CDC has received over 900 influenza isolates or original specimens. Annually, influenza surveillance data are provided to both the US Food and Drug Administration, Vaccines and Related Biological Products Advisory Committee (VRBPAC), and WHO to assist in the strain determination of the annual influenza vaccine. Since the year 2000, AFIOH has contributed 1 influenza isolate to the seasonal influenza vaccine and strongly influenced 3 other vaccine component decisions: The seed virus for the Influenza A/Panama/H3N2 component of the 2000-2001 influenza vaccine for the Northern Hemisphere was provided by AFIOH. This strain was used through the 2004 season; The first A/New Caledonia/H1N1 isolate outside of New Caledonia was first detected among Peruvian Naval cadets in 1999 and

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led to the addition of this strain in the vaccine from 2000 to the present; Identification of an A/California/H3N2-like strain from Nepal in 2004 was instrumental in the determination that this strain should be a component of the 2005-2006 vaccine; and, Identification of B/Malaysia-like strains obtained from both Nepal and Arizona in 2005 was instrumental in the determination that this strain should be a component of the 2006-2007 vaccine.

Naval Health Research Center Activities

The US Navy's adenovirus surveillance program was expanded in 1996 to include febrile respiratory illness (FRI) and is managed by the Naval Health Research Center (NHRC) located in San Diego, California; it forms the basis for population-based influenza surveillance within DoD. The NHRC is a participating laboratory of the CDC Laboratory Response Network (LRN) and is College of American Pathologists (CAP) certified. This year the NHRC has also added a separate BSL-3 (Ag) laboratory capability to be able to handle highly pathogenic avian influenza virus and other high-level containment respiratory pathogens. The populations monitored for FRI include 8 military training centers (representing all service branches) throughout the US; at least 21 deployed ships; civilian populations at 2 US clinics located on the border of California and Mexico (San Ysidro and Calexico counties); select deployments; and, outbreaks of concern within active duty populations. Specimens are obtained if the patient meets the case definition of FRI: fever (oral temperature) of $\geq 38^{\circ}\text{C}$, and a respiratory symptom (e.g. sore throat or cough). Samples from military populations that are highly vaccinated against influenza (such as military recruits) are particularly important to identify coverage of the current seasonal influenza vaccine against wild circulating strains. Annual vaccine effectiveness calculations are performed through data collected via this surveillance. Influenza (and other respiratory pathogen) specific infection rates are monitored on a weekly basis at the 8 military training centers (Figure 5). The annual respiratory surveillance submission workload is roughly 3,000 specimens per year – a majority of the samples are positive for adenovirus; Influenza A and B are confirmed less often (3% and 1%, respectively). All specimens that are positive for Influenza A or B are sequenced; genetic sequence data and influenza isolates are shared with the CDC. Recently, NHRC diagnostics included the addition of Triangulation Identification for the Genetic Evaluation of Risk (T-5000) platform – one of only 4 operational units in the world (remaining ones are at CDC, Johns Hopkins University, and the US Army Medical Research Institute for Infectious Diseases). This equipment is a high-throughput broad spectrum platform that can screen for at least 20 respiratory agents to include influenza, adenovirus, and group A streptococcus, among others, and is ideally suited for pathogen discovery efforts. The T-5000 uses polymerase chain reaction (PCR) along with mass spectrometry and can process 200 to 300 samples per day.

Overseas Research Laboratory Activities

The 5 overseas DoD laboratories are the US Naval Medical Research Unit (NAMRU)-2 in Jakarta, Indonesia; NAMRU-3 in Cairo, Egypt; US Naval Medical Research Center Detachment (NMRCD-Lima) in Lima, Peru; Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand; and, the US Army Medical Research Unit–Kenya (USAMRU-K) in Nairobi, Kenya. Recent key activities of the labs are discussed below. AFRIMS has sentinel influenza surveillance sites established in Nepal, Thailand, and the Philippines and at regional US Embassies that report from 11 countries in the region. They also have reporting from Thai civilian hospitals in 18 key provinces and 6 Royal Thai Army hospitals along border areas of Burma, Laos, Cambodia and Malaysia. Key activities for NAMRU-2 in 2006-07 included assistance to the local National Institute of Health for Research and Development (NIHRD) in the confirmation and referral of specimens from any suspected H5N1-infected patients and animals in Indonesia; assisting with migratory and domestic bird surveillance efforts; collaborating on a pediatric and influenza-like illness study; and, collaborating with the National Centre for Laboratory and Epidemiology in Vientiane, Laos, on the expansion of syndromic surveillance. Kenya is the largest sub-Saharan country in Africa with ongoing human influenza surveillance at 8 different sites. USAMRU-K collaborates with the CDC's International Emerging Infections Program and Kenya's Medical Research Institute (KEMRI) in the referral of specimens to the National Influenza Center, located at the Kenyatta Hospital in Nairobi. NAMRU-3 in Egypt constitutes the WHO's regional reference laboratory for the Eastern Mediterranean (EMRO) and was also recently designated as the 9th WHO H5 Reference Laboratory. As the only laboratory with full capability for H5N1 virus testing in the region, the lab provides both human and animal influenza diagnostic support to at least 19 countries in Africa, Eastern Europe, the Central Asian Republics and the Middle East. NMRCD-Lima assists with syndromic and diagnostic surveillance reporting in the Peruvian Navy and Army. In addition, plans this year are to expand such surveillance to collaborating regional militaries and Ministries of Health in countries to include Costa Rica, Colombia, Ecuador, Bolivia, Paraguay, Uruguay and the Dominican Republic. Conclusions: Influenza surveillance within DoD is essential to preserve readiness, enhance force protection, and support national and international efforts to detect new influenza viruses and to prevent or control epidemic and pandemic influenza. Currently, pandemic influenza is a significant public health concern, not only for the US military, but for all populations globally. The DoD influenza surveillance network is important in that it covers a large geographic area. Of particular importance to the network are those locations where other organizations do not have a presence; isolates identified to-date have been critical to the effectiveness of the annual influenza vaccine used in the US. DoD laboratory diagnostic capabilities, both domestically and internationally, will continue to monitor for the emergence of new infectious diseases, including pandemic

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strains of influenza virus. This DoD network will continue to play a significant role in global influenza surveillance efforts.

Figure 1. US Department of Defense Global Emerging Infections Surveillance and Response System, Influenza Surveillance Worldwide, June 2007.

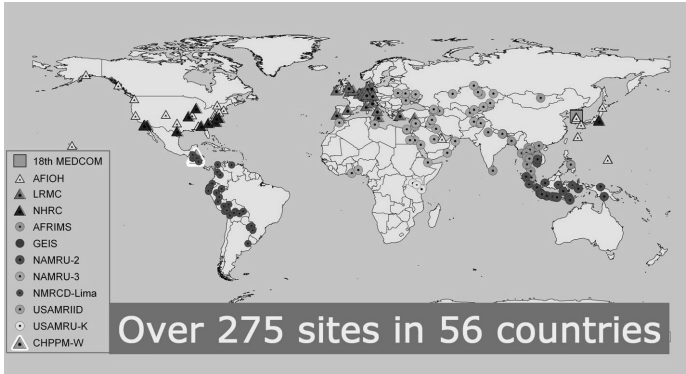


Figure 2. AFIOH Sentinel Surveillance Sites.

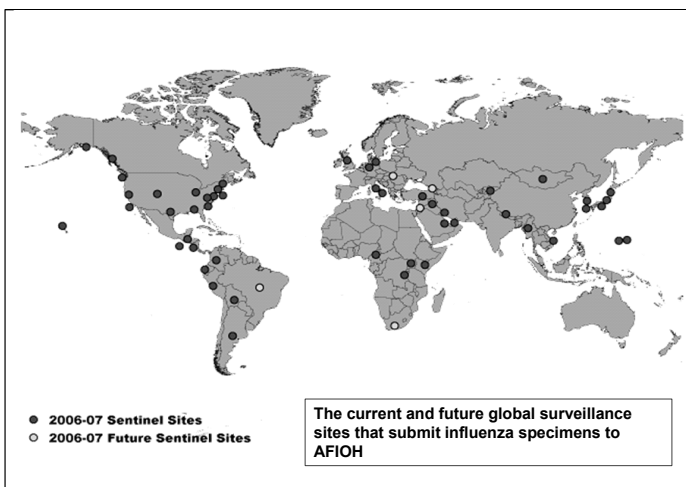
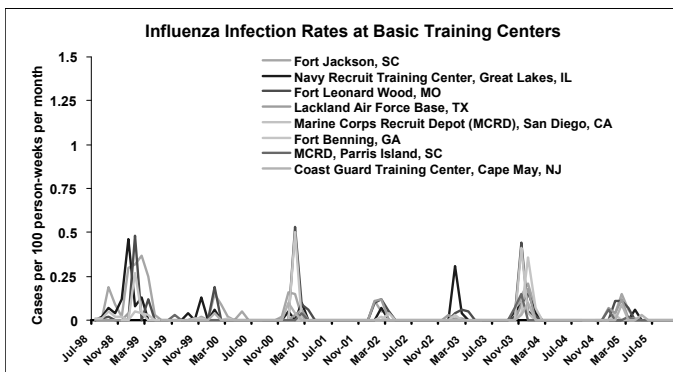


Figure 5. Influenza Infection Rates at Basic Training Centers in the US.



Enhanced Support to Countries Impact on Participation in the WHO Global Influenza Surveillance Network

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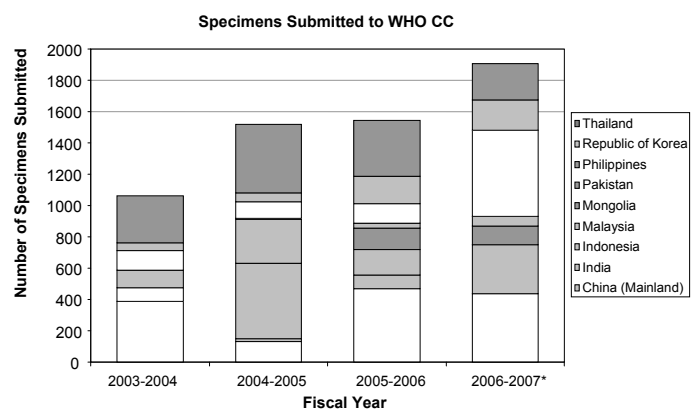
Introduction

Seasonal influenza is estimated to contribute between 250,000 and 500,000 excess deaths annually around the world. Influenza vaccines are our most effective control measure for influenza. Constant genetic and antigenic changes of influenza viruses required the composition of influenza vaccines too be adjusted bi-annually for the northern and southern hemisphere seasons. One hundred-eighteen National Influenza Centers (NICs) in 89 countries and other influenza laboratories in countries without NICs contribute isolates to the World Health Organization Collaborating Centers (WHO CCs) located in Melbourne, Australia; Tokyo, Japan; London, United Kingdom; and Atlanta, USA. Based on characterization of isolates provided to the Collaborating Centers and other relevant data, WHO recommends vaccine strains that represent most recent antigenic variants of influenza A(H1N1), A(H3N2), and B viruses circulating in humans. To enhance the WHO Global Influenza Surveillance Network (GISN) and to support countries and regions experiencing avian influenza outbreaks in their efforts to control potential pandemic influenza, the U.S. Centers for Disease Control and Prevention (CDC) began providing funds to countries through direct cooperative agreements in 2004. The purpose of these cooperative agreements was to assist countries in developing or improving their epidemiologic and virologic influenza surveillance networks. Funded countries were required to have an active National Influenza Center (NIC) recognized by WHO as a member of WHO GISN. The objectives of the cooperative agreements were as follows: 1) Establish or enhance an active influenza surveillance network that uses standardized data collection instruments, operational definitions, and laboratory diagnostic tests to enhance surveillance for influenza at three or more sites within the country; 2) Use the experience gained to expand the surveillance system to include additional sites; 3) Improve laboratory diagnostic capabilities by supporting and enhancing those local laboratories that participate in influenza surveillance; 4) Develop educational and training opportunities for local public health practitioners as part of broader efforts to improve public health infrastructure in the region; and 5) Improve communications and data exchange between laboratories and epidemiologists by expanding the network and improving the reporting of data from surveillance sites, laboratories, NICs and

WHO. Nine countries were funded in 2004—China (Mainland), India, Indonesia, Malaysia, Mongolia, Pakistan, Philippines, Republic of Korea, and Thailand. Three additional countries or territories were funded in 2005—Kazakhstan, a consortium of islands that contribute to the NIC in New Caledonia, and Viet Nam.

Methods

To examine the trends in participation in the WHO GISN, data were collected from multiple sources: 1) Four WHO CCs, 2) FluNet, 3) WHO Geneva. Data summarizing the total number of seasonal influenza isolates submitted to WHO GISN and frequency of reporting through FluNet from October 1, 2003 – September 30, 2004 served as the baseline for the funded countries, and these data were compared to data, specimens, and isolates submitted from October 1, 2004 – May 15, 2007. Data from October 1, 2006 – May 15, 2007 were annualized to determine an estimate for the fiscal year 2006-07. Data from progress reports of countries funded through CDC cooperative agreements were examined to determine whether the number of sentinel surveillance sites was expanded during the funded period. The reports were also examined to determine if laboratory training was provided or if the country experienced an improvement in laboratory capacity and capability.



Results

Baseline data provided by the WHO CCs revealed 1061 specimens were submitted to the WHO CCs between October 1, 2003 and September 30, 2004, prior to the first nine countries being funded. The same 9 countries submitted 1516 specimens in 2004-05 and 1544 in 2005-06 during the same time period. After the data received for October 1, 2006 – May 15, 2007 were annualized, the initial 9 countries are forecasted to submit 1904 specimens to the WHO CCs. This shows an increase in overall specimen submissions by countries receiving a CDC-funded cooperative agreement. Of the 9 initially funded countries, 6 countries showed an increase in the number of specimens submitted during the three years of the cooperative agreement based on data collected from the WHO CCs. Of the three countries or territories initially funded in 2005, the consortium

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of islands that contribute to the NIC in New Caledonia increased specimen submissions during the two years while receiving monies and technical assistance through a cooperative agreement. In 2003, 6 of the 9 initially funded countries reported the number of specimens processed by NICs through FluNet, as reported by WHO Geneva. During the subsequent three years of funding through the cooperative agreement, 7 countries reported through FluNet. Of the two additional countries funded in 2005, Vietnam began reporting to FluNet in 2006. Of the 9 initially funded countries, 8 countries have increased the total number of sentinel surveillance sites reporting to the WHO GISN. Of the 9 countries, all have received training and have increased laboratory capacity or capability within the country. Of the additional three countries funded in 2005, all have received training and have increased laboratory capacity or capability within the country. In addition, CDC is supporting three long-term avian influenza assignees in three of the 9 originally funded countries as well as 2 from the additionally funded countries.

Discussion

The data show a positive trend in the number of specimens submitted to WHO CCs by the majority of countries funded through the CDC cooperative agreements. With the increased number of specimens submitted, the constant genetic and antigenic changes in influenza viruses can be better monitored to provide evidence for needed changes in the seasonal influenza vaccine. Although multiple factors contribute to a country's ability to contribute specimens, CDC's cooperative agreements are one mechanism helping to enhance the WHO Global Influenza Surveillance Network. Limitations: Annualized

data were calculated for the fiscal year of October 1, 2006 – September 30, 2007 using data collected by WHO CCs from October 1, 2006 – May 15, 2007. Annualized data were compiled for 2007, making these data provisional. Delays in accessing cooperative agreement funds have hindered some countries' project progress.

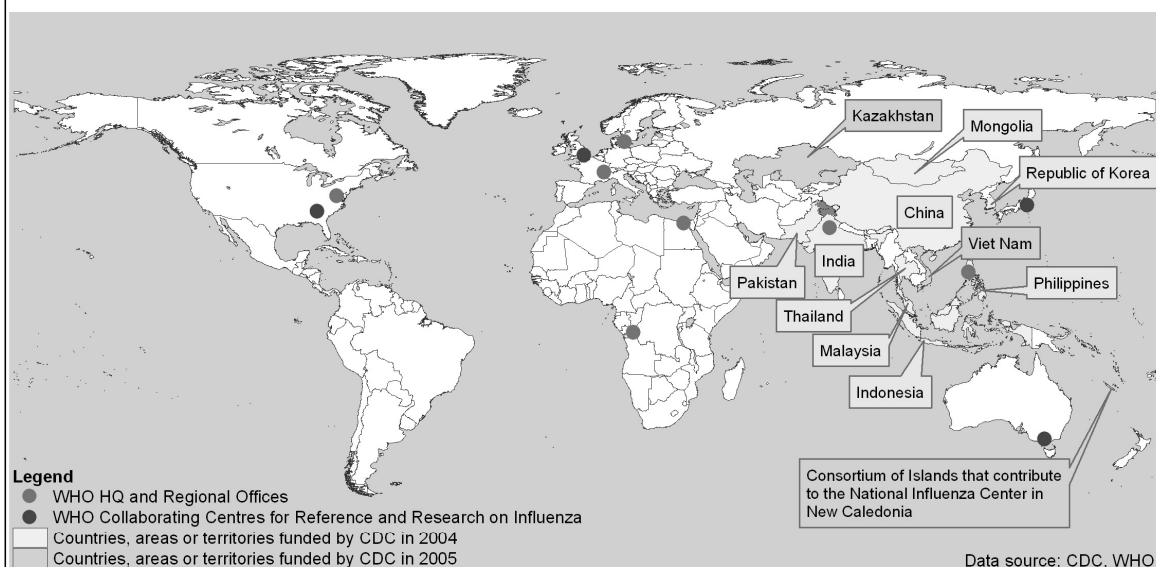
Acknowledgements

The authors thank the influenza programs in China, India, Indonesia, Kazakhstan, Malaysia, Mongolia, a consortium of islands that contribute to the NIC in New Caledonia, Pakistan, Philippines, Republic of Korea, Thailand, and Viet Nam for providing materials necessary for this study. The authors also thank Ian Barr and Ann Kelso, WHO Collaborating Center for Influenza, Melbourne, Australia; Masato Tashiro and Takato Odagiri, WHO Collaborating Center for Influenza, Tokyo, Japan; Vicki Gregory and Alan Hay, WHO Collaborating Center for Influenza, London, United Kingdom; Nancy Cox, WHO Collaborating Center for Influenza, Atlanta, USA; and support from influenza programs at WHO Global Influenza Surveillance Network and WHO Regional Offices for providing data for this poster.

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2. World Health Organization. FluNet Global Influenza Program. Available at: <http://gamapserver.who.int/GlobalAtlas.home.asp>

WHO Offices and Countries, Areas or Territories Funded for Influenza by CDC: 2004-2005



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization and CDC concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

Statistical Analysis of Clinical Manifestations in Influenza-Associated Encephalopathy

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Objectives: To examine the differences in clinical manifestations of influenza-associated encephalopathy by age and virus subtype, and to identify the risk factors for this disease. **Materials and Methods:** Between 1998 and 2002, 585 cases of influenza-associated encephalopathy were reported to the Collaborative Study Group on Influenza-Associated Encephalopathy. To analyze the differences by age, these patients were divided into three groups, <1 year, 1–5 years, and 6–15 years old. Odds ratios and 95% confidence intervals were estimated with reference to data from the 1–5 years group. The differences by virus subtype were evaluated with χ^2 tests. To analyze prognostic factors, the outcome for each patient was classified as survival or death. Predictors of death were identified using a logistic regression analysis. **Results: Differences by age.** Compared to patients aged 1–5 years, those <1 year old had a significantly higher frequency of convulsions as the initial neurological symptom, later onset of neurological signs, lower frequency of hematuria or proteinuria, and higher frequency of hemorrhage, according to brain computed tomography (CT) on admission. Compared to those aged 1–5 years, patients aged 6–15 years had a significantly greater incidence of influenza type B infection, lower frequency of convulsions, higher frequency of loss of consciousness and altered consciousness as the initial neurological symptom, later onset of neurological signs, lower serum transaminase levels, and lower incidence of sequelae. **Differences by virus subtype.** Fewer cases caused by influenza type A H1N1 virus had extremely abnormal findings upon blood examination. The cases caused by influenza type B virus had poorer prognosis than those caused by type A H1N1 virus. **Prognostic factors.** Four significant prognostic factors were detected (multivariate analysis; $P < 0.05$): elevation of aspartate aminotransferase, hyperglycemia, the presence of hematuria or proteinuria, and use of diclofenac sodium. The following variables tended to be related to a poor prognosis according to the multivariate analysis ($0.05 \leq P < 0.20$): hyperthermia, thrombocytopenia, hypoglycemia, abnormal findings on brain CT, and use of mefenamic acid.

Introduction

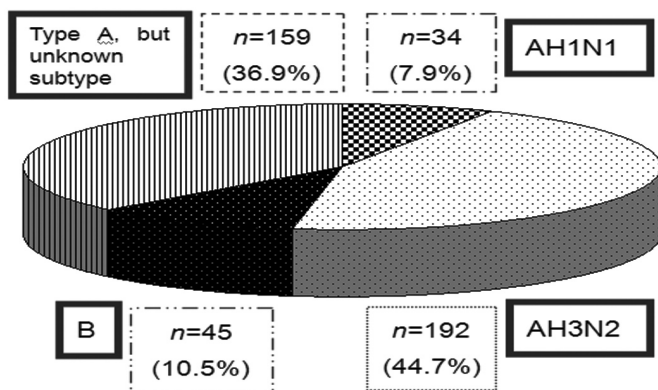
Influenza-associated encephalopathy, now reported worldwide, is common in Japan [1-3]. Although this disease was previously known to exist only in Japan, case reports from other countries have increased [4-9]. Influenza-associated encephalopathy is an abrupt disorder of the nervous system

triggered by influenza virus infection, and often leads to severe sequelae or death. Nationwide data on this disease did not exist before this study, and frontline clinicians are confronted with difficulties in identifying it and determining the course of therapy. Thus, we initiated a national survey in the winter of 1998 to investigate the various parameters of this disease in Japan. Our first comprehensive report, which contained 148 cases of influenza-associated encephalopathy in Japan, was released in 2002 [1]. In this study, which is based on data collected over 4 years, we analyzed differences in the clinical manifestations of influenza-associated encephalopathy by age and virus subtype, and studied the risk factors for this disease.

Materials and Methods

Between 1998 and 2002, 585 cases of influenza-associated encephalopathy were reported to the Collaborative Study Group on Influenza-Associated Encephalopathy, which was organized by the Japanese Ministry of Health, Labor, and Welfare. Among these cases, 472 patients aged 15 years or younger who had been properly documented were examined retrospectively. The diagnosis of encephalopathy was based on clinical signs. All patients had altered consciousness (i.e., delirium, confusion, and senselessness) or loss of consciousness (i.e., deep coma, coma, semicoma, stupor, and somnolence). Patients with meningitis, myelitis, and febrile seizures without prolonged unconsciousness were excluded. Postictal unconsciousness with prompt recovery was classified as febrile convulsion. The diagnosis of influenza infection was based on a positive viral culture, viral antigen test, or viral RNA PCR, or a fourfold or greater rise in paired serum antibody titers (hemagglutination inhibition or complement fixation test). For analyzing differences by age, the patients were divided into three groups, <1 year, 1–5 years, and 6–15 years old, with 28 (5.9%), 354 (75.0%), and 90 (19.1%) patients in the respective groups. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated with reference to the data from the 1–5 years group. For analyzing differences by virus subtype, 271 patients in whom the virus subtype was confirmed were divided into three groups: AH1N1, AH3N2, and B (Fig. 1). The differences were evaluated with χ^2 tests. A multivariate logistic regression analysis was used to identify the most important prognostic factors for death from influenza-associated encephalopathy, including 20 factors about the patient's background, symptoms, complications, laboratory findings, and medication. First, 442 of 472 patients for whom the outcome was confirmed were analyzed by univariate analysis, and then, 184 of these 442 patients for whom we had complete data were analyzed by multivariate analysis.

Figure 1. Virus subtypes distribution.



Results

Differences by age. The number of patients peaked at between 1 and 2 years of age. The youngest patient was 2 months old, although only three were under 6 months of age. The patients aged 6–15 years had the following characteristics compared to the group aged 1–5 years. More cases were caused by influenza type B virus (OR, 2.25; 95% CI, 1.06–4.72). As the initial neurological symptom, more developed loss of consciousness or altered consciousness (OR, 2.17; 95% CI, 1.21–3.90), and fewer had convulsions (OR, 0.36; 95% CI, 0.21–0.64). They had a longer time from fever onset to neurological onset (OR, 2.72; 95% CI, 1.15–6.36) and lower serum transaminase levels on admission (OR, 0.38; 95% CI, 0.19–0.76), and fewer of them had sequelae (OR, 0.49; 95% CI, 0.25–0.96). The patients younger than 1 year old had the following characteristics compared to the group aged 1–5 years. More patients developed convulsions as the initial neurological symptom (OR, 7.67; 95% CI, 1.08–154.53). They had a longer time from fever onset to neurological onset (OR, 4.61; 95% CI, 1.33–15.15). On admission, fewer patients had hematuria or proteinuria (OR, 0.21; 95% CI, 0.03–0.96), but more had hemorrhage as revealed by brain CT (OR, 14.10; 95% CI, 1.33–150.42).

Differences by virus subtype. A total of 430 patients had an identified virus type and outcome, but in 159 cases of type A influenza, the virus subtype was not identified (AH1N1 or AH3N2) (Figure 1). More patients in the 0–6 year age group had influenza caused by the AH3N2 virus than by the other two subtypes (AH1N1, $P=0.002$; B, $P<0.001$). No significant difference was observed in peak body temperature according to virus subtype. Fewer patients with influenza virus B had convulsions as initial neurological symptoms compared to those with virus AH3N2 ($P=0.004$). More patients with influenza virus AH3N2 had an elevation of aspartate transaminase (AST) compared to those with virus AH1N1 ($P=0.021$). More patients with influenza viruses AH3N2 and B had an elevation of creatinine phosphokinase (CK) compared to those with virus AH1N1 (AH3N2, $P=0.010$; B, $P=0.008$). More patients with influenza viruses AH3N2 and B had thrombocytopenia (platelet count $<1.0 \times 10^5/\mu\text{l}$) compared to those with virus AH1N1

(AH3N2, $P=0.007$; B, $P=0.008$). More patients with influenza virus B had elevated levels of NH_3 compared to those with viruses AH1N1 or AH3N2 (AH1N1, $P=0.018$; AH3N2, $P=0.033$). No significant difference was observed in the incidence of hematuria or proteinuria according to virus subtype. Patients with influenza virus B had higher mortality than those with virus AH1N1 ($P=0.037$). The descending order of mortality was B (31.1%), AH3N2 (25.5%), and AH1N1 (11.8%).

Table 1. Prognostic factors.

Significant by multivariate analysis ($P<0.05$)	elevation of AST (≥ 500 IU/L); hyperglycemia (≥ 150 mg/dl); hematuria or proteinuria; use of diclofenac sodium
Nonsignificant but relatively important by multivariate analysis ($0.05 \leq P < 0.20$)	hyperthermia ($\geq 41^\circ\text{C}$); thrombocytopenia ($<1 \times 10^5/\mu\text{l}$); hypoglycemia (<50 mg/dl); abnormal findings of brain CT; use of mefenamic acid
Important on univariate analysis	diarrhea; elevated AST (100–500 IU/L); elevated CK (≥ 1000 IU/L)

AST, aspartate transaminase; CK, creatinine phosphokinase.

Prognostic factors. Four significant prognostic factors were detected (multivariate analysis; $P<0.05$). Moreover, we determined additional, although nonsignificant, prognostic factors (multivariate analysis; $0.05 \leq P < 0.20$) and other important prognostic factors (univariate analysis; Table 1).

Discussion

Patients with influenza-associated encephalopathy often die from multiple organ failure, which is assumed to be caused by mitochondria-mediated apoptosis [10]. Hosoya *et al.* and Nunoi *et al.* reported that cytochrome *c*, a mitochondrial protein found in the intermembrane spaces, is a good marker for evaluating the clinical severity of influenza-associated encephalopathy [11, 12]. Our comparison of laboratory findings showed that patients aged 6–15 years had a lower incidence of liver dysfunction than the group aged 1–5 years. The low magnitude of apoptosis might have caused the low frequency of sequelae in the group aged 6–15 years. Analysis of differences by virus subtype revealed that the AH3N2 virus is more likely than the other types to trigger encephalopathy and have a poor prognosis, and that the cases involving B type virus were the fewest in number, but had the highest mortality with a course similar to Reye's syndrome. An analysis of the prognostic factors revealed that the use of the nonsalicylate antipyretic drug diclofenac to alleviate fever influenced disease prognosis, and the use of mefenamic acid tended to affect the prognosis. The use of acetaminophen was determined to have little effect. In May 2001, the Japanese Ministry of Health, Labor, and Welfare banned the use of these antipyretic drugs to alleviate fever in influenza infection, although it is still unclear whether these drugs are related to the pathogenesis of influenza-associated encephalopathy. Clarifying the differences by age and virus type could be helpful in diagnosing influenza-associated encephalopathy. We can provide intensive care for patients predicted to have severe disease by evaluating the severity

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early on using these prognostic factors.

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Clinical Features of Influenza C Virus Infection and Impact of Antigenicity of Hemagglutinin Esterase Protein

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Influenza C virus is a significant cause of upper-respiratory-tract (URT) illness in children <6 years old. Among influenza C viruses isolated in Japan, five distinct antigenic groups can be recognized. Outbreaks of influenza C virus infection occurred in Yamagata and Miyagi at intervals of almost 2 years between 1996 and 2004, and Yamagata/26/81 antigenic group (YA81-group) circulated from 1996 to 2001 and Kanagawa/1/76 antigenic group (KA76-group) predominated between 2002 and 2004. We revealed the clinical features of influenza C virus infection and evaluated the clinical impact caused by these different antigenic groups. Subjects and Methods: We examined the clinical data on 142 children infected with influenza C virus between 1996 and 2004 in Yamagata and Miyagi, Japan, and compared them between 60 children infected with YA81-group and 71 children infected with KA76-group. Results: Between 1996 and 2004, 51,366 respiratory specimens were obtained from patients ≤ 15 years old, and 161 specimens (0.31%) were positive for influenza C virus by cell culture system. Of 142 children, 130 (91.5%) were <6 years old. Fever (90.1%), cough (75.3%) and rhinorrhea (62.0%) were the most frequent symptoms. Overall, 93 (65.5%) were diagnosed with either a URT illness or "influenza", and 30 (21.1%) were diagnosed as having a lower-respiratory-tract (LRT) illness such as pneumonia, bronchitis or bronchiolitis. The median duration of fever was 2 days. The rate of hospital admission was 18.3% (26/142). Of the 26 hospitalized children, 14 (53.8%) were <2 years old and 18 (69.2%) had LRT illness. The clinical features (age distribution, symptom, rate of hospital admission) caused by YA81-group were similar to those caused by KA76-group, but diagnosis of "influenza" was more common for patients with KA76-group infection. Conclusion: Typical clinical symptom of influenza C virus infection is a common cold-like illness with a fever that persists for 2 days and the risk of complications with LRT illness is high in children <2 years old. The clinical impact caused by different antigenic groups was not shown. Diagnosis of "influenza" increased in 2002 and in 2004, when outbreaks of influenza C virus coexisted with those of influenza A and B viruses.

Introduction

Influenza C virus is a significant cause of upper-respiratory-tract illness in children <6 years old [1]. Serological studies have indicated that more than 80% of humans acquire antibodies to the virus by the age of 7-10 years, which suggests that influenza C virus infection is common in childhood [2].

We have surveilled influenza C virus infections in Yamagata since 1988 and in Miyagi since 1990, and have succeeded in isolating >200 strains of influenza C viruses. Among influenza C viruses isolated in Japan, five distinct antigenic groups can be recognized [3]. In this study, we revealed the epidemiological and clinical features of influenza C virus infection and evaluated the clinical impact caused by these different antigenic groups.

Materials and Methods

Specimens and virus isolation. Between 1996 and 2004, 51,366 respiratory specimens were obtained from symptomatic children ≤15 years old who visited either 1 of 4 pediatric clinics (Katsushima Pediatric Clinic, Nagai Children's Clinic, Shoji Clinic, and Yamanobe Pediatric Clinic) or 1 of 3 hospitals (Yamagata City Hospital Saiseikan, Sendai Medical Center and Tohoku Koseinenkin Hospital). The collected specimens were transported to either the Virus Research Center of Sendai Medical Center or the Yamagata Prefectural Institute of Public Health, for virus isolation. Virus isolation was carried out by means of a microplate methods as previously described [1]. A total of 161 influenza C viruses were isolated from the MDCK cell line.

Antigenic analysis. The 161 influenza C virus strains were examined in hemagglutinin inhibition test for reactivity with four different anti-hemagglutinin esterase (HE) monoclonal antibodies and were divided into four distinct antigenic groups (Yamagata/26/81 [YA]-group, Kanagawa/1/76 [KA]-group, Mississippi/80-group and Sao Paulo/378/82-group).

Clinical data. Clinical data were obtained retrospectively from the medical records. Of the 161 cases, 19 were excluded because of coinfection with another respiratory virus or incomplete medical records; therefore we examined the clinical data on 142 children infected with influenza C virus.

Results

Seasonality. Between 1996 and 2004, 51,366 respiratory specimens were obtained from patients ≤ 15 years old in Yamagata and Miyagi prefectures, and 161 specimens (0.31%) were positive for influenza C virus by cell culture system. Outbreaks of influenza C virus infection occurred at intervals of almost 2 years (Figure 1). Isolation of influenza C virus was successful with specimens obtained during each calendar month but predominantly with those obtained during winter to early summer. Influenza C virus coexisted with epidemics of influenza A and B viruses during the 1999-2000, 2001-2002 and 2003-2004 seasons. Outbreaks of YA81-group occurred in 1996, 1998 and 2000, and KA76-group predominated between 2002 and 2004.

Clinical features. Of the 142 children studied, 130 (91.5%) were <6 years old. A total of 26 (18.3%) of the 142 children were hospitalized, and 14 (53.8%) of the 26 hospitalized children were <2 years old (Table 1). Overall, fever (temperature >38°C) (90.1%), cough (75.3%) and rhinorrhea (62.0%) were the most frequent symptoms. The median duration of fever was 2 days. In the 26 hospitalized children, fever lasted longer and

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tended to have a higher maximum temperature, and other symptoms, including cough, rhinorrhea, wheezing, exanthema, conjunctivitis, cervical lymphadenopathy and convulsion, were observed more frequently. Of 142 children, 93 (65.5%) were diagnosed with either an upper-respiratory-tract (URT) illness or "influenza", and 30 (21.1%) were diagnosed as having a lower-respiratory-tract (LRT) illness such as pneumonia, bronchitis or bronchiolitis. Of the 26 hospitalized children, the most frequent diagnosis was pneumonia (50%) and 18 (69.2%) had LRT illness. We compared the clinical data between 60 children infected with YA81-group and 71 children infected with KA76-group. The clinical features (age distribution, rate of hospital admission, symptom) caused by YA81-group were similar to those caused by KA76-group. Wheezing, exanthema, cervical lymphadenopathy and headache were slightly more frequent in children infected with YA81-group than in those infected with KA76-group. The diagnosis of either a URT illness or "influenza" was present in 39 (65%) of 60 YA81-group-infected children and 47 (66.2%) of 71 KA76-group-infected children, but diagnosis of "influenza" was more common for patients with KA76-group infection.

Table 1. Clinical features of influenza C virus-infected children and comparison of them caused by different antigenic group strains.

variable	Nonhospitalized children (n=116)	Hospitalized children (n=26)	Antigenic group	
			YA81-group (n=60)	KA76-group (n=71)
Male sex	58 (50.0)	13 (50.0)	35 (58.3)	33 (46.5)
Age, mean±SD, years	3.20±2.30	2.19±1.60	2.88±2.20	3.14±2.11
Age distribution				
<2 years	32 (27.7)	14 (53.8)	20 (33.3)	20 (28.2)
2-3 years	36 (31.0)	4 (15.4)	18 (30.0)	20 (28.2)
4-5 years	36 (31.0)	8 (30.8)	19 (31.7)	23 (32.4)
>5 years	12 (10.3)	0	3 (5.0)	8 (11.3)
Maximum temperature, mean ±SD, °C	38.76±0.72	39.12±1.03	38.81±0.87	38.86±0.78
Duration of fever, mean ±SD, days	2.55±1.45	4.28±1.95	2.88±1.85	3.00±1.58
Hospital admission			11 (18.3)	15 (21.1)
Sign and Symptoms				
Fever	106 (91.4)	22 (84.6)	53 (88.3)	64 (90.1)
Cough	82 (70.7)	25 (96.2)	42 (70.0)	56 (78.9)
Rhinorrhea	67 (57.8)	21 (80.8)	31 (51.7)	50 (70.4)
Wheezing	11 (9.5)	6 (23.1)	11 (18.3)	5 (7.0)
Hoarseness	7 (6.0)	2 (7.7)	2 (3.3)	3 (4.2)
Vomiting	17 (14.7)	5 (19.2)	11 (18.3)	11 (15.5)
Diarrhea	12 (10.3)	3 (11.5)	4 (6.7)	7 (9.9)
Abdominal pain	8 (6.9)	2 (7.7)	3 (5.0)	7 (9.9)
Exanthema	7 (6.0)	6 (23.1)	8 (13.3)	4 (5.6)
Conjunctivitis	7 (6.0)	3 (11.5)	4 (6.7)	5 (7.0)
Cervical lymphadenopathy	5 (4.3)	4 (15.4)	6 (10.0)	3 (4.2)
Headache	7 (6.0)	1 (3.8)	5 (8.3)	2 (2.8)
Convulsion	2 (1.7)	2 (7.7)	1 (1.7)	3 (4.2)
Final diagnosis				
Upper-respiratory-tract illness	73 (62.9)	1 (3.8)	37 (61.7)	30 (42.3)
Influenza	19 (16.4)	0	2 (3.3)	17 (23.9)
Bronchitis	9 (7.8)	3 (11.5)	2 (3.3)	8 (11.3)
Pneumonia	2 (1.7)	13 (50.0)	8 (13.3)	7 (9.9)
Bronchiolitis	1 (0.9)	2 (7.7)	1 (1.7)	2 (2.8)
Asthma	2 (1.7)	1 (3.8)	1 (1.7)	2 (2.8)
Croup	5 (4.3)	2 (7.7)	3 (5.0)	2 (2.8)
Sinusitis	3 (2.6)	0	2 (3.3)	1 (1.4)
Otitis media	0	1 (3.8)	0	1 (1.4)
Gastroenteritis	2 (1.7)	0	2 (3.3)	0
Kawasaki disease	0	2 (7.7)	2 (3.3)	0
Henoch-Schoenlein purpura	0	1 (3.8)	0	1 (1.4)

Data are no. (%) of children, unless otherwise indicated.

Infection rate in children younger than 6 years. To compare infection rate of influenza C virus with those of respiratory syncytial virus (RSV) and parainfluenza virus (PIV) infections, we analyzed the samples of children <6 years old who visited

Yamanobe Pediatric Clinic between January and December in 2004. As shown in Table 2, infection rate of influenza C virus was 2.6% of the highest in the children aged 1 year. In contrast with RSV and PIV type 3, infection rate of influenza C virus was low (0.9%) in infants younger than 1 year. Of the 16 children infected with influenza C virus, 4 (25%) were diagnosed as pneumonia, bronchitis, or bronchiolitis. Influenza C virus infection seemed less frequently to involve the lower respiratory tract, compared with RSV infection.

Table 2. Influenza C virus infection rate in children younger than 6 years; in comparison with respiratory syncytial virus (RSV) and parainfluenza virus (PIV) type 3 infections at Yamanobe Pediatric Clinic during January-December 2004.

Age	Total no. of children	FluC		RSV		PIV type 3	
		No. (%) infected	No. (%) with LRD	No. (%) infected	No. (%) with LRD	No. (%) infected	No. (%) with LRD
<1	108	1 (0.9)	1 (0.9)	9 (8.3)	4 (3.7)	7 (6.5)	0
1	230	6 (2.6)	2 (0.9)	21 (9.1)	11 (4.8)	6 (2.6)	1 (0.4)
2	166	3 (1.8)	0	11 (6.6)	9 (5.4)	3 (1.8)	0
3	161	3 (1.9)	0	9 (5.6)	3 (1.9)	7 (4.3)	0
4	103	2 (1.9)	1 (1.0)	5 (4.9)	2 (1.9)	1 (1.0)	0
5	92	1 (1.1)	0	3 (3.3)	1 (1.1)	0	0
Total	860	16 (1.9)	4 (0.5)	58 (6.7)	30 (3.5)	24 (2.8)	1 (0.1)

LRD, lower-respiratory-tract disease

Discussion

The data obtained in the present study have suggested that influenza C virus is also a significant cause of respiratory-tract disease in children <6 years old. Outbreaks of influenza C virus infection occurred at intervals of almost 2 years, and most influenza C viruses were isolated during the winter and spring. Thus, in winter months, influenza C coexists with epidemics of influenza A and B, and different diagnosis therefore would be valuable [4]. Typical clinical symptom of influenza C virus infection is a common cold-like illness with a fever that persists for 2 days. Previously, we compared the clinical symptoms of influenza C virus-infected children versus those of influenza A virus-infected children [1]. As a result, the maximum temperature was slightly higher in the influenza A virus-infected children (mean±SD, 39.22±0.56°C) than in the influenza C virus-infected children (mean±SD, 38.38±0.61°C), and the duration of fever was significantly shorter in the influenza C virus-infected children (mean±SD, 2.13±1.19 days) than in the influenza A virus-infected children (mean±SD, 5.06±2.08 days). In the influenza season, however, it is difficult to differentiate clinically the influenza C virus infection from influenza A and B, since most children infected with influenza C virus had fever (temperature of >38°C). In the present study, diagnosis of "influenza" increased among the children infected with influenza C virus having an antigenicity of KA76-group. Outbreaks of KA76-group strains occurred in 2002 and in 2004, and these outbreaks coexisted with those of influenza A and B viruses. The risk of complications with LRT illness such as pneumonia, bronchitis or bronchiolitis was 21.1% (30/142), but this rate increased 69.2% (18/26) in the hospitalized children. Of the 26 hospitalized children, 14 (53.8%) were <2 years old,

therefore, we concluded that the risk of complications with LRT illness is particularly high in children <2 years old. However, this rate was found to be low compared with that for RSV infection.

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Influenza Activity In Poland In The Epidemic Seasons 2004/2005 –2006/2007 According To Data Collected By The SENTINEL Influenza Surveillance System

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Introduction

Influenza surveillance is essential for obtaining data on virus activity in a given season and population, selection of vaccine strains and warning in the case of epidemic. A significant role of surveillance is highly emphasized, especially since 1997 when the first human infections with avian influenza were confirmed and the risk of pandemic outbreak occurred (1, 2). Until the epidemic season 2004/2005 there were some gaps in influenza surveillance in Poland (3). The most important problem was the lack of integration between epidemiological surveillance and virological surveillance. The latter was not representative nationally or regionally and specimens were collected by only 9 to 12 physicians representing 3 to 5 cities in the central part of the country. Epidemiological influenza surveillance was nationally and regionally representative, because it was and it is still a part of the national surveillance of infectious diseases, including not only influenza. The other problem concerned reporting periods. Since autumn 2000, between October and April data were collected for 7, 8 or 9-days periods (1-7, 8-15, 16-22, 23-30/31 days of a given month). This means that the reporting periods did not always agree with the calendar weeks used in other countries and accepted by the European Influenza Surveillance Scheme (EISS). The last, but not least problem was data collection for the specific age groups. According to the EISS requirements information should be available by four age groups, i.e. 0-4, 5-14, 15-64 and ≥ 65 years. In Poland epidemiological data were collected in two age groups (to 14 years and 15 years). Considering the above gaps the National Influenza Center took some steps to form well working influenza surveillance in Poland based on the SENTINEL system (3). Epidemic season 2004/2005 was the first season of existence of SENTINEL influenza surveillance in Poland. This study presents the efficacy of this system and data collected in the last three epidemic seasons.

Material and Methods

Participants of the SENTINEL influenza surveillance system in Poland are sixteen Voivodship Sanitary-Epidemiological Stations (VSESs), family physicians and the National Influenza Center (NIC) as the coordinator. Physicians register on a weekly basis according to the calendar numbering of weeks number of cases of influenza-like illness (ILI) in the age groups: 0-4, 5-14, 15-64 and ≥ 65 years and collect specimens (usually nasal and throat swabs) from patients suspected to

be infected with influenza. They send epidemiological data and specimens to an appropriate VSES, where virological tests are performed. Depending on the laboratory capacity, VSESs isolate influenza virus on MDCK or chicken embryos or perform immunofluorescence test (IF) for influenza and other respiratory viruses, including RSV, adenovirus and parainfluenza. Testing for respiratory viruses other than influenza is not performed routinely. The results of the diagnostic tests are forwarded to the physician who collected the specimen. Weekly epidemiological reports and virological reports are then prepared and sent by each VSES to NIC. Influenza isolates are also sent by VSESs to NIC, where antigenic characteristics of the isolated strains is made to determine virus type and subtype. VSESs are informed by NIC of the results of such analysis. Then, isolated influenza strains are sent by NIC to WHO Collaborating Centre for Reference and Research on Influenza in London, UK for more detailed analysis. Next NIC, after analysis and verification of data received from VSESs, prepares weekly epidemiological and virological reports for the entire country and sends to EISS and WHO (FluNet).

Results

Presented information include data collected in the epidemic seasons 2004/2005 – 2006/2007 from week no. 36 of a given year until week no. 16 of the next year inclusive. In the epidemic season 2004/05 the number of VSESs reporting epidemiological data ranged from 6 to 10 (median: 8), and the number of VSESs reporting virological data ranged from 1 to 9 (median: 6). Number of reporting physicians ranged from 165 to 219. Within SENTINEL system 399 specimens were tested and this was 91% of all specimens. The remaining 9% of swabs were non-SENTINEL specimens collected in hospitals and received almost exclusively by NIC. Respiratory infections (influenza, RS, adenovirus parainfluenza) were confirmed in 84 cases (21.1%) of SENTINEL specimens, including 63 influenza cases. Forty-one influenza strains were isolated (23 B, 17 A/H3N2, 1 A/H1N1). Peak of incidence of influenza and ILI was observed between week no. 6/2005 (7-13 February 2005) and week no. 12/2005 (21-27 March 2005) with the highest incidence in week no. 11/2005 (14-20 March 2005) amounting to 641.7/100,000 (Figure 1). In the season 2005/06 the number of VSESs sending epidemiological data ranged from 8 to 16 (median: 16), while the number of VSESs sending virological data ranged from 6 to 15 (median: 12). Since week 45/2005 epidemiological reports were received from all 16 VSESs and virological reports from 11 VSESs. Number of reporting physicians ranged from 98 to 949 (median: 868). Number of SENTINEL specimens amounted to 949 and this was 98% of all specimens tested in this season. Respiratory infections were confirmed in 83 cases (8.7%) of SENTINEL specimens, including 47 influenza cases. Thirty-five influenza strains were isolated (27 B, 6 A/H1N1, 2 A/H3N2). Peak of incidence of influenza and ILI occurred between week no. 9/2006 (27 February to 5 March 2006) and week no. 14/2006 inclusive (3-9 April 2006) with the highest incidence registered in week no. 12/2006 (20-26 March 2006) and amounting to 229.7/100,000 (Fig. 1). In the season 2006/07 from 8 to 16 VSESs

sent epidemiological data (median: 16) and from 10 to 16 VSEs sent virological data (median: 15). Since week 40/2006 epidemiological reports were received from 16 VSEs, and virological reports were received from 14 VSEs. Number of reporting physicians ranged from 696 to 1054 (median: 1017). Number of SENTINEL specimens amounted to 1195 and this was 97% of the total number of swabs tested in this season. Respiratory infections were laboratory confirmed in 63 cases (5.3%) of the SENTINEL specimens, including 37 influenza cases. Seventeen strains of influenza virus were isolated (12 A/H1N1, 5 A/H3N2). Peak of incidence of influenza and ILI was observed since week no. 06/2007 (between 5 and 11 February 2007) until week no. 12/2007 inclusive (19-25 March 2007) with the highest incidence registered in week no. 09/2007 (between 26 February and 4 March 2007) and amounting to 265.8/100,000 (Figure 1).

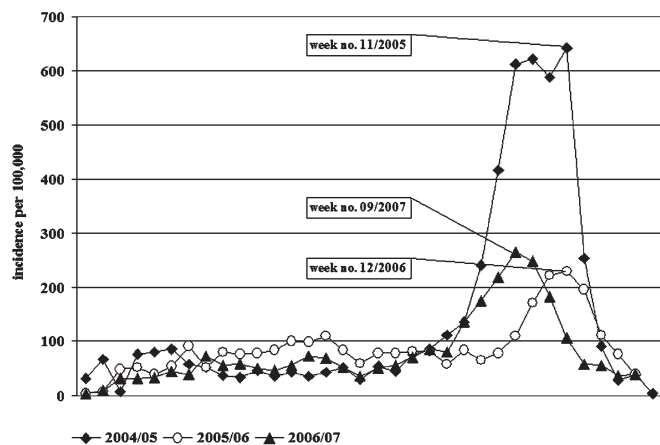
Discussion and Conclusions

Surveillance of influenza in Poland has a long history, dating back to the forties of the last century, however its quality was various, especially in the case of virological surveillance (4). Setting up of the SENTINEL made a significant difference in this area and ensured for Poland nationally representative and integrated virological and epidemiological influenza surveillance, uniform surveillance with other European countries according to EISS recommendations as well as full membership of EISS. Epidemiological data collected by the SENTINEL system during the last three epidemic seasons showed that activity of influenza in Poland in the season 2006/07 was similar to season 2005/06, but was significantly lower than in the season 2004/05. Depending on the epidemic season different types/subtypes of influenza viruses caused infections and illnesses. Virological influenza surveillance showed that in the season 2004/05 majority of influenza cases were caused by A/H3N2 and B strains, in the season 2005/2006 a dominant type was type B, while in the season 2006/2007 infections were caused by strains of subtype A/H3N2 and A/H1N1 and only a small percentage of cases was confirmed as influenza B. As it was mentioned in *Introduction* until the epidemic season 2004/2005 the major problem with influenza surveillance regarded virological part and a very small number of specimens collected from patients suspected to be infected with influenza. Limited virological information on influenza not representative for the entire country became especially disturbing in the last few years when one of the most important global priorities for influenza and public health experts is preparedness for the next influenza pandemic. Efficient influenza surveillance is one of the essential components of each of the national influenza pandemic preparedness plans (2, 4). The epidemic seasons 2004/2005-2006/2007, which were the first seasons of the SENTINEL existence in Poland, clearly showed that SENTINEL is an effective system for influenza surveillance. It is obvious that this system is not perfect as yet. Nevertheless, there is a big improvement, especially in the virological part of the surveillance, in comparison with the previous epidemic seasons. It is worth to emphasize that the number of the processed specimens in

the season 2004/2005 was almost 3 times higher, in the season 2005/2006 – over 6 times higher and in the season 2006/2007 – 8 times higher than in the epidemic season 2003/2004, when only 151 specimens were tested. Moreover, since the season 2004/2005 the specimens are obtained from different regions being representative for the entire country. On the one hand the establishment of the SENTINEL influenza surveillance in Poland significantly improved the collection of data on influenza activity, but much work is still required, especially in the area of laboratory testing and its quality. This is due to the fact that the percentage of positive results of laboratory testing is rather low and decreased since the epidemic season 2004/2005 to the season 2006/2007. There are two possible reasons for the above situation: the negative results were true – they were actually negative or some conditions essential for proper and valid specimen processing were not fulfilled. Therefore, the awareness of laboratory staff and also physicians of factors that may have influence on a quality of laboratory testing and results of such testing as an appropriate specimen collection and transport, time when the specimen is collected from patient, appropriate viral transport medium, should be necessarily increased (5). Other important issue is to ensure and maintain integration between epidemiological surveillance with virological surveillance (6, 7). It was found that the number of physicians have been gradually increased, even up to over 900 physicians reporting in one week. Nevertheless, this increase was not always connected with active participation of physicians in virological surveillance, and consequently with the simultaneous increase of the number of clinical specimens collected for testing. Further improvement of SENTINEL influenza surveillance system should lead to: cooperation with possibly the same family physicians season by season; effective encouragement physicians to participate in the SENTINEL despite the volunteer basis of this system; better qualified and experienced laboratory staff to ensure the highest quality of laboratory processing; improved laboratory capacity; better control and coordination of the surveillance in the individual regions (Voivodships); year round influenza surveillance according to the EISS plans; publishing detailed national reports similar to EISS annual reports with information on the effects of influenza surveillance in a given epidemic season (6).

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Figure 1. Incidence of influenza and influenza-like illness registered by the SENTINEL influenza surveillance system in Poland in the epidemic seasons 2004/2005 – 2006/2007.



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Viral Circulation and Latitude in Argentina

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Introduction

Because Argentina is a Southern Hemisphere country with temperate climate extending from latitudes $-21^{\circ}46'$ to $-55^{\circ}03'$, influenza seasons occur usually from May to September. Data obtained from influenza-like illness (ILI) notification to the Ministry of Health confirm this seasonality. Occasionally, influenza outbreaks have been observed outside the expected seasonality, mainly in cities located in extreme latitudes. The availability of data provided by the laboratory diagnosis of respiratory viruses (RV) during 7 consecutive years in four Argentine cities located in different latitudes will be analyzed in order to establish whether there is any relationship between geographical location and the characteristics of the virus circulation period.

Materials and Methods

Virological influenza surveillance in Argentina is based on the data and the respiratory samples provided by the laboratories participating in the National Network of Influenza and Other Respiratory Viruses Laboratories (NIRVL). They perform the RV diagnosis by immunofluorescence (IF). Once an influenza positive sample is detected, the sample is sent to the Reference Laboratory (RL) in the Buenos Aires National Influenza Center (NIC) in order to isolate viruses on MDCK cells, and to characterize them. From the 27 NIRVL, we selected results provided by four laboratories because of their geographical location and the availability of data for the entire period between 2000 and 2006: Salta city (latitude $-24^{\circ}48'$); Buenos Aires city ($-34^{\circ}38'$); Neuquen city ($-38^{\circ}55'$) and Ushuaia city ($-54^{\circ}48'$). Normal seasonality (NS) based on notification data from the Ministry of Health was considered when the peak occurs between May and September; early peak (EP) when it occurs from January to April, and late peak (LP) from October to December. The average number per year of respiratory samples analyzed at the local laboratories are as follows: Salta $n=1114$, Buenos Aires $n=8058$, Neuquen $n=2664$, Ushuaia $n=171$.

Results

The comparison of the curves of influenza circulation detected during the period shows that laboratory diagnosis confirms the ILI notification data with influenza circulation predominantly occurring during winter months. In addition, extended curves have been observed in different years with minor early peaks

in Salta and Ushuaia and a prolonged influenza season until spring in Neuquen city. Salta city, located at the Northern border, demonstrates a viral circulation similar to that observed in tropical areas, showing EPs in 2003 and 2004 (single); but also NS in 2003 as a second peak, in 2005 (single) and 2006 (first peak); and LPs in 2001 (single), 2002 (single) and 2006 (second peak). Buenos Aires city never experiences an EP; a NS was observed during all periods analysed, usually peaking in June-July, except for years 2003 and 2004 peaking in May. Mild LPs were observed in 2000, 2004 and 2006 as a second peak occurring in September-October. Neuquen city demonstrates a similar profile as Buenos Aires city with the NS peaking always in June. LPs occurred in 2002, 2003 and 2004 as second peaks. Ushuaia city experienced an EP in 5 out of 7 years usually followed by a second peak either as NS or LP. In 2005, there was a single EP in March. Viral characterization from EPs in extreme latitudes was only available in a few years. Data for Buenos Aires and Neuquen have been consequently obtained these data are presented in Table 1.

Table 1. Viral characterization in four cities, Argentina, 2000-2006.

YEAR	EARLY PEAK EP	PREDOMINANT ISOLATES NS	LATE PEAK LP
2000	USHUAIA Influenza A (CNA)	A/New Caledonia/20/99 (H1N1) B/Yamanashi/166/98	USHUAIA Influenza A (CNA) BUENOS AIRES B/Yamanashi/166/98??
2001	(-)	A/Panamá/2007/99 (H3N2) A/New Caledonia/20/99 (H1N1) B/Sichuan/379/99	SALTA A/Panamá/2007/99(H3N2) Influenza B (CNA)
2002	(-)	A/Panamá/2007/99 (H3N2) B/Hong Kong/330/01	SALTA A/New Caledonia/20/99 (H1N1) NEUQUEN B/Hong Kong/330/01
2003	SALTA A/New Caledonia/20/99 (H1N1) USHUAIA A/Panamá/2007/99 (H3N2)	A/Panamá/2007/99 (H3N2) A/Fujian/411/02 (H3N2) A/New Caledonia/20/99 (H1N1)	NEUQUEN A (H3) (CNA)
2004	SALTA A/Fujian/411/02 (H3N2) USHUAIA A/New Caledonia/20/99 (H1N1)	A/Fujian/411/02 (H3N2) B/Sichuan/379/99 (predominant) B/Hong Kong/330/01	USHUAIA B/Sichuan/379/99 BUENOS AIRES B/Sichuan/379/99 (predominant) B/Hong Kong/330/01
2005	USHUAIA A/California/07/04 (H3N2)	A/California/07/04 (H3N2) B/Shanghai/361/02 B/Hong Kong/330/01	(-)
2006	USHUAIA Influenza A (CNA)	A/New Caledonia/20/99 (H1N1) A/New York/55/04 (H3N2) (California-like) A/Wisconsin/67/05 (H3N2) B/Shanghai/361/02 B/Malaysia/2506/04	NEUQUEN B/Malaysia/2506/04

CNA: characterization not available

Discussion

Influenza circulation in Argentina is usually observed as a major winter peak. In the cities located at extreme latitudes, influenza also peaks during summer or early autumn and late peaks are observed universally in six out of seven years. Although the information is based on different numbers of samples studied in each of the four cities, these are in relative to the local populations: the predominating strains in each season are roughly influenced by those obtained in Buenos Aires because this city is home to one third of the total population of

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Argentina. We also show that the LPs are usually produced by the circulation of influenza B strains. However, co-circulation of influenza A and B was also observed. If we analyze the antigenic characterization of the strains obtained for the year 2003, the EP in Salta was produced by the circulation of A/New Caledonia/20/99 (H1N1)-like strains followed by the co-circulation of A/Panama/2007/99 (H3N2) in May-June. These data and the circulation of A/Panama in April in Ushuaia did not predict the appearance of A/Fujian-like strains in Buenos Aires and Neuquen as NS peaks. This new variant of influenza H3 caused a severe influenza season with a huge impact in public health due to high rates of hospitalizations and mortality. So, we could infer that new variants are not introduced in the country following a climatic spread from temperate areas, but by way of introduction through trafficking of people from outside regions. More extensive surveillance in Salta and Ushuaia will be necessary for to better our understanding of the behavior of influenza circulation and spread in the rest of the country. A higher number of viral isolates obtained early in the year will improve the accuracy of these previous conclusions.

Proceedings Topic #2

**Virus Structure/Function
and Receptor Binding**

**Poster
Presentations**

Some Events in the Biogenesis of Influenza Virus Nucleocapsid Protein

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We have earlier shown that in addition to folded compact NP oligomers the incompletely-folded NP-NP complexes, designated "NP multimers", are detected in influenza virus infected cells. Here we report on the properties of two different forms of intracellular NP polymers. It was shown that NP multimers are detected in 5% stacking gel of SDS-PAGE as retarded and loose structures, heterogeneous in size. NP multimers are more sensitive to heating and to protease than NP oligomers. The results obtained indicate that in contrast to compact NP oligomers, NP multimers are not a highly ordered structure, probably due to incomplete folding of their NP subunits. It was also shown that NP multimers appear at early stages of NP synthesis preceding compact NP oligomer formation. The NP multimers are partially chased into faster migrating compact NP oligomers. The excess of NP multimers not converted into compact NP oligomers accumulates in cells and partially degrades. NP multimers are associated with cytoplasm whereas compact oligomers migrate in nucleus.

Introduction

We have shown previously that in influenza virus infected cells stable compact NP oligomers (dimers and larger structures) are revealed approximately 20 minutes after translation [7, 10]. Compact NP oligomers of avian viruses are stable in the presence of urea, proteases, at high ionic strength, basic pH, and react with conformational monoclonal antibodies (mAbs) with high efficiency. They dissociate in the presence of SDS at +80°C [7]. All these data indicate that compact NP oligomers are the final mature structures in NP biogenesis, possessing properties of folded proteins. However, since the formation of compact NP oligomers takes place posttranslationally, a preceding immature, incompletely folded state of NP may be predicted. In the present study we have shown that at the early stages of influenza virus infection, the incompletely folded loose NP multimers, are formed. Some properties of two forms of NP polymers and their relationships were studied and discussed.

Materials and Methods

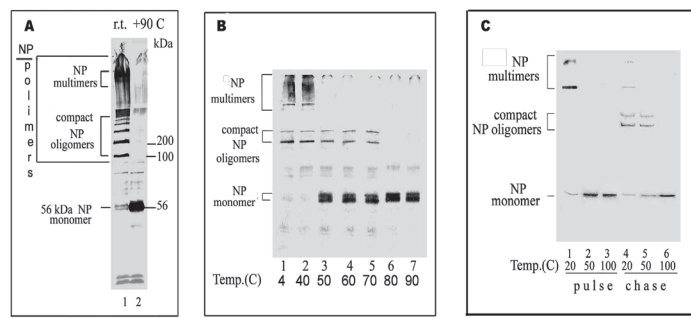
A/Duck/Germany/49(H10N7) influenza virus and the continuous MDCK cell line as well as the slices of chorionallantoic membranes were used. Monoclonal anti-NP antibodies (mAbs) were kindly given by professor L. Stitz (Institute of Immunology, Tübingen, Germany). The infected cells were labeled with [³⁵S]-methionine after 5 hrs p.i. In pulse-chase experiments the cells were shortly labeled and then chased in a methionine-free medium. The cytosols were subjected to immunoprecipitation by RIPA using the pooled anti-NP mAbs which bound to both

compact NP oligomers and NP multimers. Immunoprecipitates were dissolved in a Laemmli sample buffer. Before SDS-PAGE the analyzed lysates were divided into two portions: one portion was left unheated (r.t.) to preserve NP polymers and the other one was heated at 90°C to dissociate NP polymers into NP monomeric subunits. Western blot with pooled anti-NP mAbs was also used. Because of the thermosensitivity of the studied NP-NP complexes, SDS-PAGE was carried out at +4 °C and at low current, preventing the heating of the gel.

Results

For preservation of protein quaternary structures we used relatively weak dissociating conditions of SDS-PAGE without any pre-heating of the samples, and at minimal power supply [10]. Figure 1A shows the typical SDS-PAGE pattern of immunosorbed NP-containing structures obtained from the infected chorionallantoic membranes labeled with 35S methionine for 3 hrs at 5 hrs. p.i. Reducing SDS-PAGE containing 15% acrylamide in the resolving gel and 5% in the stacking gel was used. As shown in Figure 1A, in unheated sample (lane 1) different SDS resistant NP polymers were detected ranging from dimers to larger structures. As it was previously shown [7,10] the compact NP oligomers were localized in the 15% acrylamide resolving gel. In addition to compact NP oligomers large and loose NP-polymers possessing a retarded mobility and localized in 5% stacking gel were also detected. We designated these retarded NP polymers as "NP multimers". NP monomers were almost absent in the unheated sample (lane 1) and appeared only after heating (lane 2) as a result of all NP polymer dissociation. The ability of the detected NP polymers to penetrate into 5%-15% acrylamide suggests that they are not RNP. The dissociation of the heated NP oligomers and NP multimers only into NP monomers suggests that both structures are homo-polymers. Next, the stability of NP multimers and compact NP oligomers was compared. Figure 1 B shows the melting of NP polymers forming in the infected MDCK cells at 5 hrs p.i. and analyzed by non-reducing SDS-PAGE after labeling for 30 min. As shown in Figure 1 B, in non-reducing conditions the NP multimers of the used avian influenza virus were localized in 5% stacking gel as irregular, heterogeneous in size structures (lanes 1,2) which dissociated into monomers at +50°C (lane 3), whereas the compact NP oligomers dissociated only at +80°C (lane 6). Interestingly, NP multimers of A(H1N1) viruses are more thermo-sensitive in comparison with NP multimers of avian and human A(H2N2) and A(H3N2) viruses and dissociate even at room temperature [10]. In all studied influenza virus strains NP multimers were also relatively more protease sensitive than compact NP oligomers and the protease sensitivity increased in this order: compact NP oligomers>NP multimers>NP monomers (not shown). The data presented suggest that in contrast to compact NP oligomers, NP multimers possess a loose quaternary structure characteristic for incompletely folded proteins.

Figure 1. Intracellular NP multimers and compact NP oligomers revealed by non-dissociating SDS-PAGE. A- pattern of different immunosorbed NP polymers in the slices of chorionallantoic membranes labeled for 3 hrs at 5 hrs p.i. and detected by reducing SDS-PAGE. B-relative thermostability of NP multimers immunosorbed from the infected MDCK cells labeled for 30 min. at 5 hrs. p.i. and detected by non-reducing SDS-PAGE. C- Pulse-chase analysis of the relationships between NP multimers and compact NP oligomers in MDCK cells. The immunoprecipitates were incubated in a sample buffer for 3 min at indicated temperature before SDS-PAGE containing 15% acrylamide in running gel and 5% one in the stacking gel.



To study the relationships between different intracellular forms of NP polymers, MDCK cells were pulse labeled with [³⁵S]-methionine for 5 min at 5.5 hrs p.i., and then chased for 20 min. The cell lysates were subjected to RIPA with pooled mAbs binding both the NP oligomers and NP multimers. As shown in Figure 1 C, after a 5 min pulse NP multimers were localized in the stacking gel (lane 1), and they dissociated after heating at +50°C (lane 2). After a 20 min chase, the decrease in the amount of NP multimers correlated with the appearance of compact NP oligomers (lanes 4 and 5). The results obtained suggest that NP multimers appear at early stages of NP synthesis, preceding compact NP oligomer formation. The NP multimers are partially chased into compact NP oligomers, and therefore they probably may play the role of precursors of the compact NP oligomers. Association of intracellular NP polymers with cytoplasm and nucleus was also studied. For this aim pulse-chase experiments with cell fractionation were carried out [11]. It was shown that both NP multimers and compact NP oligomers were detected in cytoplasm. Compact NP oligomers were also detected in nucleus after pulse labeling, continuing to increase in the nuclear fraction during the following chase (not shown here). In contrast, NP multimers were not revealed in the nucleus. In the course of infection the excess of NP multimers non-converted into compact oligomers were partially degraded in cytoplasm [10]. The comparative studies of NP polymers obtained from the infected cells and NP polymers obtained from purified virions were carried out. The results obtained suggest that the structural design and probably the type of inter-subunit linking is different in intravirion NP polymers and in the most abundant species of intracellular NP polymers. It is possible that either intracellular NP polymers change their conformation after being incorporated into virion, or that only a small fraction of intracellular NP polymers is incorporated in the packaging RNP [11].

Discussion

On the basis of the presented results here and in earlier published data [7, 10, 11] a hypothetical model of influenza virus NP biogenesis may be suggested. The nascent NP chains released from polysomes are incompletely folded and possess free hydrophobic surfaces, which are able to interact with one another to form NP multimers. Transient disulfides are formed in these early stages of NP synthesis to protect unfolded NP chains against proteases and to prevent non-specific spontaneous NP-NP association [9]. After release from polysomes NP multimers lose disulfides and bring incompletely folded NP molecules into close contact with each other for post-translational folding and formation of compact NP oligomers. Hence, one fraction of NP multimers plays the role of a transient intermediate. The other fraction of NP multimers, non-converted into compact NP oligomers, remains in the cytoplasm and degrades. Proteolytic degradation of incompletely folded NP multimers together with NP truncation [8] may be responsible for the generation of NP peptides and its presentation to T cells, in addition to defective ribosomal products studied by Yewdell et al [1]. Conformational maturation of proteins via formation of multimeric intermediates was described earlier for the G protein of VSV [3], reovirus cell attachment protein [5] and Coxsackie virus 2B protein [2]. We have also shown that compact NP oligomers are transported into nucleus, and probably they take part in RNP formation. At the same time the accumulation of a fraction of NP polymers in cytoplasm (NP-multimers), resistance of NP polymers to RNase and their penetration into a high concentration acrylamide suggest that a large fraction of intracellular NP polymers are RNA-free. It is well known that *in vivo* intracellular NP is capable of self-associating to form large RNA-free homo-polymeric complexes [4, 6, 12], which are morphologically similar to intact viral RNP. As influenza virus NP is a multifunctional protein [6], it may be suggested that in addition to encapsidation of virus RNA intracellular NP polymers possess many other activities not known at present.

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Effect of the Addition of Oligosaccharides on the Biological Activities of Influenza A Virus Hemagglutinin

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Influenza A/H3N2 viruses have developed an increased number of glycosylation sites on the globular head of the hemagglutinin (HA) protein since their appearance in 1968. We have previously constructed seven mutant HAs of A/Aichi/2/68 virus with one to six glycosylation sites on the globular head, as found in natural isolates, and analyzed the biological activities of the molecule. The glycosylation sites of mutant HAs correspond to representative A/H3N2 isolates (A/Victoria/3/75, A/Memphis/6/86 or A/Sydney/5/97). The results showed that mutant HAs containing three to six glycosylation sites decreased receptor binding activity but retained cell fusion activity. Here, we attempted to produce recombinant viruses possessing these mutant HAs to investigate their biological activities. The recombinant viruses expressing HAs with one to six glycosylation sites were recovered. However, titer of mutant viruses with HAs having four to six glycosylation sites was much lower than that of wild type virus. Some of these mutant viruses had amino acid changes in the neuraminidase (NA) protein. Recently, evidence has been presented that indicates that a balance of receptor binding and receptor destroying activities is strictly required for efficient replication of influenza A virus. The NA activities of mutant viruses were reduced as the number of oligosaccharides on the HAs increased. The results showed that a decrease in the receptor binding activity of HA was accompanied by a concomitant decrease in the receptor destroying activity of NA. Therefore, it seems likely that a decrease in the receptor binding activity of the HA with additional oligosaccharides may result in the loss of the balance of receptor binding and receptor destroying activities.

Introduction

The hemagglutinin (HA) of influenza A virus is a homotrimeric glycoprotein with an ectodomain composed of a globular head and stem region. Both regions carry N-linked oligosaccharide chains. The acquisition of new oligosaccharides is an important mechanism underlying the antigenic drift of HA. Based on analysis of the HA sequences of A/H3N2 viruses isolated from 1968 to 2006, the oligosaccharide chains on the globular head show large variations in number among different A/H3N2 isolates. Most of the A/H3N2 viruses that circulated between 1968 and 1974 (represented by A/Aichi/2/68) had only two oligosaccharides at residues 81 and 165 on the globular head of the HA. However, viruses isolated in 1975 (represented by A/Victoria/3/75) had lost a glycosylation site at residue 81 and gained two new sites at residues 63 and 126. The 1986

isolates (represented by A/Memphis/6/86) had acquired a new carbohydrate attachment site at residue 246, and the 1997 isolates (represented by A/Sydney/5/97) had obtained two additional sites at residues 122 and 133. Recent isolates (represented by A/Panama/2007/99) had obtained a novel site at residue 144. Thus, the A/H3N2 viruses recently circulating have seven glycosylation sites on the globular head of the HA, although whether these are glycosylated is not known. These observations suggest that the addition of new oligosaccharides to the globular head of the HA may provide influenza viruses with an increased ability to prevail among humans. We have previously constructed seven mutant HAs of A/Aichi/2/68 virus having one to six glycosylation sites on the globular head and analyzed the biological activities of the molecule [1]. The mutant HAs have glycosylation sites as found on the HA of representative A/H3N2 isolates (A/Victoria/3/75, A/Memphis/6/86 and A/Sydney/5/97). The results showed that mutant HAs containing three to six glycosylation sites decreased receptor binding activity but retained cell fusion activity. In this study, we attempted to produce recombinant viruses possessing these mutant HAs to investigate their biological activities.

Materials and Methods

Virus and cells. The A/Aichi/2/68 strain of influenza A/H3N2 virus was grown in the allantoic cavities of 10-day-old embryonated hen's eggs. 293T cells and MDCK cells were maintained in DMEM supplemented with 10% FCS and MEM containing 10% FCS, respectively.

Plasmid construction. The wild type and mutant HA gene cDNAs encoding one to six N-glycosylation sites of A/Aichi/2/68 virus were amplified by PCR using the recombinant pME18S plasmids containing a wild type or mutant HA gene as a template [1]. The wild type NA gene cDNA of A/Aichi/2/68 virus was synthesized from viral RNA and amplified by PCR. The PCR products were excised by digestion with BsmBI and cloned into the BsmBI sites of the pHH21 vector [2].

Generation of recombinant viruses. 293T cells were transfected with eight RNA polymerase I plasmids (HA and NA genes were derived from A/Aichi/2/68 virus and the remaining six genes, were derived from A/WSN/33 virus) and four plasmids expressing PA, PB1, PB2, and NP proteins derived from A/WSN/33 virus as described elsewhere [2]. At 48 h posttransfection, a 200 µl of the supernatant was inoculated onto MDCK cells. The virus titers in the supernatant were determined in MDCK cells after 3 days postinfection.

Hemagglutination inhibition test. Rabbit antiserum against egg-grown A/Aichi/2/68 virions was prepared as described previously [1]. Hemagglutination inhibition tests were done in microtiter plates with a 0.5 % suspension of chicken erythrocytes.

Neuraminidase assay. Different amounts of viruses were incubated with 4-methylumbelliferyl-N-acetyl-α-D-neuraminic acid (4-MU-NANA) ammonium salt for 30 min at 37°C. The NA activity was calculated by measuring the fluorescence of released 4-MU.

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Results

Mutant viruses expressing HAs with one to six glycosylation sites were recovered. However, the titer of these viruses with HAs having four to six glycosylation sites was much lower than that of wild type virus. The hemagglutination inhibition titers against mutant viruses were reduced as the number of oligosaccharides on the HAs increased. These observations raised the possibility that the addition of four to five oligosaccharide chains considerably affects the antigenicity of the HA molecule. The mutant viruses with HAs having four to five glycosylation sites had an amino acid substitution in the neuraminidase (NA) protein. The NA activities of mutant viruses were reduced as the number of oligosaccharides on the HAs increased.

Discussion

Recently, evidence has been presented that indicates that a balance of receptor binding and receptor destroying activities is strictly required for efficient replication of influenza A virus. A decrease in the receptor binding activity of the HA was accompanied by a concomitant decrease in the receptor destroying activity of the mutant virus NA. Therefore, it seems likely that a decrease in the receptor binding activity of the HA with additional oligosaccharides may result in the loss of the balance of receptor binding and receptor destroying activities.

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Genomic Structure of Highly Pathogenic Influenza H5N1 Viruses. Identification of Ebola Like Immunosuppressive Domain in NS2 Protein

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Introduction

During the latter half of 2005 a widespread outbreak caused by highly pathogenic H5N1 influenza virus among wild and domestic birds occurred in Russia. Sequence data are available for many of H5N1 strains but many of them only concern HA and NA genome segments. Recent studies have shown that other segments as well have a role to play in determining pathogenicity and host specificity [1, 2], which makes full genome sequencing a necessity. Sequencing and analysis of entire genomes of influenza H5N1 viruses isolated in Kurgan region (Siberia) was performed.

Materials and Methods

Virus isolation. Two viruses A/chicken/Kurgan/05/2005 and A/duck/Kurgan/08/2005 were isolated from dead poultry in the Kurgan region from the first passage of the cell culture MDCK and from the chicken embryos. Hemagglutination indices were 64 – 128 (MDCK cells). TCID₅₀/0,2ml of the isolated viruses were 7-8 lg and EID₅₀/0,2ml were 8-8,5 lg.

Viral RNA isolation. Viral RNA was isolated using commercial kits RNAzol® (“GibcoBRL”) and RIBO-sorb (Central Research Institute of Epidemiology, Moscow, RF).

cDNA synthesis. Reverse transcription was carried out by a standard method using the universal oligonucleotide primers (5'AGCAAAAGCAGG3', 5'AGTAGAAACAAGG3').

Sequencing of DNA fragments. Pairs of primers, suggested by E.Hoffmann et al. [3], were used in PCR to obtain DNA fragments. Sequencing of fragments was carried out on ABI PRISM 3100-Avant Genetic Analyzer (“Applied Biosystems”, USA) with BigDye Terminator Cycle Sequencing Kit. **Phylogenetic analysis** was performed by neighbor joining method using the program MEGA 2.1 (PSU, USA) and GeneDoc 2.6. International data base *GenBank* was used. All the obtained primary sequences were submitted to the *GenBank* data base. The access codes for strains A/chicken/Kurgan/05/2005 and A/duck/Kurgan/08/2005 are DQ449632-DQ449639 and DQ449640-DQ449647.

Results

HA receptor-binding site of both Kurgan strains had Gln and Gly at positions 222 and 224 respectively, which is characteristic of avian influenza viruses. All isolates contain an identical cluster of positively charged amino acid residues at the HA cleavage site: QGERRRKKR. Isolated strains should be classified as highly pathogenic judging from their biological

properties. Predicted amino acid sequences of HA molecule of these strains contained amino acid residues typical of both high and low pathogenic influenza virus strains in functionally relevant positions. In four out of six positions the Kurgan isolates had amino acid residues typical of highly pathogenic viruses. Neuraminidase structure analysis of the H5N1 isolates suggested that they belong to the Z genotype. Amino acid residues typical of avian strains were revealed in 38 out of 40 sites in viral internal genes determining host specificity. One of the isolated strains contained a lysine in position 627 of the PB2 protein which has been linked to virulence and course of infection [4, 5]. Kurgan isolates was shown to have a rimantadine-sensitive genotype. Glutamic acid was found at position 92 of NS1 protein in both strains suggesting virus resistance to interferon. By phylogenetic analyses, the Kurgan isolates were classified into subclade II of clade II of HPAI H5N1 viruses. Figure 1 depicts all functional elements of NS1 and NS2 protein primary structure of influenza virus. Table 1 shows amino acid residues in NS1 crucial positions of different strains.

Figure 1. Linear map of NS1 and NS2 proteins functional elements of Kurgan strains.

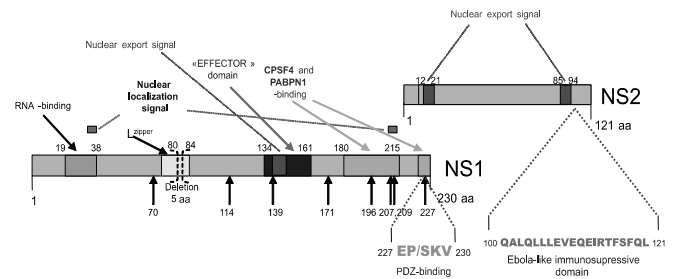


Table 1. Amino acid residues in NS1 crucial positions of different influenza strains.

Strain	AA substitution							
	70	114	139	171	196	207	209	227
A/BrevigMission /I/1918(H1N1)	K	S	D	D	E	N	N	K
A/Leningrad/134/57(H2N2)	-	P	-	N	K	-	-	R
A/HongKong/168(H3N2)	-	P	-	I	K	-	-	R
A/HongKong/156/97(H5N1)	E	-	N	E	-	D	-	E
A/Netherlands/033/03(H7N7)	E	-	-	-	-	-	D	E
A/HongKong/1073/99(H9N2)	E	P	N	-	-	D	-	E
A/Thailand/5/494/2004(H5N1)	E	-	-	G	-	D	D	E
A/Vietnam/CL115/2005(H5N1)	E	-	-	G	-	D	D	E
A/Goose/Guangdong/1/96(H5N1)	-	G	-	T	-	D	-	E
A/duck/Kurgan/08/2005(H5N1)	E	-	-	N	-	D	D	E
A/chicken/Kurgan/05/2005(H5N1)	E	-	-	N	-	D	D	E
A/Indonesia/CDC759/2006(H5N1)	E	-	-	G	-	D	D	E

Discussion

Due to lack of print space we would like to concentrate on the structure and supposed function of NS proteins. Belonging to Z genotype Kurgan isolates have a typical 5 amino acid deletion in NS1 protein. The predicted protein structure can be suggested to recover a perfect L-zipper motif in this region (data not shown). Fine comparative analysis of NS1 sequence data provides new insight on potential function of protein C-terminus. R. Krug [2] discussed differences in C-terminal sequences of NS1 protein of HPAI and LPAI viruses and their role in contribution to pathogenicity of avian flu

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viruses. The consensus sequence at NS1 protein C-terminus which is defined as a PDZ-binding domain is a tetrapeptide: ESEV or EPEV (Fig 1). H5N1 nonvirulent strains contain RSKV in that region. The presence of these short C-terminal domains strongly correlates with pathogenic phenotype. Isolates of 1918 "spanish" strains contain KSEV tetrapeptide. In our isolates this tetrapeptide is EPKV (A/chicken/Kurgan/05/2005) or ESKV (A/duck/Kurgan/08/2005), which is closer to the motif of low-pathogenic virus sequence [1]. A study by Obenauer et al. showed the interaction of PDZ-domains with C-terminal tetrapeptides of NS1 of all pathogenic avian isolates [1]. This domain interferes with PDZ-directed pathway of intracellular signaling. Interference with the control of signaling along PDZ-dependent pathways could be more damaging for innate cellular immunity than inhibition of dsRNA-dependent protein kinase. In this regard, it could be supposed that single mutation could not be programmed. Appearance of this mutation is time consuming and casual. Domain ESEV/EPEV is encoded by a nucleic acid sequence which does not overlap with NS2 protein coding part of segment 8. To understand the mechanism of generation of pathogenic and non pathogenic type of PDZ-binding domains we superposed this domain coding sequence on secondary structure of NS gene. As a result we identified a very specific position of short sequence coding for this tetrapeptide. The location of the sequence inside the bulge of the loop with coordinates 696-707 may lead to high frequency mutations due to errors of viral polymerase from the loop to the bulge transition. Spontaneous frequent mutations could generate sequence diversity in this site. Further analysis of nucleic acid and protein sequences in segment 8 of H5N1 Russian isolates showed that there is a very specific domain inside NS2 protein that should have special attention. The domain is located between amino acid residues 100-121 at C-terminus of this protein (Fig 1). This "immunosuppression" consensus was found in Kurgan isolates and 1918 "Spanish" strains. This domain was first identified in Ebola virus protein [6, 7]. Later similar sequences were identified among retroviruses [8]. It was proved that homologous peptides of Ebola or retroviral proteins are strongly immunosuppressive and make a significant contribution to pathogenicity of highly pathogenic viruses as Ebola. We have synthesized peptides representing a part of this domain according to the sequence of A/Chicken/Kurgan/05/2005 strain and tested them using standard system for immunosuppressive peptides. Comparative analysis of immunosuppressive activity of peptides from VP protein of Ebola and influenza viral peptides showed both strong immunosuppression and specific binding with its receptor (data not shown). Using extended database of NS1 protein from past and recent isolates we recognized additional "immunosuppression-like" domain in the middle part of NS1 protein of "Spanish" flu isolates. Thus, in segment 8 in NS1 and NS2 genes we have identified new features on the level of potential recombination events, which may control the sudden appearance of highly pathogenic H5N1 strains and a new domain in NS2 protein, which is similar to the Ebola virus

immunosuppression domain. Immunosuppression domain is an intrinsic property of many influenza viruses, including H3N2 and H1N1 viruses causing seasonal flu and used for flu vaccine design. Live attenuated vaccines produced by old technologies are not so safe as it is required in pre-pandemic period. Design of novel vaccines based on NS1-deletion technologies, so called replication defective vaccines [9] is an important step ahead to safe vaccines that lost internal features of pathogenicity. Deep understanding of internal properties of HPAI strains is of particular importance for further improvement of genetic construction of pandemic vaccines.

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Heterosubtypic Cross-Neutralization of Influenza A Viruses By a Novel Hemagglutination-Inhibiting Monoclonal Antibody

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Influenza A virus hemagglutinin (HA) is the major target protein inducing antibodies that neutralize virus infectivity. Sixteen antigenically different HA subtypes (H1-H16) of influenza A viruses have been identified in nature. It is generally believed that the neutralizing antibodies to HA are not cross-reactive among HA subtypes. However, we generated a novel monoclonal antibody specific to HA, designated MAb S139/1, which showed heterosubtypic cross-reactive neutralization and hemagglutination inhibition (HI) of influenza viruses. S139/1 was obtained by immunizing mice intranasally with intact formalin-inactivated A/Aichi/2/68 (H3N2) virus. MAb S139/1 showed neutralization and HI activities against some strains of the H1, H2, H3 and H13 subtypes of influenza A viruses. In enzyme-linked immunosorbent assay, MAb S139/1 showed broad specificity to many other virus strains, including H1, H2, H3, H5, H9, and H13 subtypes. Six escape mutants of A/Aichi/2/68 (H3N2) were selected by MAb S139/1, and sequence analysis of HA genes of those escape mutants revealed amino acid substitutions at position 156, 158 or 193. A molecular modeling study showed that these amino acids were located in the HA surface on the globular head and formed a conformational epitope adjacent to the receptor-binding domain of HA. Four and three escape mutants selected from A/Adachi/2/57 (H2N2) and A/WSN/33 (H1N1), respectively, all had amino acid substitutions at position 193 (H3 numbering). These results suggest that MAb S139/1 neutralizes virus infectivity by affecting the common antigenic site shared among different subtypes of HA. The present study suggests the possibility of antibody therapy for heterosubtypic immunity against multiple subtypes of influenza A virus.

Introduction

Neutralizing antibody plays a critical role for protection from influenza virus infection. Inactivated vaccine-induced neutralizing antibodies only prevent infection of the homologous strain or closely related strains. Most of neutralizing antibodies recognize epitopes on the hemagglutinin (HA) which exists on the surface of virus. There are 16 HA subtypes in nature, and these subtypes are antigenically distinct [1, 2]. Therefore, cross-reactive HA-specific antibodies among HA subtypes have been rarely reported [3]. HA is a trimeric membrane protein and consists of two distinct regions, a globular head composed of HA1 and

a stem region composed of mainly HA2. There is considerable amino acid variability in the globular head region, while structure of the stem region is highly conserved. The HA is responsible for virus entry into target cells. The globular head and stem regions contain receptor binding site and fusion peptide, respectively. The antigenic structure of H3 subtype is well characterized [4]. Five antigenic sites (A to E) have been identified on the HA1 globular head [5]. Monoclonal antibodies (MAbs) recognizing epitopes on the receptor binding site mostly neutralize virus infectivity *in vitro* and inhibit the hemagglutination by the virus [6]. Although cross-neutralizing MAb between H1 and H2 subtypes was reported, which recognize a conformational epitope consisting of HA1 and HA2 in the middle of the stem region [3], there have been no report of MAb showing heterosubtypic cross-neutralizing activity to influenza virus of multiple HA subtypes. In this paper, we demonstrate a novel cross-neutralizing MAb which recognizes a common epitope among variety of HA subtypes.

Materials and Methods

Viruses. The influenza virus strains used in this study are shown Table 1. Viruses were grown in embryonated chicken eggs or MDCK cells. Purified viruses were prepared by sucrose gradient centrifugation of infected allantoic fluids.

MAbs. Six-week-old BALB/c mice were immunized intranasally with the formalin-inactivated purified A/Aichi/2/68 (Aichi) virus with cholera toxin B. The spleen cells from these mice were fused with mouse myeloma cells. Hybridoma cells producing anti-HA MAb were screened by enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) assay. MAb was purified from mouse ascites using protein A agarose columns.

Biological assay. Reactivities of MAbs were analyzed by ELISA, HI assay and virus neutralization test. HI titers are expressed as the lowest concentrations of purified MAb S139/1, which completely inhibited hemagglutination. Neutralization titers were determined as the concentrations of MAb S139/1 causing a 50% reduction of plaque-forming units using MDCK cells.

Sequence analysis of escape mutants. Escape mutants were selected by culturing A/WSN RG/33 (WSN), A/Adachi/2/57 (Adachi) and Aichi strains in MDCK cells in the presence of MAb S139/1. Nucleotide sequences of the HA genes were determined by direct sequencing of the RT-PCR products from viral RNA.

Molecular modeling. MODELLER was used for homology modeling of HA structures. The predicted HA structures of WSN (H1) and Adachi (H2) strains based on the crystal structures of H1 (PDB code: 1RU7) and H5 (PDB code: 2FK0) subtypes, respectively.

Results

Characterization of MAb S139/1. Table 1 shows the reactivity of MAb S139/1 to various subtypes of influenza virus strains. MAb S139/1 exhibited high titers of HI to some strains of H1 and H3 subtypes, and relatively lower activity to H2 and H13 viruses, but not to H5 and H9 and type B strains. Accordingly, MAb S139/1 neutralized infections of H1, H2, H3 and H13 viruses. In ELISA, MAb S139/1 reacted with all influenza A virus strains

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tested (H1, H2, H3, H5, H9 and H13 subtypes). In particular, MAb S139/1 showed higher binding activities to WSN (H1), Adachi (H2), Aichi (H3) and Maryland (H13) strains, consistent with HI and neutralizing activities.

Table 1. Reactivity of MAb S139/1 to influenza virus strains.

Virus	ELISA	HI titer ($\mu\text{g/ml}$)	Neutralization titer ($\mu\text{g/ml}$)
A/PR/8/34 (H1N1)	+	> 50	> 100
A/WSN RG/33 (H1N1)	+++	1.56	0.14
A/Adachi/2/57 (H2N2)	+++	12.5	4
A/duck/Hong Kong/836/80 (H3N1)	ND ^a	≤ 0.39	ND
A/Aichi/2/68 (H3N2)	+++	0.78	≤ 0.036
A/Memphis/1/96 (H3N2)	++	> 50	> 100
A/swan/Hokkaido/67/96 (H5N3)	+	> 50	> 100
A/duck/Hong Kong/W213/97 (H9N2)	++	> 50	> 100
A/gull/Maryland/704/77 (H13N6)	+++	12.5	21
B/Lee/40	ND	> 50	ND

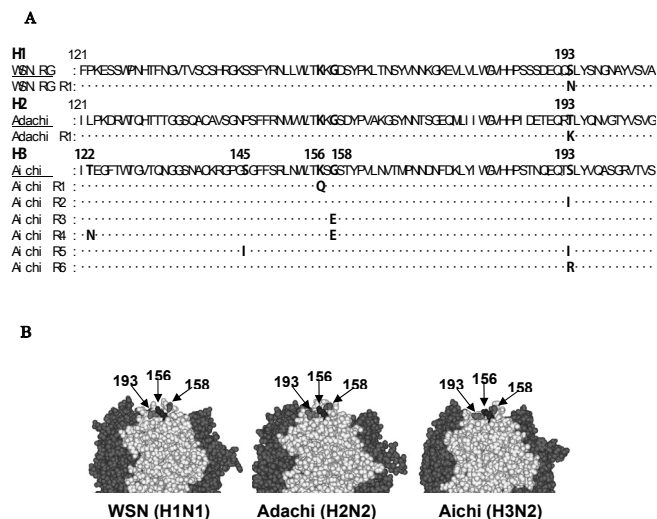
Identification of the epitope recognized by MAb S139/1. We obtained escape mutants of WSN, Adachi, and Aichi by propagating the viruses in the presence of MAb S139/1, and determined deduced amino acid sequences of HAs of the mutants. We found five amino acid substitutions at positions 122, 145, 156, 158 and 193 (H3 numbering) in these mutants (Fig.1A). Three escape mutants of WSN and four mutants of Adachi had the same substitution at position 193, S to N and T to K, respectively. Six escape mutants of Aichi had the amino acid substitutions either at positions 156 (K to Q), 158 (G to E), or 193 (S to I or R). Substitutions at positions 122 and 145 do not seem important. By three-dimensional structure analysis, positions of the amino acid substitutions were mapped in the globular head of H1, H2 and H3 HAs (Figure 1B). Three amino acids at positions 156, 158 and 193 were located on the antigenic site B in the surface of globular head and formed a conformational epitope adjacent to the receptor-binding domain.

Discussion

We showed the characteristics of a novel MAb S139/1 that reacted with HA of various subtypes of influenza A viruses. MAb S139/1 bound to all strains of H1, H2, H3, H5, H9 and H13 subtype viruses tested in ELISA, however, MAb S139/1 showed neutralization and HI activities to some but not all strains of the viruses. Since we assume that MAb S139/1 recognizes a single epitope on the HA of any virus subtypes, it is most likely that the different binding affinity of MAb S139/1 to each HA subtype influences the neutralization and HI activities. Indeed, our data demonstrated that there were significant correlations between

binding affinity and HI or neutralization titer of MAb S139/1. By sequence analyses of the escape mutants obtained from H1, H2 and H3 viruses, we identified a common neutralizing epitope among different subtypes of HA. The present study suggests the potential use of cross-reactive MAbs such as MAb S139/1 for the antibody therapy that gives heterosubtypic immunity against influenza A virus infection of multiple subtypes.

Figure 1. Amino acid substitutions found in the HA1 molecules of escape mutants (A), and their positions in the globular head of HAs on the three-dimensional structure (B).



Acknowledgements

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Proceedings Topic #3

Outbreak and Pre-Pandemic Response

Poster Presentations

Laboratory Surveillance in Russia at Phase 3 of Influenza Pandemic

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Epizootic of influenza A (H5N1) in many countries of South East Asia, Europe, Near East, Africa continued to be reported. Human infection continued to be registered in affected countries. Necessity to monitor the situation closely using both local and regional epidemiological analysis and laboratory surveillance has appeared. Laboratory tests for influenza A(H5N1) surveillance in Russia include virus isolation, direct immunofluorescent assay and RT-PCR with following genome sequencing. Hemagglutination inhibition and microneutralisation test has been used in serological surveillance for avian influenza.

Introduction

Epizootic of influenza A(H5N1) in Russia, Ukraine, Azerbaijan and many countries of South- East Asia, Europe, the Near East, Africa continued to be reported. This infection became enzootic in certain areas of the world [1, 2]. Human infection resulting from direct contact with infected birds continued to be registered in affected countries. Separate cases of human-to-human transmission were revealed in Indonesia where the highest mortality rate was registered [3]. Besides, during the last years infection of humans with avian influenza viruses of H7 and H9 subtypes was detected on multiple occasions as a result of evolution of the viruses and acquisition of the capacity to infect humans directly without prior adaptation in mammalian hosts. Current global situation determined by WHO as Phase 3 (Pandemic Alert) could be evaluated as growing threat for public health [4]. Strengthening of influenza surveillance is one of primary objectives of preparedness to a next pandemic. These activities will facilitate an early detection of unusual influenza virus emergency and spread in human population. Rapid isolation of a novel pandemic virus will provide for facilities of early preparing of vaccine strain for timed issue of the first vaccine lots and urgent protection of high-risk groups.

Materials and Methods

Influenza laboratory surveillance and morbidity registration in Russia is under regulation of the Federal Service "Rosпотребнадзор" at Ministry of Public Health [5] and is carried out in specialized RBL of FSI CHE having experience in virus isolation in MDCK cells, virus identification, direct immunofluorescent assay (IFA). FITC-conjugates of antibodies to influenza A (nucleoprotein), A(H1N1), A(H3N2), A(H5N1) and B viruses, parainfluenza (types 1-3) viruses, adenoviruses and respiratory syncytial virus (RSV), produced by Diagnostic

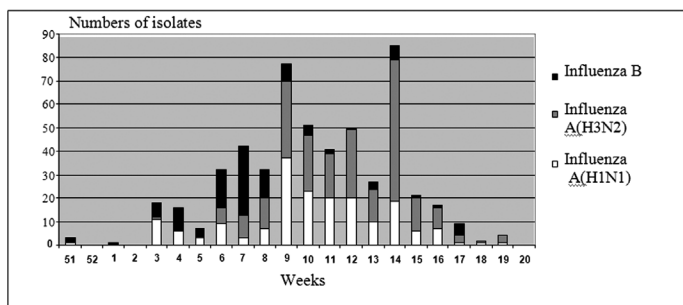
Reagents Production Venture (St.Petersburg) and licensed in Russia were used in this work. These investigations conducted at RBL in accordance with Guidelines developed in Russia [6,7]. Implementation of RT-PCR (Amplisens-Influenza A/H5/H7 kits, InterLabService, Moscow) began in RBL during 2006-2007. Investigation of infectious A(H5N1) virus (BSL-3 condition), genome sequencing, phylogenetic analysis of new viruses and determination of specific antibodies to influenza A(H5N1) virus by microneutralization-ELISA test were conducted at RII. Apathogenic influenza A(H5N1) virus (NIBRG-14 vaccine strain prepared by reverse genetic technology and kindly presented by Dr. J. Wood, NIBSC, UK, to RII) was used in serological surveillance for avian influenza.

Results

Influenza and other ARVI morbidity data, virus isolation and laboratory diagnosis results are reported on a weekly basis by RBL to the FCI (St. Petersburg) collaborating with 49 RBL and to the Centre of Ecology and Epidemiology of Influenza (Moscow) interacted with other 10 RBL and two affiliated NIC where antigenic and genetic structure of viruses is investigated. The results of analysis are reported on a weekly basis to the Ministry of Public Health, WHO, WHO CC and back to RBL. Additionally Centre for Reference Diagnosis and Study of Highly Pathogenic Influenza Viruses was organized in Novosibirsk in 2007. Enhancement of investigations on virus isolation and identification is an important task of influenza surveillance. Increased number of clinical materials was investigated during the 2006-2007 season in RBL. A total of 9531 clinical specimens from patients with ILI were investigated using MDCK cells for virus isolation in 23 RBL located in different regions of the country. In most regions circulation of both influenza A(H1N1), A(H3N2) and B viruses was registered. A total of 504 influenza viruses including 173 (34,3%) influenza A(H1N1) viruses, 221(43,8%) influenza A(H3N2) viruses and 110 (21,8%) influenza B viruses were isolated and identified during the last season. For comparison, number of isolates during previous epidemic seasons 2000-2001, 2001-2002, 2002-2003, 2003-2004 and 2005-2006 was estimated as 119, 152, 169, 141 and 395 viruses, correspondingly. Virus isolation rate in the last season varied depending on the stage of epidemic process from 0,08% (pre-epidemic period) to 6,0-9,9% in epidemic period (weeks 6-16.2007) and peaked (19,4%) on the week 14.2007 (fig.1). Number of isolated viruses depended on intensity of epidemic in separate regions being the highest in Khabarovsk, Astrakhan, Samara, where epidemic thresholds were exceeded in all age groups of population, but other conditions such as sensitivity of MDCK cell sub-line available, quality of used reagents, intensity and qualification level of the work was of great importance. Analysis of epidemic dynamics indicated that at the early stages influenza A(H1N1) and B viruses predominated but since week 6 along with these agents influenza A(H3N2) viruses started to be isolated regularly with prevalence in March-April 2007. According to the results of HA antigenic analysis the strains with distinct drift from reference strain A/Solomon Islands/03/06

were revealed in population of influenza A(H1N1) viruses. The genetic analysis of predicted HA sequences showed existence of the following groups of isolates in population of viruses of the 2005-2006 season: closely related to vaccine strain A/New Caledonia/20/99 or to its drift variant A/Hong Kong/2367/04. Isolates of 2004-2005 season were shown to belong to A/New Caledonia/20/99 group and had changes L69V, Y252F, V314A localized in Cb antigenic site and C-terminal end of HA-1 protein. However, some isolates of 2005-2006 season evolutionary related to this vaccine strain did not possess such changes and formed separate subclade on the H1 phylogenetic tree. The next group included isolates with 3 amino acid changes T82K, Y94H, R145K in Cb and Ca antigenic sites characteristic for the so called Hong Kong group of A(H1) influenza viruses. Viruses isolated in Russia during season 2005-2006 had the change W251R.

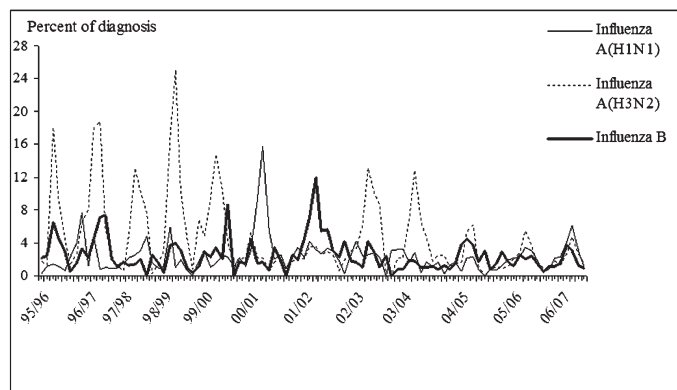
Figure 1. Weekly data on influenza virus isolation in base Laboratories of National Influenza Center (St. Petersburg) located at different regions of Russia, season 2006-2007.



No new potential sites of glycosylation were detected in HA molecule. Heterogeneity of influenza A(H1N1) viruses remained in population of 2006-2007 season with appearance of viruses with amino acid changes typical for A/Solomon Islands/03/06 strain. Influenza A(H3N2) and B viruses were mostly similar to reference strain A/Wisconsin/67/05 and B/Malaysia/2506/04, correspondingly, recommended to be included in influenza vaccine composition for the season 2007-2008 for the Northern Hemisphere. A small proportion of influenza B viruses belonging to Yamagata sub-lineage was revealed in Siberia (Novosibirsk) and Far East (Khabarovsk) but main population was presented by the Victoria-like strains. Investigation of susceptibility of influenza A viruses to rimantadine widely used in Russia for prophylaxis and treatment of influenza showed a growing proportion of strains resistant to this drug reaching up to 69-71% during epidemic seasons 2005-2006 and 2006-2007 while among isolates 2004-2005 this rate was estimated as 38% only. Analysis of nucleotide sequences of genome region coding for M2-protein transmembrane domain of rimantadine-resistant strains revealed mutations leading to amino acid change in 30, 31 positions responsible for rimantadine resistant phenotype as well as sense mutations in adjacent regions of M2 protein. Recent advances in epidemiology and virology as regards to the problem "Strengthening of Global Influenza Surveillance" require development of new, simple and inexpensive

methodological approaches which could be used for practical network activity of virological laboratories. The focus will remain on implementing sustainable technologies. IFA as a sensitive test recommended by WHO [8] for diagnosis of influenza and five other clinically important respiratory viruses was used for many years in Russia for determination of etiology of both sporadic ILI and outbreaks of respiratory morbidity. This test provides for possibility of determination of etiology of about 30%-40% of ARVI. A total of 23 264 patients with ARVI were examined by IFA for the season 2006-2007 in 45 RBL. Results of IFA monitoring for influenza correlated well with the results of virus isolation but beginning of circulation of influenza viruses recognized by virus antigens detection in clinical materials from patients with ARVI was registered at low level (0,1-1,5%) long before the first cases of influenza virus isolation. Rate of IFA diagnosis increased at peak of epidemic up to 5,6-25,1 % in different seasons. Analysis of IFA data obtained for the several years showed a decrease of influenza diagnosis rate (down to 6-8%) during the last three epidemic seasons (from 2004 to 2007) in comparison with the period since 1995 to 2001 when the rate of influenza diagnosis reached 15-25% (fig.2).

Figure 2. Etiological monitoring of season influenza using IFA data in Russia for period since season 1995-1996 to season 2006-2007.



Epidemic events developed on the background of uninterrupted circulation of parainfluenza viruses, adenoviruses and RSV, diagnosis rate of which was estimated in different seasons as 7,5-10,4%, 5,4-8,9% and 4,6-5,8% of investigated patients correspondingly. The panel of new MAb to epitopes in HA1 molecule of influenza A(H5N1) virus with high specific activity both in hemagglutination inhibition test, IFA and ELISA was developed at RII recently. These MAbs were used in IFA for rapid virus antigen detection of influenza A(H5N1) in infected cells and could be used for identification of influenza A(H5N1) virus in HI and ELISA as well. In addition, broadly reactive MAb to NP of influenza A and B viruses suitable for IFA and ELISA were developed taking into account both antigenic drift of contemporary influenza A virus, high probability of new pandemic subtype emergence and co-circulation of two evolutionary sub-lineages of influenza B virus needed to be indicated. "Amplisens" kits for RT-PCR of influenza A, H5 and H7 testing and determination of NA subtype were developed at

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the Central Institute of Epidemiology (Moscow) and distributed widely in Russia. Early identification of A(H5N1) virus during epizootics among birds in Siberia and European part of Russia in 2005-2007 was performed successfully. Determination and analysis of full genome sequences revealed similarity of Russian isolates to Quinghai group of influenza A(H5N1) viruses (clade 2, subclade 2) [9,10,11]. Supply of laboratories with materials and equipment for real-time RT-PCR along with conducting of seminars for laboratory staff training will contribute to further improvement of laboratory surveillance and will increase preparedness level to future influenza pandemic.

Discussion

During 2006-2007 season strengthening of influenza surveillance in Russia was registered in comparison with previous period 1995-2005. As a result of improvement of investigations on virus isolation number of isolates was increased 2-3 times and geographical coverage of surveillance was enlarged. Obtained regular data on diagnosis of influenza and other ARVI testify for existence of some correlations between morbidity growth, increase of rate of influenza virus isolation and virus antigens detection by IFA at the beginning of epidemic. Prognostic significance of laboratory data as regards to early determination of epidemic start requires further investigations. Both IFA and RT-PCR results can supplement each other in different conditions in practice and would be of great importance for early recognizing of pandemic virus emergence and spread especially in regions of Russia where investigations on virus isolation are not yet conducted. Necessity to monitor the situation closely during phase 3 of pandemic using both local and regional epidemiological analysis and laboratory surveillance exists [12]. Use of apathogenic influenza A(H5N1) vaccine strain opened the additional possibility to conduct serological surveillance for avian influenza in laboratory practice which can be performed in BSL-2 condition using hemagglutination inhibition and microneutralisation test in addition to RT-PCR and IFA analysis.

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NAMRU-3 Outbreak Response to Seasonal and Avian Influenza

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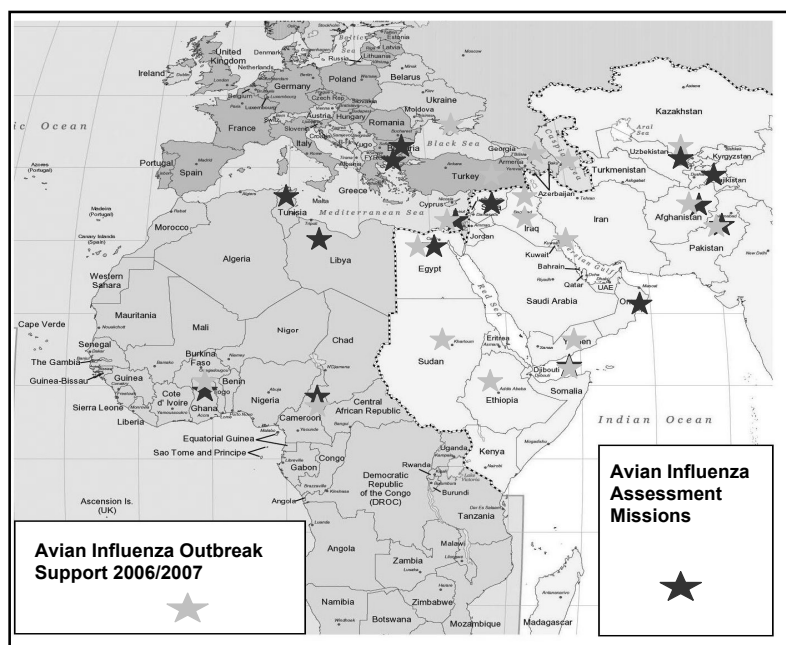
The U.S. Naval Medical Research Unit No. 3 (NAMRU-3) serves as the World Health Organization - Eastern Mediterranean Region Influenza Reference Laboratory and H5 Reference Laboratory. As such, NAMRU-3 has developed outbreak support platforms to project diagnostic capacity to countries within Africa, the Middle East, and the Central Asian Republics. As a member of the World Health Organization Global Outbreak Alert and Response Network, NAMRU-3 is continually poised to provide support to those countries in need. NAMRU-3 is comprised of diverse scientific staff (i.e. physicians, veterinarians, epidemiologists, microbiologists, etc.), which allows for a country-specific response based on individual scenarios. In addition to personnel, NAMRU-3 deploys diagnostic supplies, personal protective equipment, and the Idaho Technology Incorporated Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.[™]) real-time polymerase chain reaction (RT-PCR) platform as it has proven reliable in harsh environments. From January 2006 through February 2007, NAMRU-3 has supported 16 outbreak investigations with varying levels of support. Most noteworthy was a three-week response mission in Azerbaijan beginning in March 2006 resulting in the rapid identification of five positive H5N1 cases as judged by RT-

PCR. In addition to avian influenza, NAMRU-3 provides on-site diagnostics for seasonal influenza outbreaks, such as was experienced in Jordan in November of 2006. Over the course of two months, NAMRU-3 trained Jordanians and collected 138 specimens resulting in 24 (17%) influenza isolates all typed as Influenza B (B/OHIO/01/2005-LIKE). NAMRU-3 is positioned centrally in a region of the world that has limited advances in terms of laboratory diagnostic infrastructure. Nevertheless, there is a clear need for support as highly pathogenic avian influenza continues to devastate the poultry industry, poses a health risk to exposed humans, and elevates the chances of development into an eventual pandemic strain. By providing outbreak support, NAMRU-3 has been able to readily identify the etiological agent while simultaneously providing training, bio-safety upgrades, assessments of preparedness, and specimen collection kits. These initiatives are geared to initiate sustainable surveillance systems utilizing NAMRU-3 as a regional reference lab. Efforts such as these should serve as a model for other capable laboratories worldwide.

Introduction

Over the past 1-2 years, highly pathogenic avian influenza (AI) H5N1 has spread across Southeast Asia, Central Asia, Africa, the Middle East, and parts of Europe. To reduce the spread of AI, it is imperative that countries establish rapid laboratory diagnostics and implement control measures as needed. Diagnostic capacity throughout the region is significantly lacking and thus it is critical that organizations like NAMRU-3 be prepared to provide forward laboratory diagnostic support. The U.S. Naval Medical Research Unit No. 3, originally founded in 1946, is the largest overseas military medical research facility

Figure 1. NAMRU-3 regional avian influenza outbreak support, 2006 - 2007.



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and is located in Cairo, Egypt. NAMRU-3's mission is to conduct infectious diseases research, including the evaluation of vaccines, therapeutic agents, diagnostic assays, vector control measures, and to carry out public health activities, principally aimed toward improved disease surveillance and outbreak response assistance. Current activities include human seasonal and avian influenza surveillance, outbreak support, migratory bird surveillance, training initiatives, laboratory design consultation, and biosafety enhancements in over 20 countries in the Middle East, Central Asia, and Africa. NAMRU-3 serves as a World Health Organization Influenza Reference Laboratory serving the Eastern Mediterranean Region (WHO-EMRO) and has recently been named the 9th WHO H5 Reference Laboratory worldwide. In support of an outbreak response, current equipment and staffing allow for up to four diagnostic teams to be deployed simultaneously with sufficient reagents and cold storage to last up to three weeks prior to re-supply. In addition to a molecular-based diagnostic AI response, NAMRU-3 provides support for suspected seasonal influenza outbreaks. This support includes personnel with culture and viral characterization expertise, required materials and reagents and onsite training on case definition enrollment, specimen collection, handling, and storage. Over the last ten years, NAMRU-3 has provided outbreak support for 69 disease outbreaks in 25 different countries.

Azerbaijan Avian Influenza Outbreak. On March 4th 2006, six patients were referred to the Scientific Research Institute of Lung Diseases Hospital in Baku for suspect avian influenza. Two days later, the Ministry of Health (MoH) in the Republic of Azerbaijan reported an additional cluster of nine suspect cases of human avian influenza (HAI), including two deaths, to the WHO Regional Office in Europe. The suspect cases, eight from the same family and one from a neighboring family in Daikyand settlement in Salyan District, became ill over a two-week period with dates of onset of illness between February 15th and March 4th. On March 13th, a NAMRU-3 field laboratory with real-time polymerase chain reaction (RT-PCR) capacity to detect influenza A/H5 virus was dispatched, which subsequently established a forward site at the Anti-Plague Station in Baku. On the same day, throat swabs and/or blood samples were obtained from 11 suspect HAI cases. Samples from an additional 20 suspected individuals and 32 contacts were tested. All samples were also sent to the National Institute for Medical Research for confirmation (NIMR), Mill Hill, United Kingdom for further analysis. Influenza A/H5 virus infection was detected in samples from five deceased individuals (including Salyan and Tarter district) and from eight of the suspect AI cases.

Seasonal Influenza Outbreak in Jordan. On December 11th 2006, the MoH in Jordan requested laboratory support in evaluation of a suspect influenza outbreak. NAMRU-3 responded by sending supplies and personnel to conduct training on the case definition, throat swab collection, and proper storage of specimens in viral transport medium. Specimen collection started on December 12th in two primary healthcare centers, one

in Amman and the other 75 Km north of Amman, as both sites serve densely populated areas. Throat swabs were obtained from patients with influenza-like illness and transported to NAMRU-3 through courier service. Specimens were inoculated in MDCK cells and incubated at 37°C in CO₂ incubator. Influenza isolates were sub-typed using hemagglutination inhibition (HI) kits provided by WHO Collaborating Center for Reference and Research on Influenza, Centers for Disease Control (CDC), Atlanta, GA. Isolates were subsequently sent to the CDC to be included in the seasonal influenza vaccine decision-making process. Between December 12th 2006 and January 21st 2007, a total of 138 specimens were collected and isolates were obtained from a total of 24 (17%) specimens and all confirmed as Influenza B (B/OHIO/01/2005-LIKE).

Conclusions

The threat of pandemic influenza combined with the potential socioeconomic and public health impact of avian influenza highlights the need for rapid identification and control programs as containment strategies are time dependent. This makes the development of regional and in-country rapid response teams required for a greater chance of success.

NAMRU-3 has developed an inventory list for a forward molecular laboratory outbreak support for avian influenza including the following equipment and supplies: *AI Molecular Diagnostic Forward Lab equipment.* Ruggedized Advanced Pathogen Device (R.A.P.I.D.); Microfuge; Dry shippers; Reagents/Supplies for a three week mission; Qiagen RNA extraction kit; Personal protective equipment; Additional support gear. NAMRU-3, through a trilateral agreement with WHO-EMRO, is responsible for culturing and characterizing seasonal influenza virus circulating each year and to send these isolates to CDC to be included in the seasonal vaccine decision-making process. As part of the agreement, NAMRU-3 supplies participating countries with collection material and WHO HI reagents. During the 2006 seasonal influenza outbreak, NAMRU-3 provided additional training as well as the following equipment for sample collection:

Seasonal influenza inventory for outbreak support. Viral transport medium; Throat swabs; Patient clinical form; Liquid nitrogen containers; Personal protective equipment; Dry shippers. The above data describe NAMRU-3's capacity to respond to outbreaks in both a timely and efficient manner. NAMRU-3's capacity to respond to outbreaks has significantly increased over the past several years while mobilizing resources and conducting field and lab assessment in more than 14 countries.

Disclaimer

The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Department of the Navy, Department of Defense, the United States Government, or the Ministries of Health of Jordan and Azerbaijan.

Development of a Clade 2.3 H5N1 Avian Influenza Virus for Pre-Pandemic Vaccine Clinical Trials

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As a result of the continuous circulation of H5N1 among poultry and other livestock, the H5 hemagglutinin has evolved into multiple distinct antigenic groups. The most recently identified group, named clade 2.3, caused human infections in Anhui province of China and Lao People's Democratic Republic. Reassortant virus Anhui/01/2005(H5N1)-PR8-IBCDC-RG5 (Anhui/PR8) was produced in compliance with WHO guidelines and under Good Laboratory Practice (GLP) specifications. The virus contains 6 internal genes from A/Puerto Rico/8/34 (PR8) and the NA and modified HA genes from A/Anhui/01/2005(H5N1). Anhui/PR8 reassortant virus grew to very high titers in embryonated eggs and was trypsin-dependent for the formation of plaques in chicken embryo fibroblast (CEF) cells. Pathogenicity of the reassortant virus was evaluated using mammalian (ferrets) and avian (chickens) animal models and was confirmed to be non-pathogenic. In conclusion, our results suggest that Anhui/PR8 can be used as a candidate vaccine virus for potential use as pre-pandemic vaccine.

Materials and Methods

Influenza virus A/Anhui/1/2005 (H5N1) is a highly pathogenic avian influenza first isolated from human in China. All correlative viral study was performed in BSL-3 laboratory. The reassortant virus was rescued by transfection of the 2 viral plasmid DNAs which are a modified HA and NA with 6 PR8 internal genes into certified Vero cells. Anhui/PR8 reassortant virus was tested trypsin-dependent assay on chicken embryo fibroblast (CEF) cells. Pathogenicity of the reassortant virus was evaluated using mammalian (ferrets) and avian (chickens) animal models.

Results

Grown characteristics of reassortant virus Anhui/PR8-RG5. The reassortant virus Anhui/PR8-RG5 has a high yielding characteristics on eggs. The HA titers of reassortant virus are 1:640, 10^{8.5} EID₅₀/mL, 10⁹PFU/mL.

Sequence analysis of the reassortant virus HA gene. The HA and NA genes of rescued virus are identical to those of the parental virus except for the modified HA cleavage site where 4 basic amino acid (R-R-K-R) were removed.

Trypsin-dependent plaque formation on chicken embryo fibroblast (CEF) cells. The reassortant virus had approximately the same PFU as the wild type virus on CEF cells in the presence of trypsin. In contrast, the reassortant virus failed to form plaques on CEF cells lacking of trypsin.

Table 1. Trypsin-dependent plaque formation on chicken embryo fibroblast (CEF) cells.

Virus	Plaque formation on CEF cells			
	With Trypsin		Without Trypsin	
	PFU/ml	Diameter (mm)	PFU/ml	Diameter (mm)
Anhui01-PR8-RG5	10 ⁹	3	≤1	NA*
A/Anhui/01/05	10 ^{9.3}	2-3	10 ^{9.3}	2-3

Pathogenicity testing in chickens. All eight of the eight chickens inoculated with Anhui01/PR8-RG5 were alive at the end of the 14-day observation period. In contrast, the mean death time for the wild type group was 1 day. All dead birds showed pathologic signs typical of HPAI.

Table 2. Pathogenicity testing in chickens.

Virus	Morbidity	Mortality
Anhui01/PR8-RG	0/8	0/8
A/Anhui/01/05 Wild type	8/8	8/8 (1day)
A/PR/8/34-RG	0/8	0/8

Safety testing in ferrets. None of the ferrets infected with either Anhui01/PR8-RG5 or PR8-RG showed signs of lethargy, anorexia, respiratory or neurological symptoms over the 14 day post infection. The Anhui01/PR8-RG5 virus was not detected in the spleen, brain, or whole blood of any inoculated ferrets.

Conclusion

The Anhui/PR8-RG5 virus has a high yielding characteristic in eggs, which meets an important criteria for pandemic vaccine. The low pathogenic characteristics was confirmed in vitro and in vivo. In summary, the reassortant virus can potentially be used as a pre-pandemic vaccine.

How is Influenza Transmitted? A Review of the Scientific Literature

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This paper examines the modes of transmission of influenza as indicated in the scientific literature. A systematic review of the evidence for aerosol transmission of influenza identified twelve studies, none of which were able to demonstrate that influenza was spread through this route. Observations of the spread of influenza in household and hospital settings show a pattern consistent with droplet transmission. High household attack rates and transmission in the absence of close contact have not been shown for influenza as they have for other well established airborne viruses. Taken together, this evidence indicates that influenza is transmitted through the droplet/contact route and not through aerosols.

Introduction

Infection control in health care settings should be based on modes of transmission of infectious agents in human populations and in these settings. Despite having a longstanding presence in our communities, controversy remains as to how influenza is transmitted from person to person. Several authors have concluded that droplet and contact transmission are the primary modes of spread of influenza [1-3]. Droplet spread of influenza involves the transmission through larger respiratory particles that do not remain airborne for long. Respiratory particles of > 5µm are generally considered droplet-sized [2]. Because these particles settle quickly, close contact is required for transmission, with close contact usually being defined as exposure within approximately 1 meter [1-4]. Contact transmission involves direct transfer of influenza virus from an infected person or fomite. Other authors emphasize the importance aerosol spread as a possible mode of transmission of influenza [5-9]. Spread of infection via the aerosol route involves transmission through the smallest-sized respiratory particles that are less subject to gravitational deposition, and can remain infectious and travel over long distances. The hallmark characteristic of an aerosol spread infection is transmission in the absence of the requirement for close contact. A small number of infections are believed to be capable of being spread via aerosol (e.g. measles, tuberculosis, smallpox). If influenza is transmitted through the aerosol route, N95 respirators (fit-tested to all health care workers) would be required in order to filter out small-sized respiratory particles in order to prevent transmission. Special air handling systems and UV radiation are also components of infection control for aerosol-spread infections. In contrast, infection control for droplet and/or contact-spread pathogens is relatively straightforward through droplet precautions, which

include hand hygiene and the use of gloves, surgical mask and other protective clothing for health care workers involved in patient care. The differences in resources for infection control entailed by the different modes of transmission are substantial. As such, how influenza is transmitted is a key question for pandemic planning. This paper reviews the relative importance of modes of transmission of influenza.

Methods

A search strategy was developed and executed in three electronic databases (MEDLINE (1950-2006) EMBASE (1980-2006) and BIOSIS (1969-2006)) in order to identify all English language studies published worldwide that raised the possibility of spread of influenza via the aerosol route. Studies were identified from both electronic records and a manual search of the reference lists of review papers. Original research reports from both experimental and observational settings were included in this review. The evidence for aerosol spread provided by each study was examined in detail. Second, patterns of spread of influenza, documented in the scientific literature, were analyzed. By way of comparison, patterns of spread of influenza were compared to long term patterns of transmission for varicella (chicken pox), an infection well-established as transmitted through the aerosol route.

Results

Twelve studies evaluated the possibility of spread of influenza via the aerosol route.

Experimental evidence. Three sets of studies examined transmission animal models. Loosli et al. [9] found that mice became infected with influenza virus when placed in a chamber that had been sprayed 24 hours prior with the aerosolized virus. This experiment, however, could not rule out contact as influenza virus has been shown to survive on non-porous surfaces (such as a chamber wall or floor) for periods of up to 24 hrs [10]. Transmission studies in mice by Schulman [11, 12] and in ferrets by Andrewes and Glover [13] were at best able to demonstrate that transmission could take place in absence of direct contact in these animal models. Schulman found equal rates of transmission of influenza (i) in mice housed in direct contact (ii) where uninfected mice were separated from infectors by two ¾ inch wire mesh screens. Transmission via direct contact and large respiratory secretions would have been prevented by the wire mesh. Schulman [12] also found reduced rates of transmission in mice that were housed in direct contact under high airflow. Andrewes and Glover [13] observed where the uninfected ferret was housed three feet above an infected one and between ferrets housed at each end of S and U shaped ducts at low airspeeds over a total distance of 3 meters. Large droplets were argued not to be able to travel the required distance necessary to induce infection, suggesting a role for transmission via small respiratory particles. Notwithstanding that their results come from animal models, these studies do not rule out transmission via respiratory particles in the droplet-size ranges. In Schulman's experiments,

droplet-sized particles would have been able to pass through the mesh screens. Similarly, droplets > 5µm size range could have traveled the necessary distance to induce infection in ferrets in the experiments described by Andrewes and Glover [1]. Two experimental studies that involved human subjects were identified. Alford et al. [14] subjected 23 'volunteers' recruited from correctional institutions to 10 litres of artificially aerosolized influenza via copper tubing attached to a mask. The differences between this experimental set-up and transmission in a real world setting are clear. Little et al. [15] found that subjects inoculated with influenza virus via large drops into the nose experienced a milder illness compared to those infected with wildtype virus in the natural environment. Deposition of large drops of solution containing influenza virus to the nasal passages was argued to model contact and/or droplet transmission. The inconsistency between infection through this method and clinical features of wildtype influenza has suggested to some [8] that lower respiratory tract must be the site of infection for influenza. Since the lower respiratory tract is only accessible via small respiratory particles, aerosols are argued to be important in transmission [8]. However, the differences in the symptoms between the two groups is more likely explained by differences between laboratory-adapted strain versus the actively circulating wildtype strain, than the methods of inoculation. Additionally, Little's study does not rule out transmission of influenza via droplet-sized particles accessing the mid-respiratory tract. Histopathological studies documenting the larger tracheobronchial airways as the main area of inflammatory response and pathological change associated with influenza infection [16,17] indicate that the mid-respiratory tract is the site of infection. This area is accessible by droplets of the > 5 µm size range. Furthermore, that the sialic acid alpha 2,6 galactose receptors for human influenza virus are located along tracheobronchial tree is also supportive of a site of infection along the mid-respiratory tract [18].

Observational evidence for aerosol transmission. Three influenza outbreaks on airplanes made mention of or were cited as evidence for aerosol spread. Moser et al [19] reported an outbreak in which a single index case infected as many as 38 of 52 other passengers (attack rate =72%) over a 4.5 hour period of exposure aboard a grounded aircraft. This outbreak is frequently cited as evidence of aerosol transmission with the argument that a single index case could not have infected so many others over such a short period of time if influenza was spread through a route other than aerosol. Yet, investigators report that the position of the highly symptomatic index case aboard the aircraft, adjacent to the lavatory, coat closet and buffet, put her within 1 m distance of the other passengers, who frequently accessed these areas during the period the aircraft was grounded [20]. In this situation, droplet transmission was as or more likely. Two other high attack rate aircraft outbreaks have been reported since Moser's 1979 outbreak [22,23]. Neither demonstrated aerosol-spread due to a high number of symptomatic cases aboard the aircraft [22] and lack of

laboratory confirmation of influenza infection for any persons involved, including the index case [23]. The outbreak situation reported by Moser has not been repeated over many years of aircraft travel, as could be expected if aerosol transmission were possible. Four studies were identified from health care settings. MacLean [23] found that only 2 percent of patients in a hospital ward equipped with UV lighting developed influenza as compared with 19 percent of patients in an adjacent nonUV ward (and 18 percent of health care workers) in the 1957/1958 epidemic. Some authors [8] contend that UV light would have only disinfected aerosol-sized particles. Since disinfection of only aerosol-sized particles prevented the outbreak, this report is argued to speak to the importance of aerosol transmission. However, it is unknown whether the UV lighting would have only disinfected aerosols. Furthermore, the difference in rates of infection between the UV and nonUV wards could have easily be explained by other factors or to chance as MacLean's findings are from a single outbreak in a single institution, rather than from a formally controlled study. The three other outbreak reports [24-26] from health care settings mentioned the possibility of aerosol transmission in their discussion but none were able to establish if or how transmission via this route occurred.

Patterns in transmission of influenza: evidence from household and hospital setting. Patterns of spread of influenza in the household and health care settings provide insight as to how this virus is transmitted. Six randomized controlled trials of influenza antiviral prophylaxis in home based family studies were identified that provided good estimates of influenza attack rates in the household. The household attack rates of influenza in the placebo arms ranged from 14-19% [27-32]. In contrast, estimates of attack rates of varicella in susceptible household contacts are in the range of 61-100%. An attack rate is a function of contact patterns and level of immunity, in addition to mode of transmission. Yet, the difference in attack rates cannot be attributed to contact patterns as estimates for varicella and influenza come from the household setting. The difference in attack rates is also not likely attributable to differences in pre-existing immunity as the influenza antiviral trials reported only a minority of subjects having a prior level of immunity [27-29, 31, 34]. The difference in rates can likely be attributable to transmission.

Transmission of influenza in the hospital setting. Several reviews of influenza outbreaks in hospitals [3, 38, 39] have noted the rarity of such events despite the widespread prevalence of this disease in the community every year. A detailed review of four of the largest reported nosocomial outbreaks [40-43] indicates the same general pattern of spread: from health care workers to patients, likely through close contact involved in patient care. There were no observations of spread between patients in absence of close contact, as would be expected if transmission via the aerosol route were possible. In contrast, transmission of varicella in hospital has been confirmed in absence of close contact and in situations where the index case was completely isolated from all secondary cases [44,45].

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Conclusions

There is little reliable data to indicate that spread of influenza via the aerosol route is possible. In contrast, well-documented observations of the spread of influenza in household and hospital settings demonstrate a pattern consistent with droplet spread. The relatively low household attack rates for influenza are in stark contrast to those for varicella [14-19 vs. 61-100%]. The pattern of spread of influenza in hospitals, i.e. of close contact transmission propagated by health care workers involved in patient care, is also most consistent with droplet spread. Additionally, transmission in absence of close contact is well documented in the hospital setting for varicella, while this has never been seen for influenza. Given the long historical record of influenza in the human population, if transmitted via aerosol route, even occasionally, conclusive evidence to this effect should already have been established. Infection control measures which would normally be implemented to prevent aerosol transmission are not warranted based on the scientific evidence.

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Barriers to the State and Local Public Health Management of Pandemic Influenza

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Introduction

Since, 2001, the United States has dedicated significant financial and personnel resources preparing the public health system for the next bio-emergency, including pandemic influenza. However, recent events in the United States following the September 11, 2001 terror attacks including, anthrax, SARS, Katrina, vaccine shortages, emerging food safety issues, ongoing threats of bioterrorism, as well as the possibility of influenza pandemic, have led to the questioning of America's ability to manage such a crisis. Public health and private medical practitioners agree on the need for the necessity of preparedness. However, pandemic influenza 'fatigue' is spreading across the nation, resulting in less focused attention on planning efforts on the part of medical practitioners, the public health community, politicians and the public. Pandemic planning involves the need for rapid vaccine development, enhanced surveillance methodologies, strategies to contain or slow the epidemic and carefully crafted and consistent risk communication messages. The most urgent planning need is development of a strategy to rapidly produce an effective vaccine for the country and the global populace. In addition to the need to respond humanely to a global crisis, the interconnectedness of the global economy today mandates that preparedness efforts transcend geographic boundaries. Although there are many reasons to applaud the hard work and financial resources dedicated to planning efforts to date, response to other crises, including natural disasters within the recent past, suggest that the system may not fare as well as one would hope in the face of an influenza pandemic.

Pandemic Influenza Vaccine Master Program

An effective vaccine should serve as the cornerstone of our large-scale preventative strategy against pandemic influenza. In the United States, it has been suggested that a master program for pandemic vaccine development be established that matches the scale of the Manhattan project. The purpose of the Manhattan project was to develop, test, produce and deliver a weapon to win World War II. Although the instructive analogy is somewhat troubling, it is clear that maintaining the "business as usual" approach to vaccine development that has characterized pandemic vaccine development to date, needs to be abandoned if we are to win the war against a pandemic. The widespread use of a pandemic vaccine should be the central strategy for protection of human health during a pandemic event. The federal government should lead this effort by working with public and private partners and engaging the international community to outline a comprehensive approach

that will systematize, coordinate, and strengthen vaccine research and development for a universal influenza vaccine, increase production capacity, accelerate licensure, guarantee equitable global distribution, and monitor vaccine performance and safety. Further, there is a need to think globally within our vaccine manufacturing and distribution efforts. A vaccine strategy cannot be limited to use within the industrialized countries. The "have not" countries must be ensured they too will have equitable access to the interventions that will be needed. At the public health level of implementation, revamping of influenza priority groups and adherence to vaccine priority group recommendations will be a challenge. The biggest hurdle for public health will be in determining how to operationalize a vaccine priority ranking, especially when there is an insufficient vaccine supply to cover the priority groups. It will be traumatic to turn those designated as prioritized persons away from vaccine clinics. Further operational challenges will include such questions as whether documentation of occupation or age will be required at the point of service. In a mass vaccination setting, it is also questioned whether it will be possible to verify chronic medical conditions that may qualify a person as a priority vaccine recipient in the absence of a medical record. At the health care level, flu shots for all health care workers should remain a goal: all health care workers should be required to get influenza vaccinations each year, or opt out in writing. Currently only 40 percent of health care workers are vaccinated—What sort of a message does that transmit to the patients we serve? "Health care professionals should be healers, not vectors".

Surveillance

In order to effectively address the existing, emerging, re-emerging and novel infectious disease threats including agents of bio-terrorism that challenge us today, we need to have active surveillance systems in place to monitor not only human health, which has been our traditional approach, but to implement systems capable of monitoring and integrating the health of animals and the environment around us. An active, viable, global early warning surveillance system of influenza like illness (ILI) is critical in identifying seasonal influenza trends and the emergence of a novel virus and its subsequent spread. Effective surveillance systems can help foster cooperative networks between public health, clinical providers and the animal health community. However, as valuable as new surveillance tools and newly evolving syndromic surveillance methodologies may be, we also need to remember that a rapid response to recent infectious disease threats, including anthrax, monkey pox and West Nile virus, was not achieved by a syndromic disease surveillance system, but by the fortunate observations of a single clinician, who understood the significance of his observation, and rapidly reported the event to public health authorities. Given the nonspecific nature of the ILI syndrome, public health practitioners are further challenged in their efforts to maintain the medical community's interest in disease reporting of unusual occurrences of what appear to be "just the flu". Resources are needed for the development and/or

enhancement of standardized surveillance systems including those that can monitor the severe end of the spectrum of ILI infection including hospitalizations and deaths. Traditional surveillance systems need to be strengthened by linking outpatient and inpatient data with laboratory reports and existing electronic health data. Despite the technological strides made in the business arena, similar advances have not made their way into the health care field. By adopting state of the art information technology, surveillance methodology could adopt a standardized and rapid assessment of morbidity and mortality that would allow for the necessary interpretation of disease trends across geographical boundaries. Attention also needs to be focused on expanding and supporting America's public health laboratory capacity, a critical element in detecting and establishing epidemiological links of any infectious disease entity. However, the following concerns and inadequacies need to be addressed before we can feel secure with the laboratory component of our surveillance system: a rapid point of care diagnostic test is nonexistent; PCR capability is only available within the Laboratory Response Network (LRN); an excessive number of clinical specimens are expected to be submitted for laboratory diagnostics in the early stages of a pandemic; anticipated shortages or unavailability of laboratory reagents is likely to ensue; a lack of surge capacity of laboratory personnel and equipment can disrupt our diagnostic efforts and current inadequacies in our clinical specimen transport system could pose significant disruptions in timely laboratory diagnostic procedures.

Community Mitigation Strategies

Recommended strategies utilized to contain or slow the epidemic will include cough etiquette, respiratory hygiene, social distancing, spatial separation; chemoprophylaxis; and work and school closures. However, the effect of such interventions will be unpredictable due to a lack of scientific data on efficacy. Given the lack of firm data to support many of these strategies, to the extent possible, such strategies should be based on evidence or expert consensus to better support the recommended concepts. Implementation challenges will include the potential 'burn out' of populations affected and the significant length of time, ranging from weeks to months, for which such measures will need to be implemented. Public cooperation and understanding of public health recommendations should not be taken for granted. Isolation and quarantine challenges will demand our concerted efforts to address. Continued attention is required to strengthen leadership, lines of authority, collaboration, issuance of national standards and guidance to enhance our intervention strategies. Given the inevitable insufficient supply of antivirals, we can also anticipate implementation challenges in the adoption of recommendations for priority group designation and distribution. Discussions surrounding priority groups must be made in an open and transparent forum, such that the public can have a better understanding of limited supplies of antivirals, as well as other medical resources. Establishing the

optimal venues for storage, distribution and administration will be required for pharmaceuticals designated for prophylactic use as well as for treatment.

Risk Communication

Nationally, we can't allow "the 100 flowers blooming" concept to evolve. It is time and resource intensive to develop individual and unique messages at the state and local level. Mixed and conflicting messages will be a risk communication catastrophe, with different messages being transmitted and heard across geographical boundaries. Conflicting information will contribute to public hysteria and confusion and result in further mistrust of government officials. Given the challenges that will be encountered in implementing vaccine and antiviral priority groups; isolation and quarantine recommendations and other community mitigation strategies, it is clear that public dialogue prior to the crisis will be crucial in fostering a more trusting, understanding and well informed public. Because many of the mitigation strategies will be controversial in nature, national leadership is needed to help resolve such issues by convening expert panels in an attempt to establish a consensus. Failure to address the need to proactively engage the local communities that will be affected prior to the event will result in an inability to adhere to the control strategies, including vaccination, community mitigation and treatment. In sum, control of seasonal or pandemic influenza will depend upon the public's understanding, acceptance and aggressive implementation of a multifaceted approach (to the extent they are available), including active surveillance, immunization, chemoprophylaxis, cough etiquette and respiratory hygiene, social distancing and spatial separation.

Conclusion

Many of the challenges in pandemic influenza planning are generic in scope and should be considered in dual preparedness planning efforts. Public health efforts have been major contributors to recent improvements in health status and can contribute even more as we approach a new century with new challenges. Pandemic planning involves building relationships and collaboration with new partners, with the potential for more attention focused on the control of seasonal influenza. The inevitable outcome of successful pandemic planning will yield an improved response to existing emerging, reemerging and intentionally transmitted infectious disease threats and other public health emergencies. Like earthquakes, hurricanes and tornados, influenza pandemics are recurring natural disasters. However, as the pandemic fails to evolve after several years of public, political and media attention, planning "fatigue" will result. In addition, a fog of confusion has settled over crucial questions such as how one measures preparedness and answering the economic costs of being prepared compared to the costs of being less prepared. Despite the many challenges posed by the next bio-emergency, we cannot afford to turn away from the difficult questions, issues, problems and potential solutions awaiting our concerted attention. Further,

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our preparedness activities cannot be a one-time effort, but must remain a dynamic, ongoing process that will need to be continually reviewed to reflect evolving scientific knowledge, technologic advances and successful international strategies and approaches. The timing and severity of the next influenza pandemic is uncertain but the danger remains very real. In a 1918 like scenario, no one state, nation or continent will succeed on their own, as our joint survival will depend on our global collaborative approach. A global outbreak of pandemic influenza will require a global approach.

Canada's National Surveillance Strategy for Pandemic Influenza and Emerging Respiratory Infections Response

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Introduction

Lessons learned from the severe acute respiratory syndrome (SARS) outbreak and avian influenza (H5N1) involving humans have underscored the importance of strengthening surveillance infrastructure for early detection and ongoing monitoring of emerging respiratory infections (ERI). Canada's pandemic and ERI surveillance strategy has focused on developing capacity for responding to outbreaks of severe respiratory illness (SRI) and implementation of pandemic plans. Developed following the World Health Organisation (WHO) guidelines during the post-SARS outbreak period, Canada's emerging and re-emerging respiratory infections surveillance strategy has evolved in order to reflect the current situation, namely developing capacity for the changing demands and information needs of other emerging SRI outbreaks, such as pandemic influenza. The objectives of Canada's national strategy for surveillance of emerging respiratory infections are: to detect, in a timely manner, unusually severe morbidity and mortality caused by both unknown and known respiratory pathogens (e.g. influenza, avian influenza, SARS-associated coronavirus [SARS-CoV]) that have the potential for large-scale epidemics or pandemics; to rapidly detect possible cases of emerging or re-emerging respiratory infections (e.g. SARS, novel influenza virus); to provide an early warning mechanism in order that available control measures may be implemented at the appropriate time to minimize transmission; and to ensure appropriate systems are in place to detect sporadic cases and clusters of emerging or re-emerging respiratory infections as required by the International Health Regulations (IHR).

Methods

To develop Canada's national pandemic and ERI surveillance strategy, a formal evaluation of the routine influenza surveillance system (FluWatch) was conducted, an SRI protocol was created, and the surveillance annex of the Canadian Pandemic Influenza Plan was developed in collaboration with Provincial and Territorial stakeholders. To assess the feasibility of the pandemic and ERI surveillance strategy, current Provincial/Territorial surveillance plans were reviewed, minimum national data elements and reporting processes were drafted, potential data collection sites were discussed, and capacity building for front line health care professionals was planned.

Results

A 3-fold early warning strategy was developed linking together routine influenza surveillance, enhanced recommendations for front line vigilance specific to evolving pandemic alert levels, and hospital-based surveillance for SRI-not yet diagnosed (SRI-NYD).

Routine Influenza Surveillance. Canada's routine influenza surveillance system is a national population-based monitoring program whose objectives are to: signal the arrival of influenza and monitor the spread and character of annual epidemics across Canadian jurisdictions; monitor circulating influenza virus strains for antigenic drift, antiviral resistance and detection of potential emerging subtypes; and contribute representative influenza virus isolates to WHO Global Influenza Surveillance Network for vaccine composition decision-making. It is a multi-component system comprising: Influenza-like illness (ILI) monitoring in the general population; laboratory detections of respiratory viruses, influenza virus subtyping and strain characterization; provincial/territorial reporting of influenza activity based on ILI, lab confirmations, and outbreaks (some school/worksite absenteeism); and sentinel paediatric hospitalizations and deaths.

Table 1. FluWatch coverage.

Per capita sentinel practitioners for monitoring of ILI	~1/130,000 population	Weekly, year round
Per capita influenza laboratory testing (avg. 00/01 – 06/07 seasons)	234/100,000 population	Weekly, year round
Proportion of influenza isolates with strain identification (avg. 02/03 – 06/07 seasons)	10.2% (range 7.4 – 15.7%) Avg N=875	Weekly, year round
Influenza activity level assessment	P/T regional level	Weekly, year round
Real-time severity indicator	Sentinel paediatric mortality & hospitalisations	Weekly, during active season

Enhanced recommendations for front line vigilance. Public health Agency of Canada alerts ('PHAC Alerts') are structured messages concerning suspected or confirmed disease outbreaks or events of public health importance that are disseminated first to provincial/territorial ministries of health and from there to front line public health professionals. These alerts contain national recommendations for public health action which may include, but are not limited to, increased awareness, increased vigilance, active surveillance, notification, or intervention. Additionally, an early detection and reporting course for health professionals is being developed to ensure front line health professionals in Canada have the capacity to detect emerging and re-emerging infectious diseases in a timely fashion and to meet new IHR requirements on the reporting of infectious diseases that could have a national or international impact.

Hospital-based detection of severe events. An SRI surveillance protocol and reporting form have been developed to outline processes for the systematic hospital detection and reporting of severe events (http://www.phac-aspc.gc.ca/eri-ire/pdf/02-SRI-Surveillance-Protocol_e.pdf). The protocol is intended for public health purposes and details the goals, objectives,

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methods and definitions for reporting SRI alerts through enhanced surveillance activities. These recommendations outline minimum national standards and guidelines, developed through a consensus process, involving public health surveillance partners at the federal and provincial/territorial levels. Pandemic Preparedness: The results of a cross country survey indicated that the development of provincial/territorial surveillance plans for pandemic influenza consistently focus on: maintaining/enhancing routine surveillance activities (FluWatch) with the expectation of some natural drop-off in participation due to a combination of absenteeism and increased demands; consideration of adapting surveillance activities to non-traditional health care delivery sites (e.g. influenza assessment centres); exploring options for collecting real-time severity indicators (e.g. hospital, mortality surveillance); and establishing/maintaining linkages between and across public health, laboratory, hospitals, etc. Challenges: Several challenges have been identified in outlining the strategy for emerging respiratory infections. Determining the balance between sensitivity and specificity for monitoring during the pandemic alert period for optimal use of public health resources has been one of the most important. Other challenges include: balancing the need for outbreak investigation and research versus routine monitoring of epidemic activity (conducting special studies versus population-based strategies); ramping up and scaling back of surveillance activities; and managing demand and competing priorities for surveillance information (e.g. public health/health care professionals, media, public, policy makers, decision makers).

GPHIN Teams; Collaborating Provincial Sentinel Networks/recruiters; Participating sentinels; Sentinel ILI systems: College of Family Physicians of Canada; National Research System.

Conclusions

The development of Canada's pandemic and ERI surveillance strategy has provided an opportunity to build on current infrastructure and establish and reinforce communication links between primary care providers and public health and laboratory professionals at local/provincial/territorial and federal levels. The system is not yet seamless; ongoing consultations and evaluation of the system will be needed to ensure plans are implemented, accepted and tested and performance barriers are identified. The immediate next steps will be to address gaps and strengthen routine surveillance activities and develop real-time mortality surveillance systems. Long term steps include: strengthening laboratory-public health-front line linkages; evaluating and testing pandemic influenza surveillance plans; developing strategies for translating guidelines into practice; continuation of the development of standardised electronic reporting methods; and operationalizing data sharing protocols and processes.

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Public Health Response to an Outbreak of Low Pathogenicity H7N3 Avian Influenza in UK, Including an Associated Case of Human Conjunctivitis

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Background

The pathogenicity of avian influenza H7N3 in humans is less well documented than for H5N1. However, there is concern about the ability of H7N3 infection to spread between wild birds and farmed poultry, and its potential for mutation from low to high pathogenicity amongst birds^{1,2}. Concerns also exist about possible interspecies transmission and the possibility of co-infection which can contribute to pandemic strain selection by genetic adaptation or re-assortment³. H7 infection was first recorded in the UK in birds in 1963 and a single case of human conjunctivitis caused by H7N7 was reported in the 1980's in the UK in an individual who had contact with domestic ducks⁴. More recently in 2004, an outbreak of H7N3 amongst poultry in Canada resulted in two human cases of conjunctivitis^{5,6} and in Italy, immunological evidence of prior H7N3 infection was found among poultry workers⁷ after several poultry outbreaks of avian influenza between 1999 and 2003. We describe an outbreak of Low Pathogenic Avian Influenza (LPAI) A/H7N3 in poultry in the UK where the first ever large scale public health response with mass oseltamivir prophylaxis was initiated by the Health Protection Agency (HPA) working collaboratively with the Department for Environment Food and Rural Affairs (Defra) and the National Health Service (NHS), UK. The Incident: In April 2006, an outbreak of avian influenza was identified on three poultry farms in Norfolk, England⁸. On the evening of 25 April 2006 Defra notified HPA of an outbreak of H7 avian influenza on a poultry farm in Dereham, Norfolk, UK. At this time the N subgroup was unknown. The farm reported a drop in egg production and a four-fold increase in poultry mortality rate among an indoor laying flock of 34,500 chickens. Restrictions were placed on the farm the previous night, along with the establishment of a control zone around the farm. On 27 April 2006, Defra established a 3 km protection zone and 10 km control zone around the Infected Premises (IP). The farm routinely operated high biosecurity measures, including 'shower in-and-out' facilities for the staff. The possible source of infection was unknown at the time. Two further infected free range poultry farms close by, each holding about 8,000; birds were later identified as infected on the 28 April 2006. The virus strain on the first farm was subsequently confirmed as low pathogenic avian influenza A/H7N3 (LPAI H7N3) by PCR from blood samples and cloacal swabs and by serology from birds on the free range farms.

Public Health Response

Decisions on the response to protect public health were co-ordinated nationally. Interventions based on current evidence were agreed upon notification after discussion between Defra and different divisions of HPA including the Centre for Infections (CFI), Centre for Emergency Preparedness and Response (CEPR) and Local and Regional Services (LaRS) Division. Local Health Protection Units (HPUs) are a part of LaRS, which is the service delivery arm of the HPA to the local population. Control of infection in poultry was co-ordinated centrally by Defra. Input from the HPA into decisions by Defra was given centrally from CEPR to the National Disease Control Centre (NDCC), and locally from LaRS to the Local Disease Control Centre (LDCC). Delivery of public health interventions to protect human health within the HPA was led locally by Norfolk, Suffolk and Cambridgeshire Health Protection Unit (NSC HPU) supported by Norfolk NHS staff. Interventions were initiated within 24 hours of notification to the HPA. Public health response to the incident involved risk assessment to humans (Figure 1), pre and post exposure prophylaxis with oseltamivir, including treatment doses for those with symptoms, vaccination with seasonal flu vaccine, 7 day follow up for symptoms of influenza like illness (ILI) and testing of acute and convalescent sera for evidence of infection in those potentially exposed. Individuals at risk were assessed by a physician from NSC HPU or from the local NHS. During this assessment, a poultry worker from the first IP was diagnosed with acute conjunctivitis on 26th April.

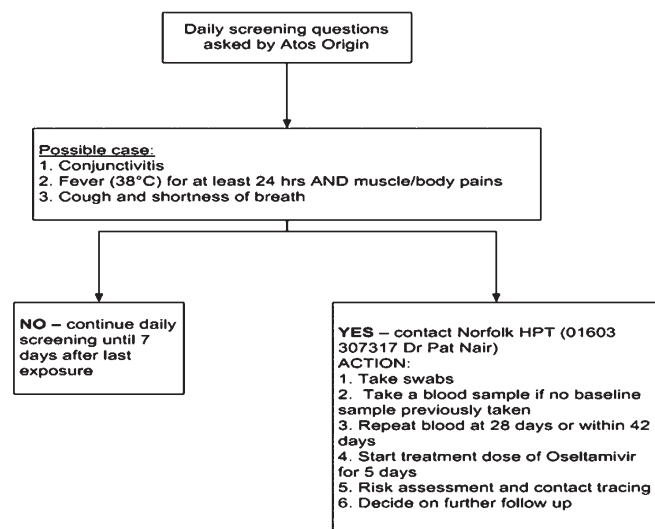
Figure 1. Risk assessment framework for exposure prone areas/activities.

- Figure 1. Risk Assessment Framework for Exposure Prone Areas/Activities.**
- All enclosed (indoor) laying / rearing areas
 - Working in environments where dust and/or aerosols might be created from infected poultry material, including slaughter and processing areas of implicated flocks
 - Primary (on-farm) sorting, grading and egg storage facilities
 - Areas where free-range rearing / grazing occurs (including a 1 metre cordon)
 - Storage area for untreated poultry litter (including: a 1 metre cordon if outdoors)
 - Care and management of poultry in enclosed sheds
 - Catching & culling of infected poultry
 - Collecting and loading of culled infected poultry for transport to disposal
 - Other activities considered to be exposure prone (eg gross contamination with poultry faecal material)

Case finding in those exposed was through 7 day symptom surveillance (Figure 2) in collaboration with Defra, whereby individuals were telephoned and asked about symptoms associated with influenza-like infection (ILI) including conjunctivitis. In addition, enhanced surveillance was initiated through local clinicians to identify others who may have had contact with the infected premises and developed ILI and/or conjunctivitis during the outbreak. Similar risk assessments and control measures were offered for individuals who were involved in the clean up process of the farms.

Options for the Control of Influenza VI

Figure 2.



Results

One poultry worker was confirmed as being infected with LPAI H7N3 using the case definition for the incident (Table 1). Conjunctival and throat swabs from this individual tested positive for LPAI H7N3 infection by PCR⁸. The case was a 29 year old immune competent worker who responded to treatment with oseltamivir. Antiviral use was contraindicated in the sole household contact, however hygiene advice given by HPA was followed and no secondary transmission occurred. Sequential swabbing was carried out for this individual and two negative samples were required from the case before returning to work.

Table 1. Case definitions for LPAI H7N3 infection in those who had contact with infected premises.

<p>Confirmed case Laboratory confirmation of LPAI H7N3, with or without symptoms.</p> <p>Possible case Individuals with the following symptoms, in the absence of laboratory evidence:</p> <ul style="list-style-type: none"> ▪ Conjunctivitis - Redness of the eyeballs with or without sticky discharge OR ▪ Fever > 38 degrees Celsius and muscle/body pains OR ▪ Cough and shortness of breath OR ▪ Other symptoms of upper respiratory tract infection (URTI)

In total, 142 individuals were considered exposed to potentially infected birds, feathers or chicken litter. Most were Defra/State Veterinary Service (SVS) staff (n=27) and independent contractors (n=32) hired by Defra for catching and culling activities. Other groups of individuals potentially exposed included incinerator workers (n=24), farm workers (n=17), private veterinary staff (n=9), residents within the 1km restriction zone (n=6), lorry drivers who transported carcasses for incineration (n=5) and additional workers involved in the clean-up of the farm (n=3). The occupation of two individuals was not determined. Of 142 individuals, 87 were exposed on an

IP, 50 off an IP. For 5 people, nature of exposure was unknown. Oseltamivir prophylaxis was taken by 90% (n=128/142) of individuals. For 9% this was post exposure, 63% on the same day as exposure and 28% pre-exposure. Seasonal flu vaccine was received by 80% (n=114/142) of exposed individuals. Of 102 individuals for whom information was available, 46 reported one or more symptoms of influenza-like illness in the week after receiving oseltamivir prophylaxis: body/muscle pain n=23, sore throat n=22, red/sore eyes n=19, cough n=25, runny nose n=16, shortness of breath n=8, temperature n=5. Fourteen people (8%) had eye, nose and throat swabs taken; 10 of whom reported influenza-like illness (including red/sore eyes (n=8) and ILI without eye involvement (n=2)) whilst two reported no symptoms. No clinical information was available on the remaining two subjects (Table 2).

Table 2. Symptoms reported during 7-day follow up.

Symptoms reported 7-days post-exposure [N=103]	
Conjunctivitis	19
Influenza like illness	41
Body/muscle pain	23
Sore throat	22
Runny nose	16
Cough	15
Shortness of breath	8
Temperature	5

H7N3 influenza virus was isolated from the throat and eye swabs of the index case which was similar to the low pathogenicity strains found in poultry in the surrounding area and was low pathogenic for chickens. Newcastle Disease Virus was detected in the material from one individual with conjunctivitis. No virus was detected in the remaining swabs. Sera were available from 91 individuals. This included acute and convalescent serum pairs from 32 people, acute sera only from 50 and convalescent sera only from 9. The convalescent serum from the index case was positive for H7N3 antibodies by microneutralisation and haemagglutination inhibition assay. No evidence of H7 infection was demonstrated from other samples. *Implications and Lessons identified.*

1. Multiagency Planning: Further discussions/actions are recommended around roles of statutory agencies to improve collaborative working locally and centrally, for decision making on interventions and delivery of such interventions. Mutual understanding of roles essential.
2. Information Technology: Development of AI contact tracing module for use nationally is a priority.
3. Staff resources for managing such outbreaks need further consideration.
4. Training: Training material to be developed to help other units to understand issues and able to respond to similar incidents.

5. Development of national guidance on all aspects of management, identify gaps and plug them.
6. Link between HPA and Defra at local level to be discussed further.

Summary

This was an outbreak in poultry affecting three farms in Dereham, Norfolk, UK, caused by low pathogenic avian influenza H7N3, that resulted in the culling of large number of birds in one laying flock and two free range farms. The source of infection was not confirmed but is thought to have been possibly introduced by wild birds into the farm⁹. This outbreak was very resource intensive, and saw the first ever large scale public health response in UK from the HPA working collaboratively with Defra and the NHS. Of the 142 individuals exposed to the infection, a 29 year old immune competent individual developed a laboratory confirmed H7N3 conjunctivitis which responded to treatment with oseltamivir without further complications. No secondary transmission occurred. Interventions offered included antivirals and seasonal flu vaccination and serological tests for evidence of transmission to humans. Nobody who received prophylaxis developed the infection and there was no evidence of person to person transmission of infection in this outbreak. There were no reported cases of ILI in the local community during this period. The major risk factor for reporting ILI in the exposed population during the surveillance period was incomplete use of PPE, details to be reported elsewhere. Prompt public health response from the HPA and the NHS may have contributed to preventing further bird to human transmission. Mutual support of different divisions of the HPA was essential for deciding on effective public health interventions of the outbreak. Collaboration between NSC HPU, local NHS and East of England ambulance trust was key in delivering the agreed interventions and preventing potential further spread. The joint HPA/Defra plan for response to avian flu was revised in the light of the lessons learnt from the first incidence of mass prophylaxis following an avian flu outbreak in UK. Other recommendations had been acted upon.

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Molecular Characteristics of Swine Influenza A(H1N1) Viruses Isolated From Humans and Animals

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Introduction

Now the world awaits the next influenza virus pandemic, that is concerned as an inevitable event. Therefore, efforts of researchers from different countries go in search for features pointing to the emergence of the next pandemic strain and look for its possible source. Different authors suggest H1N1-“swine-like” and H2N2 influenza A viruses to be potentially pandemic pathogens [1, 2]. In the past decade, besides A(H5N1), cases of influenza virus H9N1 infection [3] were recorded in humans in China, and in July 2005 a new unclear etiological agent that caused massive deaths of pigs was detected in Sichuan, 17 lethal cases among the workers of pig farms also took place [4]. There are some reports on the cases of sporadic isolation of “classical SIVs” from patients that had contacted to pigs [5-7] and also from patients that had never contacted to pigs [8-11]. Such isolates were not enough contagious to cause epidemic morbidity. However, “swine-like” H1N1 viruses were able to produce local outbreaks of influenza in 1976 in New Jersey, USA [12]. Similar “swine-like” H1N1 viruses together with H3N2 initiated seasonal increase of influenza virus sickness rate in humans in Alma-Ata, Kazakhstan in 1984. Goal and Objective: The goal of this research is to study structural-molecular characteristics of Alma-Ata H1N1 «swine-like» strains, isolated in epidemic and inter-epidemic seasons from humans and animals during several years in order to define mechanisms of activation and persistence of these strains in natural conditions.

Materials and Methods

Influenza virus isolation. Two methods were used: standard WHO method and method of interrupted infection developed in biochemistry laboratory of Institute of Microbiology and Virology of Science Academy of Science [13-15].
Nucleotide sequence. Beckman Coulter CEQ2000XL DNA Analysis System (“Beckman Coulter, Inc.”), “Beckman sequencing Kit” were used.

Phylogenetic analysis. MEGA 2.1 (PSU, USA) and GeneDoc 2.6 [14-16] was used with method of “closest neighbors”.

Figure 1. Phylogenetic tree of A/Alma-Ata/1417/84 HA gene segment HA1.

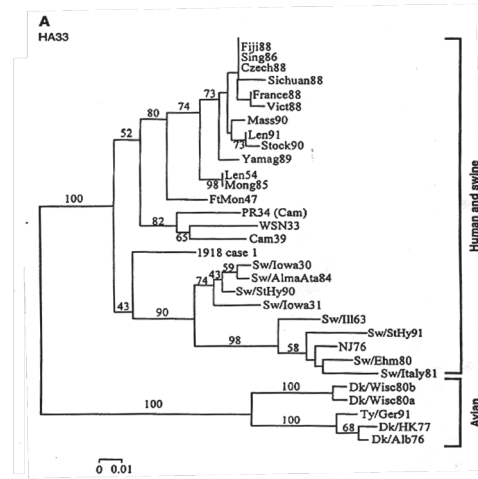
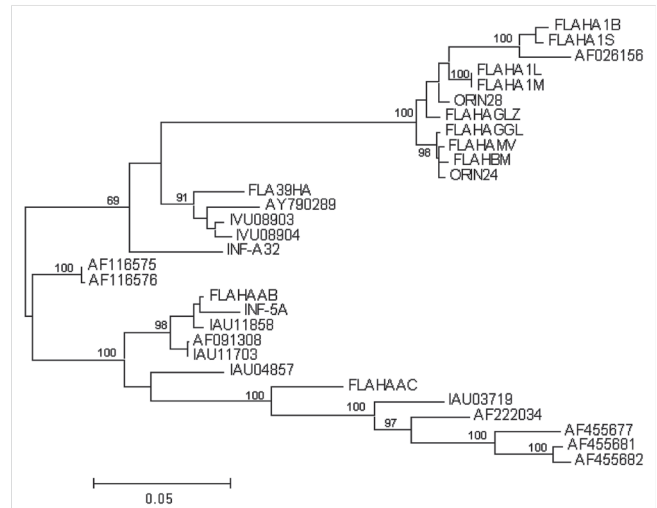


Figure 2. Phylogenetic tree of hemagglutinin gene (segment 4), A/Almaty/32/98 (INF-A32) and A/Almaty/32/98 (INF-5A).



Results

The results of oligonucleotide mapping of considerable part (1/3) of HA and NA genes of A/Alma-Ata/1417/84, A/sw/lowa/15/30, A/NJ/8/76 strains supported the results of immunological analysis of antigenic structure of surface proteins of Alma-Ata isolates. The analyzed RNA sites of the fourth segment of A/Alma-Ata/1417/84 and A/sw/lowa/15/30 strains were practically identical [15, 16] (Figure 1). Nucleotide and amino acid sequence analysis of A/Alma-Ata/1417/84 HA1 [17, 18] showed high degree of homology (98,8%) to early classical SIV A/sw/lowa/15/30 HA1. At the same time, homology between HA1 of A/Maryland/12/91 and A/sw/IN/1726/88 is 99,3% [19]. One of four amino acid substitutions in comparison to HA1 of A/sw/lowa/15/30 is localized in receptor-

binding site near Cb antigenic site. Ten amino acid substitutions are observed between HA1 of A/Alma-Ata/1417/84 and HA1 of A/sw/Saint-Hyacinthe/148/90 (including two substitutions in functional sites). Comparison of A/Alma-Ata/1417/84 HA1 with HA1 of A/South Carolina/1/18 virus revealed 25 amino acid substitutions, including 6 substitutions in functionally active sites [20, 21]. Primary HA1 structures of viruses, isolated in 1930-s and 1990-s from pigs had no substitutions of amino acids in antigenic and receptor-binding sites, whereas HA1 of human Spanish 1918 influenza and 1984 (Alma-Ata) viruses had 1 to 6 substitutions in functionally significant sites. These data may indicate possible difference between the ways of evolving H1N1 "swine-like" viruses, isolated from humans and pigs. Analysis of the variability structure of Alma-Ata (Figure 2) [22, 23] isolates against the reference strain A/PuertoRico/8/34(H1N1) let us determine most important amino acid substitutions that are localized in main antigen zones and other functionally important sites.

Discussion

The cause of "swine-like" H1N1 viruses emergence in human population in Almaty region still is not known. Taking into account the facts of emerging of pandemic influenza A type virus strains in China, the possibility of the virus penetration from the East China provinces from birds to pigs and then to humans in Almaty regions is rather high. In the third place, when it was possible to use paired sera from patients in the study, resulted in cases of positive seroconversion to "swine-like" H1N1 isolates. In the fourth place, the results were confirmed by comparison of A/Alma-Ata/1417/84 HA1 primary structure with similar hemagglutinins of H1N1 "swine-like" isolates [24]. A part of Alma-Ata H1N1 "swine-like" isolates were obtained from patients with physiological (pregnancy miscarriages, innate encephalopathies and pneumonias in newborns from mothers with virus) or acquired immunodeficiencies (post influenza serous meningitis, multifocal sclerosis, frequent respiratory diseases)[25]. The three Alma-Ata "swine-like" viruses were isolated from lung and trachea pieces of 46, 47 and 65 years old patients, died of rapid abdominal form of influenza with simultaneous cardiovascular and respiratory lesion. All the patients were countrymen and had contacted pigs [24]. Antigenic structure comparative analysis of eight Alma-Ata and two Bulgarian "swine-like" H1N1 viruses, isolated from human, and reference "swine-like" H1N1 human, swine and avian viruses, namely, A/sw/lowa/15/30, A/NJ/8/76, A/dk/Alberta/35/76, showed practically identical structure of Alma-Ata and Bulgarian hemagglutinin (HA) to A/sw/lowa/15/30, and significant differences in antigenic structure of neuraminidase (NA), which was more similar to reference human H1N1 influenza virus of 1978 [10, 7, 15, 26-28]. In the context of high homology of amino acid sequences between A/Alma-Ata/1417/84 HA1 and HA1 of swine influenza viruses isolated in 1930-s and 1990-s and HA1 of A/South Carolina/1/18 there is a question of the natural reservoir and mechanisms of long-term persistence of human influenza H1N1 "swine-like" viruses.

Genetic analysis is consistent with the above mentioned data. However, it is quite difficult to determine evolutionary links and origins of the Alma-Ata isolates. At this moment, due to absence of data on primary sequences of earlier strains, it is hard to prove and disprove these two hypotheses about the origins of the pandemic viruses and the role that circulation of Alma-Ata isolates might play in onset of new pandemic strains. This again confirms the hypothesis of re-emergence of "swine like" strains in human and swine populations and the question occurs about the natural reservoir and mechanisms of such long term persistence of H1N1 «swine like» human influenza virus.

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Multidimensional Scaling and Model-Based Clustering Analyses for the Clade Assignments of the HPAI H5N1 Viruses

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Recently, the WHO/OIE/FAO H5N1 Evolution Working Group proposed a clade designation system for highly pathogenic avian influenza (HPAI) H5N1 viruses, in which a total of 10 clades denoted as Clade 0 to 9 were defined based on the phylogenetic analysis of 884 H5N1 hemagglutinin (HA) gene sequences. In order to evaluate this clade designation system, we employed two alternative multivariate analysis methods, multidimensional scaling (MDS) and model-based hierarchical clustering, to analyze 109 HA sequences, which were selected from different representative stains and geographically diverse isolates. While the analysis results were largely in support of the clade designations defined by the H5N1 Evolution Working Group, the MDS plot showed a slightly different picture from the phylogenetic tree (i.e., there is no clear cutoff for some clades in the MDS plot). The Bayesian Information Criterion (a criterion for choosing the number of clusters) revealed the optimal number of clusters to be 10. The model-based hierarchical analysis also showed some disagreement in clade assignments defined by the Working Group. Our findings suggest further investigation of overlapping clades observed in this study might improve the proposed clade designation system.

Introduction: The highly pathogenic avian influenza (HPAI) H5N1 viruses have appeared in more than 50 countries on three continents and continue to infect humans at an unexpected rate [1]. Because of conflicting names used to describe the many lineages of the A/goose/Guangdong-like viruses, the WHO/OIE/FAO H5N1 Evolution Working Group (herein referred to as the H5N1 Working Group) recently proposed a unified nomenclature system for the HPAI H5N1 viruses based upon the HA protein gene. This unified nomenclature system is desirable since it can be applied to facilitate the interpretation of sequence/surveillance data from different laboratories and may help to avoid confusion in clade designation in future publications. Although the clade topology was found to be identical by the H5N1 Working Group regardless of which phylogenetic method (i.e., neighbor-joining (NJ), maximum likelihood (ML), maximum parsimony (MP) algorithms) was used, the groupings are not always clearly revealed by the phylogenetic tree-based method because of the algorithmic limitation of the approaches implemented for tree construction. Thus, we employed two multivariate analysis methods, multidimensional scaling (MDS) and model-based hierarchical clustering, to evaluate the proposed clade designations. MDS is a statistical method often used in data visualization for exploring similarities or dissimilarities of

samples, where the original matrix of pairwise distances is represented as being approximately equal to the distance matrix computed using the coordinates. Model-based hierarchical clustering is an agglomerative hierarchical clustering method based on maximum likelihood criteria for Multivariate Normal Fit (MVN) mixture models parameterized by eigenvalue decomposition. Both of the above methods have been applied to infer evolutionary relationships of various entities (sequences or taxa) [2-4]. The paper is organized as follows. In the Materials and Methods section, we introduce the experimental data set, the MDS method, and the model-based hierarchical clustering method. In the Results section, we present the results of the MDS analysis and the model-based hierarchical clustering analysis and compare them with those obtained using the phylogenetic tree-based methods. In the Discussion section, we discuss the implications of this study towards future research.

Materials and Methods

Experimental data set. A total of 109 sequences from the HPAI H5N1 viruses, kindly provided by the WHO/OIE/FAO H5N1 Evolution Working Group, were used for the multidimensional scaling analysis as well as for the model-based clustering analysis. These sequences were selected from vaccine strains, reference strains, many human isolates, pathogenesis study strains, and geographically diverse isolates (RO Donis, personal communication). Each sequence was approximately 1,659 nucleotides in length.

Multidimensional scaling analysis and visualization. We used the same procedure that the H5N1 Working Group used to compute the genetic distance matrix of all pairwise sequences. Briefly, the sequences were aligned with ClustalW [5] and the alignment was carefully checked and manually edited using BioEdit 7.0. The distance matrix was computed based on the Kimura 2-parameter model using MEGA 3.1 [6]. We then used the distance matrix as input to the cmdscale module in R 2.5.1 for the multidimensional scaling (MDS) analysis. The result from the MDS analysis was visualized via a 2D scatter plot, plotted using Microsoft® Office Excel 2002.

Model-based hierarchical clustering. The principle coordinates resulting from MDS were used for the model-based hierarchical clustering analysis, again in R 2.5.1 [7]. Below is an introduction of the method used. The principle coordinates are assumed to be from a mixture of multivariate normal distributions. Each distribution in the mixture represents a cluster. Given data x with multivariate observations x_1, \dots, x_n , the mixture likelihood with G clusters is,

$$L_m(\theta_1, \dots, \theta_G; \tau_1, \dots, \tau_G | x) = \prod_{i=1}^n \sum_{k=1}^G \tau_k f_k(x_i | \theta_k),$$

where f_k is the multivariate normal density of the k th cluster, θ_k are the parameter vectors of f_k . τ_k is the probability that an observation belongs to the k th cluster with $\tau_k \geq 0$ and $\sum_{k=1}^G \tau_k = 1$. θ_k consists of the mean μ_k and the covariance Σ_k

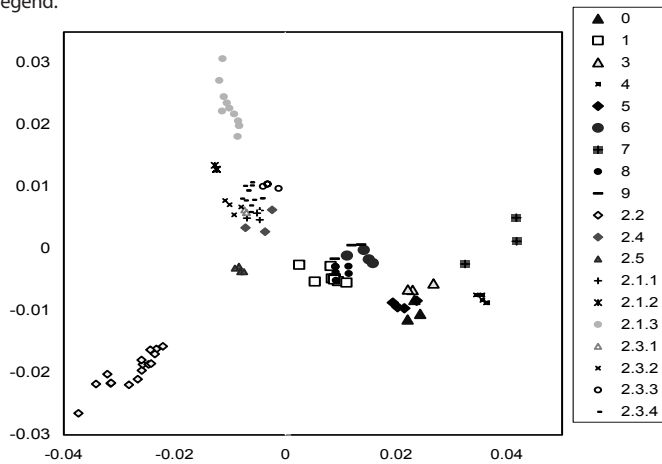
Options for the Control of Influenza VI

matrix. The covariance matrix Σ_k is parameterized by eigenvalue decomposition in the form $\Sigma_k = \lambda_k D_k A_k D_k^T$, where is the orthogonal matrix of eigenvectors, A_k is a diagonal matrix with elements proportional to the eigenvalues of Σ_k , and λ_k is a scalar. D_k , A_k and λ_k represent the orientation, shape and volume of the statistical distribution of the k th cluster, respectively. Σ_k could vary among clusters in terms of its three components. Refer to [8] for the different parameterizations of Σ_k as shown in the BIC plot. Given G and a variety of possible covariance Σ_k for the normal mixture model, the EM algorithm (the estimation-maximum likelihood) is used to maximize the mixture likelihood and estimate the parameters for the model [7]. Since each combination of a possible G and a covariance structure represents a different statistical model for the data, to select an optimal model, the Bayesian Information Criterion (BIC) is computed for each model.

Results

The multidimensional scaling (MDS) analysis. MDS analysis shows large concordance with the clade assignments proposed by the Working Group (Figure 1). For example, at the clade level, the samples from Clade 3, 4, or 7 are clustered together, but distant from samples not in the same clade (This is also true in the phylogenetic tree because these isolates are more closely related to each other due to the sharing of a common node on the tree and more distant from other clades). At the subclade level, all the samples from subclade 2.2 or 2.5 are closely clustered and all samples from sub-subclade 2.1.3 are grouped together and presented at the top of the MDS plot. It is noteworthy, however, that some clades overlap each other in the MDS plot (see Clade 0 and 5 and Clade 1 and 8).

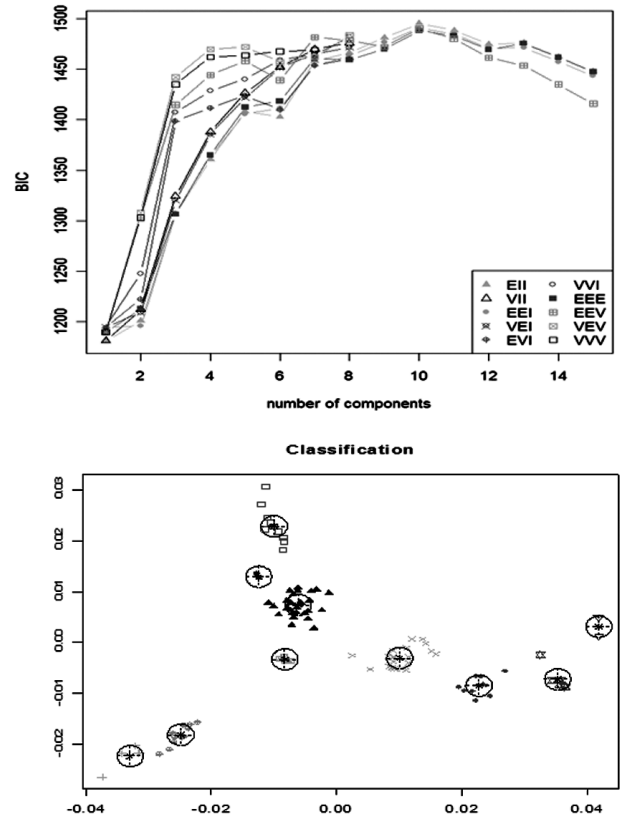
Figure 1. Multivariate scaling representation of the distance matrix of all pairwise sequences. A total of 109 samples were shown. The clades were numbered according to the clade designation of the H5N1 working group. All clades, subclades, and sub-subclades were colored, as well as shaped, as shown in the legend.



Model-based hierarchical clustering analysis. The Bayesian Information Criterion (BIC, a well-known criterion for model selection) reaches its maximum when the number of clusters

equals 10, regardless of what mode we chose (Figure 2A). Therefore, based on the BIC, the optimal number of clusters for the 109 HA sequences were 10. This observation supports the H5N1 Working Group's clade designation that defines 10 clades (Clade 0 to 9) for the HPAI H5N1 viruses. A total of 10 clusters resulted from the EII model were identified (Fig. 2B). As mentioned earlier, there was a significant correlation between the phylogenetic tree topology (data not shown) and the model-based hierarchical clustering analysis result. Regardless of whether the viral isolates were from one or more different clades, all the adjacent branches in the phylogenetic tree were found to be clustered together (e.g., Clade 0, 3, and 5). However, the 10 clusters found by the clustering analysis do not correspond entirely to the 10 clades defined by the Working Group. More specifically, one cluster defined by the model-based hierarchical clustering analysis may cross two or more clades defined by the H5N1 Working Group, and vice versa. For example, Clade 0, 3, and 5 formed a single cluster, whereas Clade 2.2 was divided into two clusters in the model-based hierarchical clustering analysis.

Figure 2. The Bayesian Information Criterion (BIC) versus the number of clusters using the normal mixture models with different covariance structures, indicating the maximum number of optimal cluster being 10 for the 109 HA sequences (A). The models are represented with character strings: For example, "EII": spherical, equal volume. The resulting plot of the model-based hierarchical clustering analysis (2B) shows 10 clusters. The EII model and the principle coordinates from MDS were used in the analysis.



Discussion

The H5N1 Evolution Working Group used different phylogenetic methods (distance-based, as well as character based) for constructing phylogenetic trees of the HPAI H5N1 viruses belonging to the A/goose/Guangdong-like lineage and developed a nomenclature system based on the resulting tree topology, while following certain conventions. In this study, we showed that by using an alignment of 109 representative H5N1 HA sequences our analysis also resulted in the grouping of the isolates into 10 clusters. Not all of the 10 clusters, however, correspond completely to the 10 clades defined by the H5N1 Working Group. Thus, our approach provides a different perspective of HPAI H5N1 clade designation and alternative models to facilitate the goal of unifying the nomenclature system to characterize these and newly emerging HPAI H5N1 viruses. In addition, the model-based clustering analysis provides a strictly mathematical point of view, while the working group clade designation relies both on phylogenetic analysis and biological support. The results obtained from the multidimensional scaling analysis and the clustering analysis are important since they suggest that further investigation of overlapping clades is warranted to develop a more clearly defined nomenclature system and may be useful in the context of H5N1 ecology, evolution, and epidemiology.

Acknowledgements

We acknowledge the WHO/OIE/FAO H5N1 Evolution Working Group for sharing the sequence data and the clade designation information. SZ and GL are grateful to the Options for the Control of Influenza VI organizers for a Travel Scholarship to attend the Conference. XF is grateful to the Dean's Office in the College of Arts and Science at the University of Nebraska at Omaha for providing the Graduate Assistantship. GL acknowledges the UCR Award from University of Nebraska at Omaha.

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Case Investigation of Suspected Human Cases of Avian Influenza in Tieviessou, Grand Lahou – Cote d'Ivoire

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Introduction

The growing epizootic and increasing number of human cases of Avian Influenza A (H5N1) has caused worldwide concern about a pandemic. Côte d'Ivoire experienced its first case of H5N1 outbreak in poultry in late April 2006. Before this current investigation, three outbreaks had been registered in Côte d'Ivoire with confirmed cases of H5N1 in poultry, but none in humans. On 6 December 2006, clinicians at Plantations et Huileries de Côte d'Ivoire (PHCI) in Tieviessou, Grand-Lahou observed abnormally high rates of patients presenting to the clinic with influenza-like illness (ILI). These patients reported the occurrence of deaths among chickens in their villages during the previous month. On 8 December 2006, the medical practitioner at PHCI informed the animal health authorities in Abidjan about the reported chicken die-offs. We report the results of an investigation into this outbreak.

Methods

Village Description. Tiéviessou/PHCI is a village with a population of an estimated 2000 persons that support timber and oil plantations situated about 100 Km from the City of Abidjan. It is a locality of 119 workers who are employed by PHCI, of which only 35 are natives of Côte d'Ivoire and the remaining majority are citizens from neighboring countries such as Burkina-Faso, and Mali.

Objective. To investigate a cluster of human cases of Influenza-like illness in the setting of chicken deaths in the village of Tieviessou. On 9 December 2006 a team consisting of a medical epidemiologist from CDC-Côte d'Ivoire, one medical epidemiologist from the National Institute of Public Health-Côte d'Ivoire and one laboratorian from the Pasteur Institute of Côte d'Ivoire travelled to investigate the cluster of cases. We interviewed the health workers of the PHCI medical care unit, consulted the medical record book in order to identify patients with ILI. We defined a case of ILI as any patient who presented to PHCI with a fever > 38°C and with respiratory symptoms such as cough, running nose or difficulty in breathing. We used a standardized questionnaire to collect information from patients who presented to the clinic with ILI on that day. Finally, we travelled to the village of Tieviessou in order to sensitize the villagers to immediately report any cases of sick or death chickens. This was done using door-to-door visits to households and group discussions with the villagers. We collected throat and nasal swabs from all patients who presented with ILI on

the day of the investigation, and from some of the patients who had presented with ILI on the previous day. Throat and nasal swabs from each of these patients were placed in the same test tube with viral transport medium, and stored in a cooler with ice packs. The swabs were then immediately shipped to the Virology Laboratory of the Pasteur Institute for testing by using the Reverse Transcriptase – Polymerase Chain Reactions (RT-PCR) technique to find out the presence of the M-Gene. The M-Gene is common to all influenza viruses and will be positive whenever Influenza A or B is present in the sample. For samples with the M-Gene positive, a Multiplex RT-PCR was conducted to find out the presence of the H5N1 subtype. Finally, positive samples were shipped to the National Institute for Communicable Diseases (NICD) of the National Health Laboratory Service (NHLS) in South Africa for cell culture on MDCK cells.

Results

From the medical record book, we observed that 109 villagers presented to the PHCI-Health Care Unit between November 29 and December 08, 2006. Of these 109 patients, 88 (81%) presented with Influenza-Like-Illness. Fifty-three (60%) were female, and thirty-five were male (40%). Of the 88 cases, the number of infants who were below 5 years of Age is 26 (30%) out of 88 patients presenting with Influenza-Like-Illness. Thirty-eight patients (43%) were aged between 5 and 20 years and, twenty four patients (27%) older than 20 (See Table 1). On the day of the investigation, eight patients presented with ILI, and during our round trip in the village, we identified a sick child with ILI. He was brought to the hospital and a throat and nasal swab was collected from him as well. Hence, nine patients were identified and throat and nasal swabs collected from each of them. A total of nine samples were shipped to the Pasteur Institute where RT-PCR was performed to identify the Influenza virus (M Gene). Of the nine samples, two samples were Influenza virus negative (M gene -), and seven samples were Influenza virus positive (M Gene +) but, H5N1 Negative. The nine samples were put into cell culture on MDCK cells at the National Institute for Communicable Diseases (NICD) of the National Health Laboratory Service (NHLS) in South Africa. Of these, two samples were identified as Influenza virus Type A, subtype H1N1, and one sample identified as Influenza virus Type B.

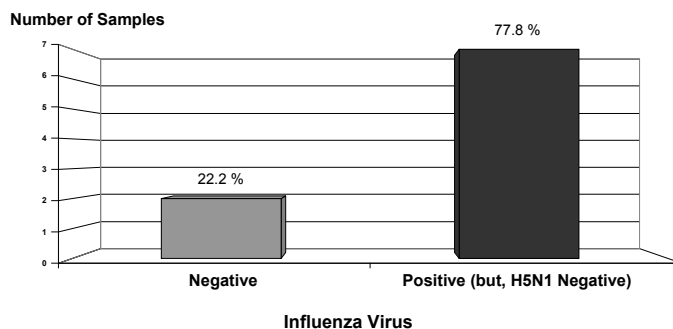
Discussions

In our investigation of a human cluster of ILI patients, in a town in Côte d'Ivoire where chickens had died, we did not identify any cases of H5N1. It is significant that some of these cases tested positive for seasonal influenza. However, avian influenza should be considered as a potential diagnosis when patients present with ILI. This is because the number of samples collected and tested was very limited (nine); we may have missed a patient with avian influenza virus. The discrepancy observed for the laboratory results between Pasteur Institute in Côte d'Ivoire and the National Health Laboratory Service in South Africa can be explained by the greater sensitivity of the RT-PCR technique

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compare to the cell culture method. Also, from the day samples were collected till their shipment and arrival in South Africa, some of the viruses may have died. Henceforth, resulting in a decrease of the viral load in test tubes before cell culture could be undertaken. The little number of samples tested during this investigation is a serious limitation to our study. Furthermore, the absence of result testing of neighborhood poultry is also a key limitation to the study. However, we could mention that, a team of veterinarians went on the field a day before our team, and all samples collected from poultry were tested negative for H5N1. Challenges are enormous when conducting an avian influenza case investigation in a rural village, in a resource-poor country. Difficulties to overcome are numerous such as; poor geographical accessibility of the village; difficulty in getting the adequate personal protective equipments (PPEs), sample collection kits with the viral transportation medium; difficulty in routing samples to the Pasteur Institute Laboratory in coolers with ice packs; difficulties in carrying out all the laboratory analysis such as cell culture, due to lack of MDCK cells; lack of a proper isolation room; challenges in sensitizing people who are relying on their chickens for protection (cultural considerations), for food and income to report sick birds without any guarantee of compensation; difficulties in communicating with the populations who think they are responsible of this situation and may be blamed; difficulty in mobilizing the investigation team within 24 hours.

Figure 1. Results RT-PCR testing for influenza virus (H5N1) of samples collected in Tieviessou.



Conclusions

Avian Influenza is and remains a great challenge in Côte d'Ivoire and in other African poor resource settings with poor human and animal surveillance systems. The deployment of a multisectorial rapid response team can be an effective approach to investigate suspected outbreaks in humans and animals.

Table 1. Age and sex distribution of patients with ILI who presented to the PHCI Health Care Unit from November 29 - December 08, 2006.

Date	Age Range			Sex		ILI # of ILI (%)
	0-4 Years	5-20 Years	> 20 Years	M	F	
11-29-06	01	02	01	02	02	04 (40)
11-30-06	03	12	03	08	10	18 (75)
12-01-06	01	02	03	03	03	06 (75)
12-02-06	00	04	02	02	04	06 (42.8)
12-03-06	01	01	01	02	01	03 (75)
12-04-06	06	05	04	06	09	15 (62.5)
12-05-06	08	05	04	08	09	17 (70.8)
12-06-06	02	02	01	01	04	05 (71.4)
12-07-06	01	03	03	01	06	07 (77.7)
12-08-06	03	02	02	02	05	07 (77.7)
TOTAL	26	38	24	35	53	88 (80.7)

Seroepidemiology of Influenza A/H5N1 Virus in the Italian Population from 1992 to 2007

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We used Single Radial Haemolysis (SRH) for detection of antibody against influenza H5 haemagglutinin, following the standard guidelines using for inter-pandemic strains. SRH assay has been standardized and validated accordingly with our internal Validation Report. A total of 1,364 sera, collected from 1992 to 2007, from Italy's population were tested against influenza A/H5N1/Vietnam/1194/2004 virus. Approximately 5% of adults and 13% of elderly were sero-protected. All positive subjects were adsorbed with influenza H1 and H3 seasonal strains and then retested, 53% of them passed from positive to negative. Our findings suggest that a small proportion of the adults and elderly population in Italy has antibodies against A/H5N1 virus. However, SRH assay probably detects antibodies against conserved regions of influenza A haemagglutinin or internal viral proteins or viral neuraminidase.

Introduction

The first documented outbreak of human respiratory disease caused by avian influenza A/H5N1 viruses occurred in Hong Kong in 1997. Avian influenza A viruses, including those that are highly pathogenic in poultry, had not previously been associated with respiratory disease in humans (1). Antibodies specific for influenza viruses are usually quantified using Haemagglutination Inhibition (HI), Virus Neutralization (VN) or Single Radial Haemolysis (SRH) (2) (3). It has been recognised that the haemagglutination inhibition assay is not sensitive for detecting human antibodies against pandemic influenza virus (4) (5). The low sensitivity of this test was demonstrated using the haemagglutination inhibition assay in subjects with confirmed A/H5N1 influenza infection, which gave a negative antibody titre, demonstrating its unreliability. Neutralization of the virus has the advantage of being a very sensitive technique, and it also has the great advantage of having to work with the live virus. The SRH test and the HI test, developed together in 1975, are currently recognized by the European Agency for the Evaluation of Medical Products (CPMP) for measuring vaccine immunity (6). The aim of the present study was to determine the presence of H5 IgG antibodies in human sera using SRH assay.

Materials and Methods

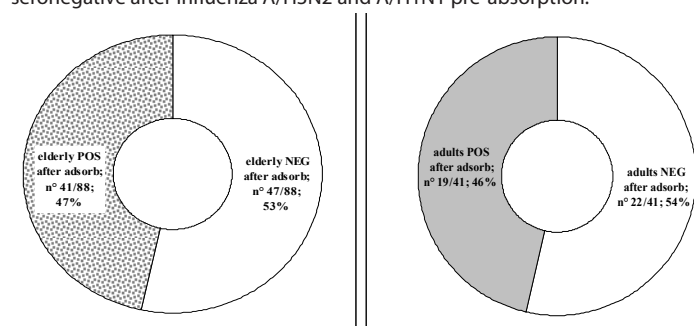
The influenza virus used in this study was the whole A/H5N1/Vietnam/1194/2004 inactivated virus at a titre of 2000 UE/ml

as the antigen for the test. The virus were propagated in embryonated hen's eggs and then purified. One thousand three hundred and sixty-four samples of sera gathered from subjects in Italy from 1992 to 2007 were studied. A minimum of 70 serum samples were collected during each year. The age of patients from which the samples were taken and analysed varied from 6 months to 92 years. The hyperimmune sheep sera A/Vietnam/1194/2004-like (H5N1), produced by the NIBSC, were used as a positive controls. Serum antibodies for A/H5N1 influenza were measured using modified Single Radial Haemolysis assay, using turkey erythrocytes instead of sheep erythrocytes. SRH immunoplates were prepared as described by Schild at all. (7). We used 0.15 mcl of Guinea Pig complement for each immunoplate. All sera were complement inactivated by heating at 56°C for 30 min, and then 6 mcl volumes were added to the wells. After incubation overnight in a moist chamber, areas of haemolysis were measured in mm². The next step of this experiment was to adsorb 6 µl of all sera with 6 µl of the mixture of A/H1N1/New Caledonia/20/99 and A/H3N2/California/7/2004 whole inactivated viruses (2000 UE/ml), in order to remove nonspecific antibodies (8). Samples with an area of haemolysis of more than 25 mm² were considered to be positive and seroprotected, if we consider the same criteria for A/H5N1 that for seasonal strains (9). The specificity of the assay was established through the titration of 20 duplicates of sera from positive controls and 20 duplicates of sera from negative controls. A pool of 10 sera from 1-year old children, gathered by laboratory analyses in 1965, were used as negative controls. The accuracy of the assay was evaluated by calculating the percentage of difference between the geometrical average of the haemolysis area expressed in mm² of the undiluted positive control sera evaluated in triplicate, and the area of haemolysis in mm² of the same sera was evaluated in triplicate and diluted at 1:2 and 1:4 in Phosphate Buffered Saline (PBS).

Results

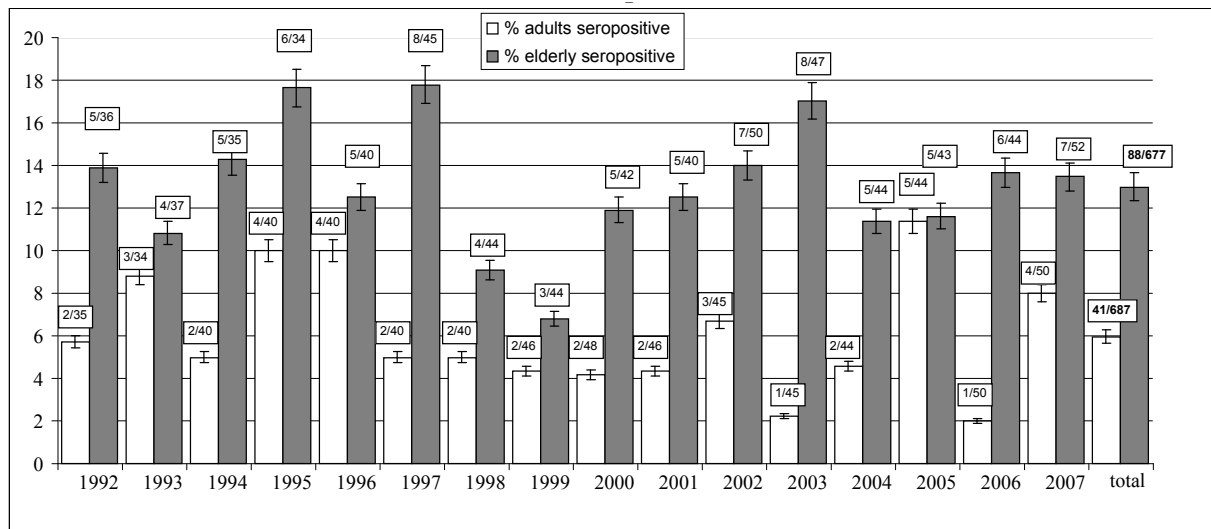
Of patients in the range of age between 0 and 64 years, 5.98 % were seropositive against the A/H5N1/Vietnam/1194/2004 virus, and this was also true for 12.99% of subjects of over 65 years of age (Figure 1). No evident difference was found between the percentages of seropositive subjects in the different years that were analysed (Figure 2).

Figure 1. Percentage of adults and elderly that passed from seropositive to seronegative after influenza A/H3N2 and A/H1N1 pre-absorption.



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Figure 2. Percentage of influenza A/H5N1/Vietnam/1194/2004 seropositivity based on year collected samples. The percentage of seropositivity for each age group is indicated. The number of serum samples positive/tested in each age group is indicated.



The seropositive subjects were analysed in order to remove non-specific antibodies, and these samples were adsorbed in a 1:1 vol mixture of A/H1N1/NewCaledonia/20/99 and A/H3N2/California/7/2004 viruses (2000 UE/ml). Of these, 53.57 % of the patients passed from seropositive to negative after this test.

Discussion

Our findings suggest that a percentage of the Italian population has antibodies against A/H5N1 virus. We have demonstrated that the SRH method probably also detects antibodies against some conserved regions of type A influenza haemagglutinin or internal viral proteins, and the role of neuraminidase in the immune response should be further investigated (10). A modified SRH assay also appears to be sensitive, specific and accurate for determining A/H5N1 antibody, but these data should be interpreted with caution. The SRH method could be used as a method for screening or confirmation. More reliable results can be obtained if they are compared with microneutralization assays.

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Report on Case of Human Avian Influenza in Shenzhen, China: Application of Field Epidemiology

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Background and Objectives

An effective surveillance system for influenza has been conducted in Shenzhen. The Ministry of Health (MOH) of People's Republic of China required all local health departments to report and manage each human Highly Pathogenic Avian Influenza (HPAI) case according to Emerging Public Health Events Ordinance (EPHEO). This paper explicated investigation on the first human HPAI in Shenzhen to clarify the application of field epidemiology in the whole process.

Methods

An emergency response system was started up to follow the probable human HPAI case detected first by the "Undefined Pneumonia Surveillance System of Shenzhen". Public health professionals administered several epidemiologic investigations and gave all contacts of the patient 7-day-long medical observation for temporally related influenza-like illness. Real-time Polymerase Chain Reaction (PCR) assay was applied to test respiratory tract samples and/or throat swabs of the patient and all his contacts specific for the hemagglutinin gene of influenza A H5N1. Hemagglutination inhibition assays and H5 antibody titer tests were carried out for serum samples from the 8th, 14th, 21st, 30th, and 60th day of disease course. H₅N₁ virus was isolated from the patient's respiratory tract samples by viral culture and genotyping for HA and NA fragments were fulfilled. Activities and strategies such as media response, notification in the public, communications with multiple related sectors, social participation and information exchange with Hong Kong and Macao were involved in field control and management.

Results

The patient was male, 31 years old, with an occupation as a truck driver for one factory, and had been residing in Shenzhen for 7 years. Starting from influenza-like illness, the patients received treatment on the 4th day of the commence from a clinic and on the 6th day from a regular hospital. On the 8th day of the disease course, he was confirmed by Shenzhen Center for Disease Control & Prevention as human avian flu case and was then transferred to Intensive Care Unit (ICU). On the 83rd day of commence, the patients was healed and released from the hospital. The patients had mainly been driving in Pearl River Triangle Region of Guangdong Province. There was no significant contact history of doubtable transmission factors

before the illness. The patient and five family members lived together, but no family member was affected. A small food market, which was under formal supervision, located near his residence. The authors tested 35 swabs from live birds and bird's coops in the market for H5 nucleic acid and all were negative. No fowl with abnormal death was proved in an 8-kilometer-range radius area near the patient's residence. During the 7-day-long medical quarantine periods, 4 contacts had fevers (above 38°C) and 2 were tested to be positive for influenza (type B). No contact had positive results for H5N1. The influenza H5N1 virus isolated for the case was named as A/Guangdong/02/2006 (H₅N₁). Characteristics of genotyping included the receptor specificity and the connecting peptide between HA1 and HA2 were revealed from avian influenza origin. Analysis on virus load showed that on the 4th week of the course virus load was almost negative. Microneutralization revealed the emergence of serum neutralization antibodies on the 15th day of commence, the peak (1:160) in the 4th week, and the decrease (1:40) on the 83rd day. Investigation process and control measures were released to the general public through the media. The case was reported via internet by the "Chinese Undefined Pneumonia Surveillance System" soon after the probable case was diagnosed by the hospital. Soon after the laboratory confirmation, information was released to the society, as well as Hong Kong Center for Health Protection. Local Departments of Agricultures, Industries & Business, and Entry-Exit Inspection & Quarantine Bureau together with the Public Health Department put up combined actions. Shenzhen Center for Disease Control & Prevention initiated computer-assisted telephone survey to investigate attitudes and knowledge of residents in town, revealing that positive atmosphere dominated and no panic existed.

Conclusions

Rapid laboratory diagnose of the virus was the key for successful treatment and survival result of the case. Still, the pathogen was from birds resources. No human-to-human transmission was observed, however infection source was unclear. Field epidemiology could offer methods for public health events response.

Food Markets With Live Birds as Source of Avian Influenza

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It has been reported that 5 patients with H5N1 infection but no history of exposure to sick or dead birds before the onset of the disease in People Republic of China. Further investigations found that 4 of these 5 patients had visited live poultry about 1-2 weeks before the disease onset. To understand the possible role of the poultry markets in transmission of H5N1 avian influenza to human, we investigated 9 markets in Guangzhou, where one of these patients had visited. Only 1 of 79 wire cages samples but none of 94 anal swabs from live birds was positive for HPAI H5N1 from the markets. Phylogenetic analysis suggested that viral hemagglutinin (HA) and neuramidase (NA) genes were very close to those identified from the patient in revolutionary relationships. One of 110 persons in the poultry business at the markets had neutralizing antibody against H5N1 virus strain whose titers against A/Hong Kong/486/97/H5N1 and A/Vietnam/1194/04/H5N1 were 1,280 and 640, respectively. This study suggests that food markets with live poultry may be a source for avian influenza infection in which healthy live birds may carry the virus.

Background

Highly pathogenic avian influenza virus (HPAI) H5N1 infected 202 persons worldwide and killed 113 as of April 30, 2006 (1). Most patients were exposed to ill or dead birds or were involved in the slaughter or preparation of birds for human food (2). However, of 19 patients with confirmed cases in the People's Republic of China, 5 had no history of direct contact with ill or diseased birds and resided in urban or periurban areas that did not have farmed birds. We studied an infected patient from Guangzhou who did not report contact with birds.

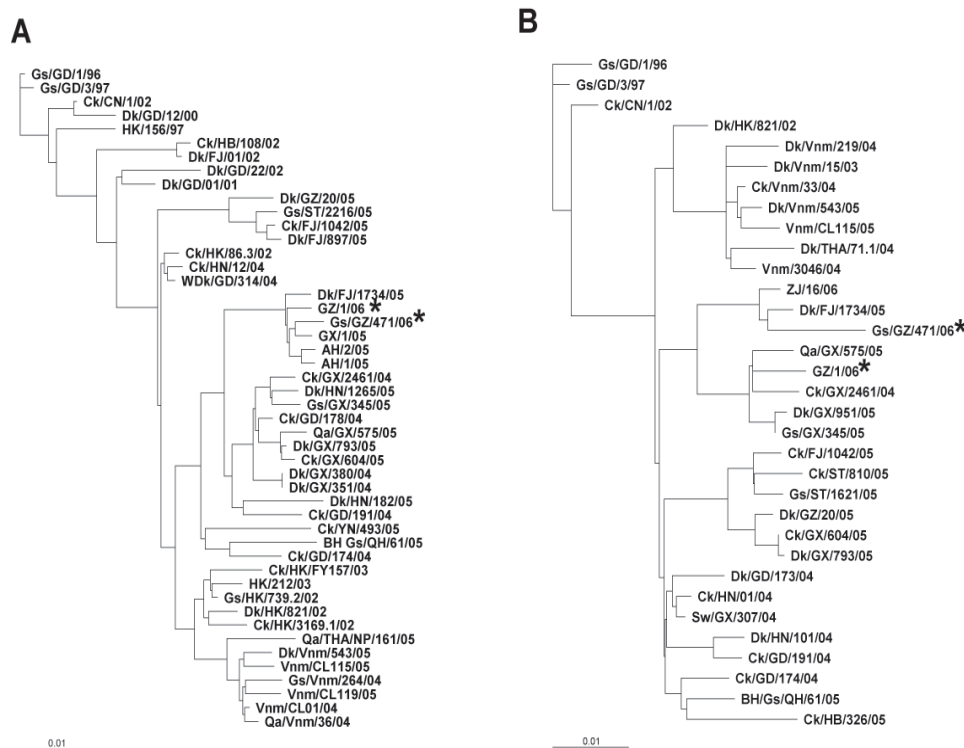
The Study

The patient was from Guangzhou, the capital of the southern province of Guangdong. A fever (39°C) developed on February 22, 2006. He was hospitalized on February 26 and died on March 2. Diagnosis of influenza virus infection was made on March 3. Throat swab specimens obtained on March 1 and 2 tested positive for HPAI H5N1 virus by reverse transcription (RT)-PCR. Virus was isolated and named A/Guangzhou/1/2006 (H5N1). Epidemiologic studies showed that the patient did not slaughter, process, or cook birds. However, while looking for

work before his illness, he visited 9 food markets that had live birds. All 9 markets were located in the central part of the city. He visited food market F twice a day from January 23 to 27 and food market G on February 17 for 30 minutes. Before his illness, he and his girlfriend (whom he lived with) shopped at markets B and F on February 20-22. He also visited food market I from February 10 to February 20. The dates he visited the other food markets could not be determined. Onset of fever occurred on February 22. The food markets were typically large, clean, and well managed and had vendors selling vegetables, fruits, raw and cooked meats, food flavorings, beverages, and other goods. They are typical of larger food markets in cities in the People's Republic of China. The only difference between markets in Guangzhou in southern China and those in cities in northern China is that more (2-9) booths are used to sell live birds in Guangzhou. Wire cages are stacked next to each other with ≈5-10 birds in each cage (chickens, geese, ducks, and pigeons). Each species of bird is placed in separate cages; chickens are the most common species. All cages are located in a closed room separated by a glass window from customers, who choose the bird they prefer. When a live bird is selected, it is slaughtered in view of the customer. Sanitation inspections are routinely performed by municipal authorities. No diseased or dead birds were observed during this investigation. Animal cages were swabbed and anal swabs of live birds were obtained at the food markets on March 3 and 4 and tested for HPAI by using RT-PCR (3) for the hemagglutinin (H5), neuraminidase (N1), and membrane (M) genes. Positive PCR results were confirmed by sequencing. None of 94 anal swabs from live birds tested positive for HPAI H5N1. However, 1 of 79 animal cage swabs tested positive for HPAI H5N1. The positive swab was from a goose cage at market I, the market that the patient visited from February 10 to February 20. The nucleotide sequences of H and M genes from specimens from this patient were compared with those from the animal cage swab and submitted to GenBank (accession nos. DQ842487-90). Forty-eight variations were found in the NA gene and 15 were found in the HA gene, which resulted in 17 NA amino acid and 3 HA amino acid changes, respectively. Phylogenetic analysis with the neighbor-joining method using the ClustalX program (4) suggested that the 2 strains are related to each other and to duck isolates (Figure). Serum samples were obtained from 110 of 121 people working at the live bird food markets and screened for antibody to H5N1 to determine if subclinical infections occurred. One of 110 serum samples was positive (titer 320) by hemagglutination inhibition assay with turkey erythrocytes (Lampire Biologic Laboratories, Pipersville, PA, USA) and H5N1 virus strains A/Hong Kong/486/97 and A/Vietnam/1194/04/H5N1 (5). Neutralizing antibody titers against the 2 strains of virus were 1,280 and 640, respectively. The positive serum sample was from a 44-year-old man who slaughtered birds for 5 years. He slaughtered ≈100 chickens/day and did not report any recent respiratory diseases. He denied any contact with ill birds.

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Figure 1. Phylogenetic relationships of representative H5N1 influenza virus strains and patient and animal cage isolates (indicated by asterisks) used in this study. A) Hemagglutinin gene (nt positions 29–1650). B) Neuramidase gene (nt positions 28–1323). Gs, goose; GD, Guangdong; Ck, chicken; CN, People's Republic of China; Dk, duck; HK, Hong Kong; HB, Hebei; FJ, Fujian; GZ, Guangzhou; ST, Shantou; HN, Hunan; WDK, wild duck; GX, Guangxi; AH, Anhui; Qa, quail; YN, Yunnan; BH Gs, brown-headed goose; QH, Qinghai; THA, Thailand, NP, Nakhon Pathom; Vnm, Vietnam; ZJ, Zhejiang; Sw, swine. (Scale bar, 0.01 substitutions per site.)



Conclusions

Our investigation suggests that the patient may have been infected by an unknown mechanism at a food market that had live birds. We detected H5N1 virus genes in a swab from a goose cage and neutralizing antibody against H5N1 in a poultry worker in 1 of the food markets the patient visited. This case from Guangzhou was not an isolated event. Five patients with H5N1 infection with no history of exposure to diseased or dead birds before the onset of avian influenza have been reported in the People's Republic of China; 4 of these 5 patients visited markets that had live birds. The first patient was a 41-year-old woman from Fuzhou, the capital of Fujian Province, whose diagnosis was made in December 2005 (6). She visited a market that had live birds 2 weeks before her illness. Another patient lived in a periurban area of Chengdu, the capital of Sichuan Province; her diagnosis was made in January 2006. She was self-employed in a shop selling dry goods at a market that had live birds in Jinhua Town in Chengdu (7). Two other patients in urban areas were reported, 1 in Shanghai and 1 in Shenzhen. Influenza was diagnosed in the patient in Shanghai in March 2006, but this patient had no history of visiting a food market that had live birds or contact with diseased birds (8). Influenza was diagnosed in the patient in Shenzhen in April 2006; this patient reported visiting a food market that had live poultry before becoming infected with influenza virus. Our findings suggest that food markets or farmers' markets that have live poultry may be a source for avian influenza infection in which

healthy live birds may carry the virus. This was previously shown in Hanoi, Vietnam, in 2001, where H5N1 virus was detected in domestic birds in a live bird market (9). Serologic investigation also demonstrated low seroprevalence of antibody against HPAI H5N1 in poultry workers from this market. However, no outbreaks of HPAI among birds were reported until early 2004 (10). H5N1 virus may be sustained in poultry largely through the movement of poultry and poultry products, especially through domestic ducks (11,12). The introduction of H5N1 virus from healthy poultry (such as ducks) may be occurring where no outbreaks in healthy flocks have been observed. Therefore, the virus is likely reintroduced at low levels and can infect persons visiting live poultry markets. The cultural preference of eating freshly slaughtered birds is not unique to the People's Republic of China; it is also common in other Asian countries. Our results suggest that the practice of selling live birds directly to consumers in food markets should be discouraged in areas currently experiencing influenza outbreaks among birds, especially in large modern cities where there may be a threat to the casual market visitor (2,13,14).

Acknowledgements

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Proceedings Topic #4

Replication and Assembly

**Poster
Presentations**

An Amino Acid on Influenza C Virus M1 Protein Affects the Virion Morphology by Altering the Membrane Affinity of the Protein

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Reverse genetics has been documented for influenza A, B and Thogoto viruses belonging to the family *Orthomyxoviridae*. In this paper, we first report the reverse genetics of influenza C virus, another member of this family. The seven vRNA segments of C/Ann Arbor/1/50 were expressed in 293T cells from cloned cDNAs, together with nine influenza C virus proteins. At 48 h posttransfection, the infectious titer of the culture supernatant was determined to be 2.51×10^3 50% egg infectious dose/ml. Second, we generated a mutant recombinant virus, rMG96A, which possesses an Ala→Thr mutation at residue 24 of the M1 protein, a substitution demonstrated to be involved in the morphology (filamentous/spherical) of the influenza C VLPs. As expected, rMG96A exhibited a spherical morphology, whereas recombinant wild-type of C/Ann Arbor/1/50, rWT, exhibited a mainly filamentous morphology. Membrane flotation analysis of the cells infected with rWT or rMG96A revealed a difference in the ratio of membrane-associated M1 proteins, suggesting that the affinity of M1 protein to the cell membrane is a determinant for virion morphology.

Introduction

For the viruses belonging to the family *Orthomyxoviridae*, successful reverse genetics was initially documented for influenza A, influenza B and Thogoto viruses [6]. On the other hand, reverse genetics of influenza C virus has not yet been reported, although we previously reported the generation of virus-like particles (VLPs) of the virus [1]. We have demonstrated that residue 24 of the influenza C virus M1 protein is involved in the formation of cord-like structures (CLS), bundles of filamentous VLPs, from the cells as well as in the morphology of influenza C VLPs [1]. Expression of M1 protein having Ala at residue 24 together with the other eight virus proteins (PB2, PB1, P3, HE, NP, CM2, NS1 and NS2) and GFP-vRNA in 293T cells resulted in the formation of CLS from the cells and the generation of filamentous VLPs, whereas expression of M1 having Thr instead of Ala at residue 24 did not lead to CLS formation, and the morphology of the VLP was spherical. However, the effect of the M1 mutation on virus replication and virion morphology remains to be clarified. To address the above question, in the present study, we first attempted to establish reverse genetics of influenza C virus. Furthermore, we report the generation of a recombinant influenza C virus possessing a mutation at residue 24 of the M1 protein, and provide evidence

that the affinity of the M1 protein to the plasma membrane affects the morphology of the virions and CLS formation from the infected cells.

Materials and Methods

Cells, viruses and antibodies. 293T, HMV-II and MDCK cells were maintained as reported previously [1, 3, 4]. The Ann Arbor/1/50 (AA/50) strain of influenza C virus was grown in the amniotic cavity of 8-day-old embryonated chicken eggs [7]. The antiserum against AA/50 virions were prepared previously [7].

Construction of plasmid DNAs. The cDNAs of each segment were synthesized as reported [1] and the whole region of each segment was PCR amplified using a pair of primers containing the BsmBI sites at their 5' ends. The obtained PCR products were digested with BsmBI, followed by insertion in antisense orientation between Pol I promoter and terminator of the vector pHH21 [2], and pPoll/PB2, pPoll/PB1, pPoll/P3, pPoll/HE, pPoll/M and pPoll/NS were constructed. Plasmids pPoll/NP and the nine plasmids for expression of the virus proteins were described previously [1]. Plasmid pPoll/M-G96A, which has a G-to-A-mutation at residue 96 of the M gene, was constructed based on pPoll/M.

Generation of recombinant influenza C viruses. To rescue recombinant AA/50, 0.5 µg of pPoll/PB2, pPoll/PB1, pPoll/P3, pPoll/HE, pPoll/NP, pPoll/M and pPoll/NS were transfected into 10^6 293T cells, together with four (PB2, PB1, P3, NP) or nine (PB2, PB1, P3, HE, NP, M1, CM2, NS1 and NS2) virus protein-expressing plasmids, according to the procedure [1]. At 48 to 60 h p.t., the culture medium was treated with 20 µg/ml TPCK-treated trypsin at 37°C for 10 min and then subjected to determination of infectious titers.

Membrane flotation analysis of infected cells. The HMV-II cells infected with recombinant viruses were resuspended in 0.3 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 5 mM MgCl₂ and a protease inhibitor cocktail) at 20 h p.i. After incubation on ice for 30 min, the cells were disrupted by repeated passage (35 times) through a 26-gauge needle. The postnuclear supernatant (0.25 ml) was dispersed into 1.75 ml of 80 % sucrose (wt/vol) in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) and placed at the bottom of the tube, and then overlaid with 6.5 ml of 65% sucrose (wt/vol) in TE and 3.25 ml of 10 % (wt/vol) of sucrose in TE. The gradient was centrifuged to equilibrium at $180,000 \times g$ for 18 h at 4°C. Fractions (1.17 ml) were collected from the top. Aliquots of the fractions were analyzed by SDS-PAGE, followed by Western blotting [1]. For immunoprecipitation, at 20 h p.i. the recombinant virus-infected cells were pulse labelled with [³⁵S]methionine for 15 min, chased for 2.5 h and treated as above. After fractionation, each fraction was added to an equal volume (1.17 ml) of 2 × immunoprecipitation buffer and immunoprecipitated with anti-AA/50 serum. The immunoprecipitates were analyzed by SDS-PAGE and processed for fluorography [7].

Options for the Control of Influenza VI

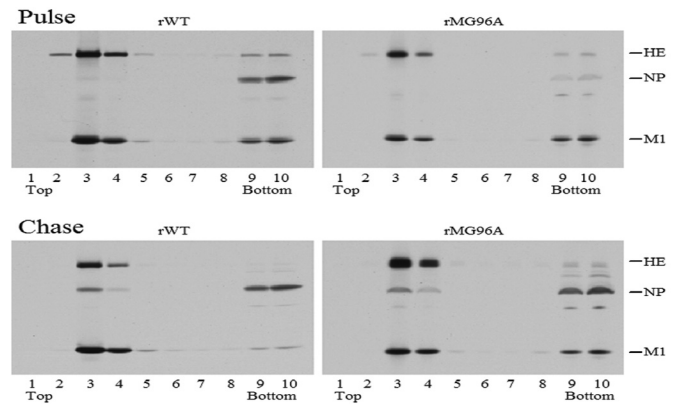
Results

Generation of recombinant C/Ann Arbor/1/50. To rescue the recombinant AA/50 (recombinant wild type; rWT), the seven Poll plasmids were transfected into 293T cells together with four virus-protein expressing plasmids. At 48 to 60 h p.t., the supernatant of the 293T cells was serially diluted and inoculated into the amniotic cavity of 9-day-old chicken eggs. After incubation for three days, the amniotic fluids from each egg were tested for hemagglutination. Three independent experiments revealed that the EID_{50}/ml of the medium was 1.62×10^1 to 1.10×10^2 . When the seven Poll plasmids and nine virus-protein expressing plasmids were transfected into 293T cells, the titers of the media were 1.58×10^3 , 2.51×10^2 and $2.51 \times 10^3 EID_{50}/ml$.

Growth kinetics and morphology of the recombinant viruses. In our previous study on the generation of influenza C VLPs, we have demonstrated that the amino acid (Ala/Thr) at residue 24 of the M1 protein is involved in the morphology of the VLPs [1]. In the present study, to investigate the role of this residue in virus replication and morphology, we rescued a recombinant virus, rMG96A, in which the amino acid Ala at residue 24 of the M1 protein was substituted to Thr. The rWT showed growth kinetics similar to that of AA/50 strain, and the rWT grew a little more efficiently (2.1×10^5 TCID₅₀/ml at 7 days p.i.) than did rMG96A (4.6×10^4 TCID₅₀/ml). As expected, the cord-like structures (CLS), demonstrated to be a bundle of filamentous virions [1,4,5], were observed on rWT-, but not rMG96A-infected HMV-II cells. The electron microscopic analyses of rWT and rMG96A showed that rWT grown in HMV-II cells exhibited a mainly filamentous morphology and a rod-like shape, whereas all rMG96A virions observed were spherical.

Membrane flotation analysis of infected cells. To detect any differences between the two recombinant virus-infected cells, the recombinant virus-infected HMV-II cells were subjected to equilibrium centrifugation, followed by Western blotting. The distribution of HE and NP proteins throughout the fractions was similar to each other. The amount of membrane-associated M1 proteins from rWT in fractions 3 and 4 accounted for 90% of the total M1 proteins present, whereas that from rMG96A-infected cells accounted for nearly 70% of total M1 protein. The infected cells were metabolically pulse-labelled, chased and analyzed by immunoprecipitation. No significant difference in the kinetics of the HE and NP proteins was observed between rWT- and rMG96A-infected cells. The majority of HE glycoproteins were recovered in the membrane fractions and the pulse-labelled NP was recovered in the bottom fractions, though some floated up to the membrane fractions during the chase period (Figure 1). On the other hand, the pulse-labelled M1 proteins were recovered both in membrane and bottom fractions. In the chase experiment, the amount of membrane-associated M1 proteins in the fractions 3 and 4 was 90% of the total M1 in the rWT-infected cells, which is higher than that (63%) in rMG96A-infected cells (Figure 1).

Figure 1. Membrane flotation analysis of infected cells. HMV-II cells infected with rWT or rMG96A were pulse labelled with [³⁵S]methionine and chased. The cells were subjected to flotation analysis. Virus proteins in each fraction were immunoprecipitated with the antiserum, and analyzed by SDS-PAGE. Membrane-bound material floats up to the interface between 10 and 65 % sucrose (fractions 3 and 4).



Discussion

In our previous report, we provided evidence that the amino acid at residue 24 of the M1 protein (Ala/Thr) is involved in the morphology of the VLPs as well as in the formation of the cords [1]. In the present study, as expected, the cords were observed only on rWT-infected cells, and the rWT exhibited a mainly filamentous morphology, whereas all rMG96A virions observed were spherical, indicating that residue 24 of the M1 protein is involved in virion morphology. Furthermore, rWT grew a little more efficiently than did rMG96A, suggesting that filamentous virions may have some advantage over spherical virions in terms of replication. The association of M1 proteins with the plasma membrane of virus-infected cells was investigated and differences in the affinity of the M1 protein to the membrane were demonstrated (Figure 1). These data suggest that the amino acid Ala or Thr at residue 24 of the M1 protein affects the morphology of the virion by altering the affinity of the protein to the cell membrane. As influenza C virus M1 protein is located beneath the membrane and may play a central role in virus budding as has been reported for influenza A virus, it is likely that the affinity to the membrane influences the morphology of the influenza C virions. The M1 protein of rWT, which associated firmly with the membrane, may have stronger propensity to virus budding, leading to filamentous virion formation. The M1 of rMG96A, which associated weakly with the membrane, may readily allow pinching off, resulting in spherical virion formation. If this is the case, the amino acid sequences around residue 24 may provide a novel motif for the late domain (L domain).

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How to Retain Type B Influenza Hemagglutinin 196/197 Glycosylation Site That is Lost During Egg Adaptation

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Embryonated chicken eggs are used to grow influenza viruses and manufacture influenza vaccines. However, recent influenza B strains from two different antigenic lineages do not grow well in eggs and adaptation of virus in eggs usually results in the loss of the 196/197 N-linked glycosylation site in the hemagglutinin (HA) protein. To determine the effect of the 196/197 glycosylation site on virus growth in eggs, paired recombinant B vaccine strains, with or without the 196/197 glycosylation site, were obtained and the glycosylation site was retained in MDCK cells. In contrast, any viruses containing the glycosylation site (except B/Jiangsu/10/03) were unable to grow in eggs and rapidly lost the glycosylation site once adapted to grow in eggs. It was identified that the Arginine at amino acid position 141 (141R) stabilized the 196/197 glycosylation site. Introduction of 141R to other influenza B viruses enabled the viruses to retain the glycosylation site in eggs. This finding has significant implication in influenza B vaccine manufacture.

Introduction

FluMist[®], the live-attenuated influenza vaccine licensed in the U.S., contains three 6:2 genetic reassortant viruses. Each 6:2 vaccine strain contains the 6 internal gene segments derived from the master donor virus of *ca* A/Ann Arbor/6/60 (MDV-A) or *ca* B/Ann Arbor/1/66 (MDV-B) to confer the characteristic cold adapted, temperature sensitive and attenuation phenotypes to the vaccine strains, and the hemagglutinin (HA) and neuraminidase (NA) gene segments from a currently circulating wild-type (wt) type A H1N1, H3N2 or type B virus provide antigen specificity. Embryonated chicken eggs are currently used to manufacture influenza vaccines. However, cultivation of influenza A and B viruses in embryonated chicken eggs frequently selects for mutants in the HA that differs antigenically and structurally from naturally occurring viruses [3]. Several studies showed that egg adaptation of influenza B viruses resulted in the loss of the N-linked glycosylation site (N-X-T/S) at amino acids 196-198 (B/Yamagata lineage) or 197-199 (B/Victoria lineage) in the HA1 region resulting in a significant change in the antigenicity of the molecule [5, 7]. In this study, we confirmed that the glycosylation site was readily lost during the growth of vaccine viruses in eggs. Furthermore, we identified a key amino acid residue that stabilized retention of the glycosylation site when grown in eggs.

Materials and Methods

Wild-type influenza B viruses used in this study, B/Malaysia/2506/04, B/Ohio/1/05, B/Florida/7/04, B/Shanghai/361/02, B/Jilin/20/03, B/Jiangsu/10/03, were originally obtained from CDC. Recombinant 6:2 vaccine viruses were rescued using the eight plasmid transfection system [1]. MDCK cells were infected with virus at a multiplicity of infection (MOI) of 0.004. After 60 minutes adsorption at room temperature, virus inoculum was removed, replaced with MEM and incubated at 33°C. The culture supernatants were collected at 3 days of postinfection and virus titers were determined by plaque assay on MDCK cells at 33°C. Embryonated chicken eggs of 10-11 days old were inoculated with 0.1 ml of virus at the amount of 10² to 10⁵ PFU as indicated in the text and incubated at 33°C for three days. Allantoic fluids were collected and virus titers were determined by plaque assay. Virus sequence was determined by sequencing the RT-PCR cDNA products amplified from viral RNAs.

Results

Egg adaptation caused the loss of the 196/197 glycosylation site in the HA1 of influenza B viruses. Nearly all recently circulating influenza B strains, including those selected for vaccine production, contain an N-linked glycosylation motif in the HA, N-X-T/S, at amino acid position of 196-198 for B/Yamagata lineage or 197-199 for B/Victoria lineage. This glycosylation site was reported to be readily lost following virus adaptation in eggs, thereby potentially altering antigenicity of the HA. To determine the effect of the glycosylation motif on virus replication in eggs, pairs of recombinant 6:2 reassortant vaccine strains (B/Ohio/1/05, B/Malaysia/2506/04, B/Florida/7/04, B/Shanghai/361/02 and B/Jilin/20/03) were constructed in which one member contained and the other lacked the glycosylation site (Table 1). All viruses amplified efficiently in MDCK cells and maintained the original sequences (data not shown). Viruses that lacked the glycosylation site grew efficiently in eggs (7.6-8.7 log₁₀ PFU/ml). However, when 100 PFU of each of the corresponding viruses that contained the NXT glycosylation site were inoculated into eggs, only very low levels of viruses were obtained (1.7-3.9 log₁₀ PFU/ml). When eggs were inoculated with a much higher quantity of these viruses (4-5 log₁₀ PFU/egg), higher titers were detected after a single passage (6.2-8.8 log₁₀ PFU/ml). However, sequence analyses of these egg amplified viruses demonstrated that they no longer contained the 196/197 glycosylation site. Either the N or T residue in the motif was substituted with a different amino acid, thereby destroying the glycosylation motif. B/Jilin/20/03 and one of the B/Florida/7/04 isolates contained Proline next to the NXT motif, which also prevented the N-linked glycosylation. These results demonstrated that 196/197 glycosylation site prevented efficient virus replication in eggs.

Arginine at the 141 position of HA stabilized the 196/197 glycosylation site. Most of the egg grown isolates did not contain the NXT glycosylation sequence at 197-199 (or 196-198) or had heterogenic nucleotide sequence at this site. However, B/Jiangsu/10/03, the vaccine strain for the 2004 to 2006 flu seasons, replicated efficiently in eggs and maintained the

glycosylation motif (Table 2). B/Jiangsu/10/03 has a unique residue Arginine residue at position 141. To test whether 141R may have stabilized B/Jiangsu/10/03 HA196 glycosylation site, site-directed mutagenesis of B/Jiangsu/10/03 HA was performed to change 141R with G. Recombinant 6:2 reassortant containing substitution of R141G rapidly lost the 196 glycosylation motif following replication in eggs (Table 2). To further confirm whether 141R was sufficient to retain the 196/197 glycosylation site for other influenza viruses in eggs, the 141G residue of 6:2 B/Shanghai/361/02 and B/Ohio/1/05 was replaced by 141R. As shown in Table 2, both B/Shanghai/361/02 and B/Ohio/1/05 that contained the 141R and 196/197 N residues maintained the glycosylation site and replicated efficiently in eggs with a titer of approximately $8.0 \log_{10}$ PFU/ml. In conclusion, the residue at the 141 position plays a critical role in influencing the stabilization and usage of the HA 196/197 glycosylation site for influenza B viruses following replication in eggs.

Discussion

In this study, we systematically investigated the effect of the 196/197 glycosylation on virus growth by comparing pairs of vaccine strains which differed only at the glycosylation site. We confirmed that the recent influenza B viruses with the 196/197 glycosylation site do not grow efficiently in chicken eggs. A single egg passage causes the loss of the glycosylation site. The 196/197 HA glycosylation site corresponds to amino acid 187 at the antigenic site B in the H3 influenza A viruses and has been found to affect antigenicity of some influenza B strains [8]. Therefore, it is important to carefully monitor the impact of sequence change due to egg adaptation on vaccine efficacy. Viruses grown in mammalian cell culture usually maintain their structural and antigenic properties [4, 6]. Our study also demonstrated that influenza B viruses remain glycosylated following replication in MDCK cells. Thus, cell culture based influenza vaccines, currently being developed as the next generation of influenza vaccines, should offer additional advantages over egg-derived vaccines. Arginine 141, which is distant from the glycosylation site, stabilizes the HA glycosylation site of influenza B vaccines derived from either Victoria or Yamagata lineage. Based on the homology modeling of the HA structure of B/Lee/40, this 141 residue is on a loop of HA1 near the HA receptor binding site [8], which may explain why this residue influences HA receptor binding and antigenicity. The G141R change in the HA was previously reported in one of an egg adapted isolate (B/EFR/83) that possessed the 196 glycosylation site [2]. Thus, replacement of 141G by 141R should provide an alternative way to grow influenza B vaccines in eggs.

Table 1. Replication of HA 196/197 glycosylation variants in chicken embryonated eggs.

Virus	Amino Acid 196-199 (197-200)	Virus titer in eggs (\log_{10} PFU/ml)	Amino Acid 196-199 (197-200) after growth in eggs ^c
B/Ohio/1/05	SETQ(G-)	8.7 ^a	SETQ
	NETQ(G+)	2.1 ^a	ND ^d
		8.8 ^b	<u>SETQ</u>
B/Malaysia/2506/04	NEAQ(G-)	8.7 ^a	NEAQ
	NETQ(G+)	1.7 ^a	ND
		7.3 ^b	<u>SETQ</u> <u>NE<u>N</u>Q</u>
B/Florida/7/04	DKTQ(G-)	8.2 ^a	DKTQ
	NKTQ(G+)	3.0 ^a	NKTQ
		6.7 ^b	<u>NKIQ</u> <u>NK<u>T</u>P</u>
B/Shanghai/361/02	DKTQ(G-)	8.7 ^a	DKTQ
	NKTQ(G+)	3.9 ^a	NKTQ
		6.2 ^b	<u>SKTQ</u> <u>DKTQ</u>
B/Jilin/20/03	NKTP(G-)	7.6 ^a	NKTP
	NKTQ(G+)	3.0 ^a	NKTQ
		6.8 ^b	<u>NK<u>S</u>Q</u>

^{a,b} Eggs were inoculated with 10^2 PFU/egg (^a) or 10^4 - 10^5 PFU/egg (^b) of the indicated 6:2 reassortant viruses amplified in MDCK cells that either had (G+) or did not have (G-) the 196/197 HA glycosylation site and incubated at 33°C for three days. Virus peak titers were determined by plaque assay in MDCK cells. The numbers represent means of at least three independent experiments with standard deviation less than 10%.

^c The HA sequence of the virus recovered from eggs were determined and amino acid sequence changes are indicated as underlined.

^d ND: Not determined.

Table 2. The 141R residue stabilized the 196/197 glycosylation site when grown in eggs.

Virus	Amino acid at		Virus Titers in eggs (\log_{10} PFU/ml)	Amino acid 196-199 (197-200) after growth in eggs ^c
	141	196-199 (197-200)		
B/Jiangsu/10/03	R	NKTQ	8.4 ^a	NKTQ
	G	NKTQ	2.4 ^a	NKTQ
			8.5 ^b	<u>DKTQ</u> <u>NK<u>T</u>P</u>
B/Shanghai/361/02	R	NKTQ	8.0 ^a	NKTQ
B/Florida/7/04	R	NKTQ	8.0 ^a	NKTQ
B/Ohio/1/05	R	NETQ	7.9 ^a	NETQ

^{a,b} MDCK cells were infected with the indicated virus at moi of 0.004 and eggs were inoculated with 10^2 PFU/egg (^a) or 10^4 - 10^5 PFU/egg (^b) of the indicated 6:2 reassortant viruses amplified in MDCK cells that either had (G+) or did not have (G-) the 196/197 HA glycosylation site and incubated at 33°C for three days. Virus peak titers were determined by plaque assay in MDCK cells.

^c The HA sequence of the virus recovered from eggs were determined and amino acid sequence changes are indicated as underlined.

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Proceedings Topic #5

**Developments in
Diagnostic and Serologic
Techniques**

**Poster
Presentations**

Is Influenza A Rendered Non-Viable Following Treatment With RNA Isolation Kit Lysis/Binding Buffers?

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Human or animal viral swab specimens which potentially contain highly-pathogenic avian influenza A virus (HPAI) may require characterization in a BSL-3 laboratory. Procedures conducted in a BSL-3 laboratory can be time-consuming and extremely costly. With the rapidly increasing need to monitor the spread and transmission of HPAI, the large number of specimens requiring processing has potential to overwhelm the capacity of our BSL-3 facilities to process samples in an effective time frame. Many protocols for typing influenza A samples rely on real-time reverse transcription PCR amplification of viral RNA isolated from samples. The ability to remove viral samples from the BSL-3 laboratory at earlier stages in the RNA isolation protocol would encourage the use of high throughput technology to speed the processing and typing of samples. To remove a samples from a BSL-3 laboratory it must be demonstrated the complete inactivation of live virus particles. In this work we demonstrate that the lysis/binding buffers from two popular viral RNA isolation kits are effective at inactivating influenza A virus. This inactivation permits the removal of treated field specimens from the BSL-3 laboratory soon after addition of the lysis/binding buffers and incubation.

Introduction

As government agencies and health care providers around the world continue to escalate surveillance for highly pathogenic avian influenza (HPAI) viruses in response to their spread, the number of isolates warranting characterization could rapidly overwhelm the capacity of BSL-3 facilities. Many agencies, having opted for the ease and speed of real-time PCR screening [1-3], will need to isolate viral RNA from a large number of potential HPAI samples. Unfortunately, high throughput sample processing protocols are more compatible with lower level containment facilities. To remove samples from a BSL-3 laboratory a team must confirm that the virus is either not a highly pathogenic strain or show, without questionable doubt, that there are no active virus particles present. The ability to demonstrate the inactivation of virus at earlier steps in the RNA isolation, enabling their removal from the BSL-3 laboratory, will greatly improve the throughput capacity of typing laboratories. While many viral RNA isolation kits allude to the ability of their lysis/binding buffers to inactivate virus, to our knowledge no published studies exist demonstrating that influenza A virus is indeed inactivated by these reagents. In this study we tested the lysis/binding reagents from two popular viral isolation kits, the QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, CA) and the MagMax™-96 Viral RNA Isolation Kit (Ambion, Austin, TX), for their ability to inactivate prototypical examples of 12 HA

subtypes of avian influenza A, three common vaccine strains of human influenza A, and three prototypical swine influenza A samples.

Materials and Methods

Eighteen Influenza A virus stock solution (Table 1) were added to QIAgen QIAamp® viral RNA isolation kit AVL buffer and Ambion MagMax™- 96 viral RNA isolation kit lysis/binding solution per the respective manufacturer's instructions. Treated virus samples were added to a 7ml 20kDa MWCO iCon™ concentrator (Pierce, Rockford, IL) pre-rinsed with 1 x PBS (Gibco Carlsbad, CA) and containing 4.5 ml 1 x PBS in the upper chamber. Concentrators were centrifuged at 2800 x g at 4°C until the volume of upper chamber was reduced to <0.5 ml. The filtrate was decanted from lower chamber of the concentrator and ~ 4.5ml of 1 x PBS was added to the upper chamber. The concentration and dilution steps were repeated 2 additional times with a final concentration step resulting in overall ~1000 fold dilution of the original buffer composition. 200 ml of the final concentrate was used to infect a 200 mm² surface area well of a 24 well tissue culture cluster plate containing a confluent monolayer of MDCK London cells and 1 ml Olsen's viral growth medium (passage 1). Plates were centrifuged at 615 x g for 30 min to facilitate efficient infection and incubated at 36°C under 5.0% CO₂ for 48-72 hours. 200 ml of cell culture media from passage 1 was used to infect fresh cultures of MDCK London monolayers in 24 well tissue culture plates and incubated as before for 48-72 hours (passage 2). Cells from passage 1 and 2 were washed two times with sterile 1 x PBS, scraped from wells into 0.5 ml sterile 1 x PBS and 25 ml was spotted onto a microscope slide. Following air drying in a BSC the slides were fixed in cold 100% acetone for 10 minutes. Fixed cells were stained with FITC labeled anti-Influenza A mouse monoclonal antibody (Chemicon/Millipore Temecula, CA) and examined under UV microscopy.

Results

The presence of cytotoxic compounds in the lysis buffers of both kits prohibit the ability to culture samples treated with these compounds on cell culture monolayers. This was overcome though a process of repeated dilution and concentration by centrifugal ultrafiltration of lysis buffer treated samples resulting in exchange of buffers with PBS equating to ~500-fold dilution of buffer components. To determine that this level of dilution of the lysis buffers was sufficient to prevent cytotoxic effects to the cell monolayers, samples containing aliquots of each lysis/binding buffer were buffer-exchanged in the manner described above and the resulting diluate used to infect cell monolayers in parallel with the experimental samples. Cell monolayers to which the exchanged lysis/binding buffers has been added looked identical to untreated monolayers thus demonstrating that the cytotoxic components of these reagents had been sufficiently removed. In addition, to determine that the buffer exchange process itself was not responsible for inactivation of virus, a sample of H1N1 Influenza A/New Caledonia/20/99 was subjected to the buffer exchange process described above.

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The resulting CPE and DFA staining seen in monolayers infected with the buffer-exchanged virus was similar to a positive control of untreated virus from the same stock demonstrating that the buffer exchange process itself did not affect viral viability. Inactivation of all viruses tested (Table 1), following treatment with lysis/binding buffers, was demonstrated by the inability to culture virus after double blind passage on MDCK London cell culture monolayers. A positive viral culture was noted by typical influenza induced CPE, compared to positively infected controls, as well as observance of FITC tagged flu specific monoclonal antibody stained cells under fluorescence microscopy. Passage of the lysis buffer treated viruses on MDCK cell culture yielded no CPE as compared to the positive control. The absence of influenza A was confirmed by an inability to detect virus by direct fluorescence antibody staining, using a FITC tagged influenza-specific monoclonal antibody of cells from the cell cultures inoculated with viral samples treated with the lysis/binding buffers. These data suggest that influenza A specimens treated with the QIAgen QIAamp® viral RNA isolation kit AVL buffer and Ambion MagMax™ -96 viral RNA isolation kit lysis/binding buffers are inactivated and would be safe to remove from the BSL-3 laboratory.

Discussion

The ability to remove influenza A specimens from the BSL-3 laboratory soon after the addition of lysis buffers has the potential to greatly speed up the capacity to process and characterize these specimens. Without this ability surveillance laboratories would need to complete the RNA isolation from the specimens before they could be removed from the BSL-3 laboratory for molecular characterization. The ability to remove specimens earlier in the process becomes even more valuable as the number of specimens increases, as would be the case in the event of a widespread influenza epidemic. For example, by our estimates, for every one hundred specimens requiring molecular characterization the ability to complete RNA isolation from specimens in our BSL-2 laboratory using automated RNA purification equipment would save at least four man-hours over having to complete these isolations by hand in the BSL-3 facility. Furthermore, the ability to inactivate specimens rapidly by the addition of the lysis/binding buffers opens up the possibility of inactivating specimens for characterization at the point of collection thus eliminating the need to ship potentially infectious materials. We are currently testing the stability of viral RNA in these buffers to determine the feasibility of using these reagents as a shipping media for viral RNA from field surveillance sites. While the study we report here tested only low pathogenic influenza strains, additional extensive testing with HPAI strains would need to be conducted to assure that the lysis/binding buffers from these RNA isolation kits could, without a doubt, inactivate HPAI strains as well before they could be removed from the BSL-3 facility. These studies are currently underway. In summary, this study suggests that samples treated with the lysis/binding buffers from either the QIAgen QIAamp® viral RNA isolation kit or the Ambion MagMax™ -96 viral RNA isolation kit

are free of active virus and are safe to remove from the BSL-3 facility upon surface decontamination of sealed tubes or 96 well plates. In addition, by inactivating virus with the lysis/binding buffers it may be possible to transport samples from the field to the laboratory without having to ship infectious materials or the need to receive the samples at a BSL-3 facility saving surveillance laboratories limited resources required to process specimens.

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Table 1. Prototypical influenza A strains tested for viability following treatment with RNA isolation kit lysis buffers.

Strain	Culture source	TCID ₅₀ /ml
Avian H1N1 Influenza A/Duck/Alberta/35	Allantoic fluid	5.62E+04
Avian H2N2 Influenza A/mallard/NY/6750/78	Allantoic fluid	5.62E+04
Avian H3N8 Influenza A/Duck/Ukraine/1/63	Allantoic fluid	1.26E+04
Avian H4N6 Influenza A/Duck/CZ/1/56	Allantoic fluid	6.82E+04
Avian H5N2 Influenza A/chucker/MN/14591-7/98	Allantoic fluid	N/D
Avian H6N2 Influenza A/turkey/MA/65	Allantoic fluid	2.16E+04
Avian H7N2 Influenza A/TKY/VA/4592/2002	Allantoic fluid	4.64E+06
Avian H8N4 Influenza A/Ty/Ontario/68	Allantoic fluid	1.78E+05
Avian H9N2 Influenza A/chicken/NJ/12220/99	Allantoic fluid	5.62E+04
Avian H10N7 Influenza A/chicken/Germany/49	Allantoic fluid	1.78E+06
Avian H11N9 Influenza A/duck/Memphis/546/76	Allantoic fluid	2.15E+06
Avian H12N5 Influenza A/duck/Alberta/60/76	Allantoic fluid	5.62E+04
Swine H1N1 Influenza A/sw/WI/238/97	MDCK cell supernatant	2.51E+05
Swine H1N2 Influenza A/sw/WI/R33F/01	MDCK cell supernatant	5.62E+05
Swine H3N2 Influenza A/sw/MN/593/99	MDCK cell supernatant	3.76E+06
Human H3N2 Influenza A/Nanchang/933/95	Allantoic Fluid	1.21E+06
Human H3N2 Influenza A/Panama/2007/99	MDCK cell supernatant	3.57E+05
Human H1N1 Influenza A/New Caledonia/20/99	Allantoic Fluid	1.17E+05

Real-Time RTPCR Assays for Universal Detection of N1 Subtype and Discrimination of HPAI H5N1 Genotype Neuraminidase Genes of Influenza A Viruses

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Type A Influenza viruses are divided into nine NA subtypes (N1~N9) based on antigenic differences of their surface Neuraminidase (NA) glycoproteins. N1 NA genes are found in seasonal human H1N1 and a number of animal virus subtypes including swine H1N1 and highly pathogenic avian influenza (HPAI) H5N1 influenza virus that occasionally infect humans causing mild to fatal respiratory disease. Thus, it is sometimes necessary to identify and characterize the NA genes of influenza viruses isolated from humans to determine if the virus is of human or animal origin. Currently available sequence data of all N1 influenza A viruses was used to design primer/probe sets based on conserved regions of the NA gene for real-time reverse transcription PCR (rRT-PCR). Assays were developed and optimized for 1) universal detection of all known N1 subtype NA genes, and 2) specific discrimination of NA genes of HPAI H5N1 genotype. These rRT-PCR assays can serve as an effective tool for rapid subtype characterization of the N1 NA gene of human and animal influenza type A viruses as well as to discriminate N1 NA genes of HPAI and other avian viruses. Furthermore, when used in combination with appropriate HA subtyping assays, these assays will allow for the detection of a reassortment event involving the N1 NA gene of human and avian influenza viruses.

Introduction

Influenza A viruses are divided into subtypes based on antigenic differences in their surface Hemagglutinin (HA) and the Neuraminidase (NA) glycoproteins (1). There are nine known NA subtypes (N1~N9) that have been identified (2,3). N1 NA genes are found in seasonal human H1N1 and a number of animal virus subtypes including swine H1N1 and highly pathogenic avian H5N1 influenza virus. Influenza viruses containing N1 NA genes have caused many severe illness and death in humans including the human H1N1 pandemic in 1918, which killed 20–50 million people as well as highly pathogenic avian H5N1 that has caused illness and death in humans and birds resulting in great economic losses and currently poses an eminent threat to emerge as a potential pandemic virus (4, 5). For surveillance and diagnostic purposes, it is important to detect and characterize seasonal human influenza viruses (A/H1N1, A/ H3N2 and B) as well as potential pandemic viruses that have been shown to infect humans, such as highly pathogenic avian influenza (HPAI) H5N1 viruses. Due to their segmented genome, influenza

viruses able to exchange genes through genetic reassortment. Indeed, pandemic influenza viruses emerged in humans in 1957 and 1968 following genetic reassortment between seasonal human and avian viruses (5). Thus, it is important to distinguish between human and animal genotypes of N1 NA in order to detect a reassortment event that may confer increased transmissibility to humans. Quantitative real-time RT-PCR (rRT-PCR) is a method for pathogen detection that provides a reproducible, high-throughput assay for simple and timely collection of surveillance and diagnostic information. In this study, rRT-PCR assays were optimized using primer/probe sets designed for 1) universal detection of all known N1 subtype NA genes, and 2) specific discrimination of NA genes of HPAI H5N1 genotype.

Materials and Methods

Viruses strains and RNA extraction. Viruses strains used in this study were grown in either MDCK cells or embryonated chicken eggs. Virus strains used to demonstrate assay performance for universal detection of N1 subtype as well as discrimination of HPAI H5N1 genotype neuraminidase genes of influenza A viruses are listed in Table 1. Viral RNA was extracted from virus stocks using RNeasy Mini kit following manufacturer's recommended procedures (Qiagen., California, CA).

Primers and probes design. Primers and probes for universal detection of N1 NA sub-typing and highly pathogenic avian influenza H5N1 NA were designed based on available nucleotide sequence data of 1,698 NA gene sequences from Genbank database of National Centers for Biological Information, NIH (NCBI) and the Influenza Sequence Database of Los Alamos National Laboratories (LANL) (Fig 1). Each primer and probe underwent nucleotide BLAST search (NCBI) analysis against the entire Genbank nucleotide database to validate their specificities and avoid non-specific reactivity. TaqMan® probes were labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher™ 1 (BHQ™1) (Biosearch Technologies, Inc, Inc., Novato, CA) at the 3'-end. Primers and dual-labeled TaqMan® probes were synthesized by the Biotechnology Core Facility, Centers for Disease Control and Prevention (CDC, Atlanta, USA).

Real-time RT-PCR (rRT-PCR). Reaction parameters were optimized using the Invitrogen SuperScript™ One-Step QRT-PCR System on the Stratagene Mx3005P or BioRad iQ iCycler platforms. All rRT-PCR reactions were performed at a volume of 25µl using primer reaction concentration of 0.8µM and probe concentrations of 0.2µM. RT-PCR conditions were as follows: 50°C for 30 min, Taq activation for 2 min at 95°C, and 45 cycles of 95°C for 15 sec and 55°C for 30 sec. These conditions are consistent with the Centers for Disease Control and Prevention Diagnostic Assay for the detection and sub-typing of influenza viruses currently in use.

Electrophoresis. Real-time RT PCR products were analyzed by LabChip® 90 Automated Electrophoresis system (Caliper LifeScience).

Results and Conclusions

Reaction efficiencies of the primer/probe sets for detection of Universal N1 and HPAI N1 genes were determined by testing against a five-fold serial dilution of viral RNA, plotting the reaction Ct values against their relative RNA concentration and estimating the slope by linear regression analysis. Universal N1 and HPAI N1 rRT-PCR assays achieved reaction efficiencies at 94.5% and 98.6%, all positive rRT-PCR reactions were confirmed by electrophoresis of amplified products. The universal N1 rRT-PCR primer/probe set was shown to detect NA genes of all N1 subtype viruses tested, including HPAI H5N1, seasonal human H1N1, PR8 H1N1, human pandemic 1918 H1N1, North America Swine H1N1, avian H1N1, and avian H6N1 viruses (Table 1). Nonspecific reactivity was not observed when RNA from other influenza A subtypes (N2, N3, N4, N5, N6, N7, N8, N9), influenza B, or nasal wash collected from uninfected humans was tested (Data not presented). The HPAI N1 genotype rRT-PCR assay detected NA genes of H5N1 influenza viruses (clade 2.1, clade 2.2, clade 2.3, and clade 1) as well as the N1 gene of non-H5N1 avian influenza A viruses that showed high similarity with HPAI H5N1 virus. The HPAI N1 genotype rRT-PCR assay did not demonstrate nonspecific reactivity against NA genes of human H1N1, human pandemic 1918 H1N1, and swine H1N1 influenza virus tested (Table 1). The rRT-PCR assays described here can serve as an effective tool for rapid subtype characterization of the N1 NA gene of human and animal influenza type A viruses as well as to discriminate N1 NA genes of HPAI and other avian viruses. The combined use of these assays will allow for the detection of a reassortment event involving the N1 NA gene of human and avian influenza viruses.

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Figure 1. Phylogenetic tree obtained by the neighbor-joining method, based on the NA gene nucleotide sequence of N1 viruses from the Influenza Sequence Database of Los Alamos National Laboratories (LANL). The scale length of each pair of branches represents the distance of % Nucleotide difference between sequences pairs. Isolates indicated in bold were used to demonstrate assay reactivity.

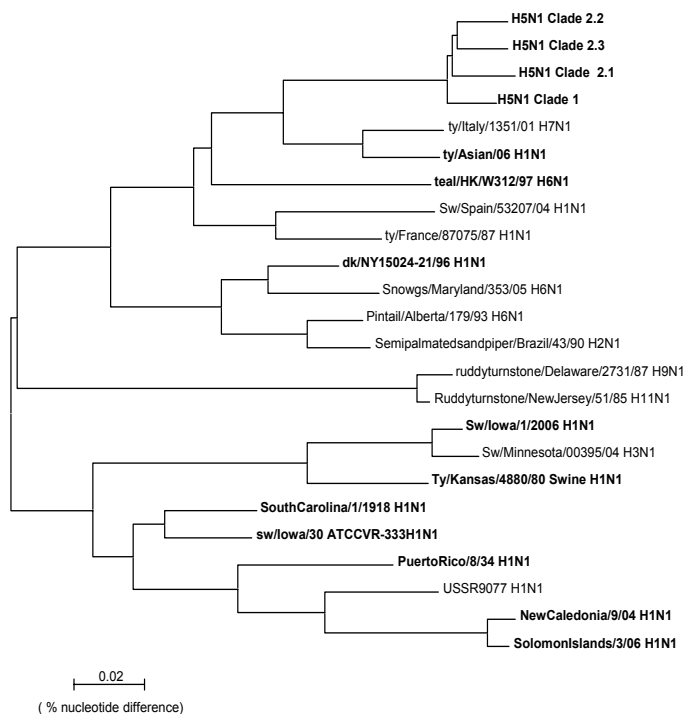


Table 1. Universal N1 NA and HPAI N1 NA assay detection of different viruses with N1 NA gene.

**Positive real-time RTPCR results are indicated as Ct values.

Virus strains	Assays**			
	Flu A	Un-N1*	HumanH1	HPAI N1
Human H1N1				
N.Caledonia/9/04	24.1	26.46	25.89	-
SolomonIslands/3/06	27.8	28.11	27.51	-
PuertoRico/8/34	17.6	17.39	27.91	-
SouthCarolina/1/1918	28.2	30.13	-	-
Swine H1N1				
Iowa/1/2006	23.2	28.91	-	-
sw/Iowa/30	21.7	20.42	-	-
ty/Kansas/4880/80	15.2	15.39	-	-
Avian N1				
dk/Asian/06 H1N1	18.3	20.16	-	24.61
teal/HK/W312/97 H6N1	21.9	28.18	-	29.49
dk/NY/15024-21/96 H1N1	19.4	18.4	-	30.23
HPAI H5N1				
H5N1 Clade 1	21.2	23.91	-	26.7
H5N1 Clade 2.1	14.1	16.17	-	17.48
H5N1 Clade 2.2	23.7	22.65	-	23.2
H5N1 Clade 2.3	11.8	12.01	-	15.86

*Un-N1: Universal N1

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Use of Pseudotyped Particles Expressing Influenza A/VietNam/1194/2004 Hemagglutinin in Neutralization Assays

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Infections with H5N1 avian influenza viruses have been occurring sporadically over the last ten years and the development of an H5N1 vaccine is considered as the primary strategy to protect humans against a possible H5N1 pandemic. Hemagglutination inhibition (HI) assays are generally used to measure antibody responses, with titers predicting protection in humans. However, since the HI test is relatively insensitive for measuring antibody responses to the recent avian strains, there is an urgent need for new assays that can be easily used to screen human sera. The micro-neutralization assay represents a valid alternative to HI, however this assay requires Biosafety Level 3 laboratories. Here we show the generation of lentiviral vector particles pseudotyped with HA from clade 1 and subclades 2 H5N1 influenza strains as safe surrogates of the whole virus and their use for the evaluation of neutralizing antibodies using a luciferase reporter gene. Results showed that using this assay we were able to detect neutralizing and cross-neutralizing antibodies in animal sera. Furthermore, pseudotype, micro-neutralization and HI titers strongly correlate in mice sera. The pseudotype-based neutralization assay is promptly adaptable to a high-throughput format for the evaluation of vaccine efficacy under Biosafety Level 2 containment.

Introduction

The influenza envelope glycoprotein hemagglutinin (HA) is the major target of protective antibodies. A correlate of protection has been established by using the hemagglutination inhibition (HI) assay, with titers predicting protection in humans [1]. However, the classical HI test is relatively insensitive for measuring antibody responses to the recent avian influenza viruses. A modification of the assay using horse instead of chicken or turkey erythrocytes results in increased sensitivity [2]. However, there is an urgent need for new assays that can be easily used to screen human sera. The micro-neutralization assay represents a valid alternative to the HI test, however this assay requires Biosafety Level 3 laboratories, and obviously access to the whole virus. Recently, pseudotyped particles have been a widely used tool to express viral glycoproteins. They are based on a process known as "pseudotyping", in which lentiviral or retroviral core proteins may interact with glycoproteins derived from other enveloped viruses, such as HA from Influenza, and assemble into infectious particles [3,4,6]. Here

we show the generation of lentiviral pseudotypes expressing influenza hemagglutinin (HA) from H5N1 avian strains as safe surrogates of the whole virus and their use to evaluate protective antibodies in a neutralization assay [5] by using sera from a sheep immunized with H5N1 VietNam/1194/04 and mice immunized with a prime-boost combination of H5N1 and H9N2 strains. The pseudotype-based neutralization assay presents the following advantages: *i*) it is promptly adaptable to a high-throughput format for the evaluation of vaccine efficacy; *ii*) it can be carried out at Biosafety Level 2, allowing the easy handling of highly pathogenic strains; *iii*) it is feasible to generate a panel of pseudotypes bearing HAs from different clades of Influenza pandemic viruses, without the need to have access to the whole virus.

Materials and Methods

Generation of pseudotypes bearing H5 hemagglutinin. Influenza lentiviral pseudotypes, expressing luciferase reporter gene and bearing HA on the surface, were generated as recently described [5]. Briefly, 293T cells were co-transfected with the following four plasmids: the gag-pol and rev constructs, the pl.18 plasmid expressing HA from Influenza A/VietNam/1194/04 (H5N1, clade 1), and the reporter plasmid expressing *renilla luciferase* gene [3,5]. In order to obtain pseudotypes bearing HA from H5N1 clade 2 strains, nucleotide sequences encoding Indonesia/5/05, whooping swan/Mongolia/244/05 and Anhui/1/05 HAs were synthesized by GeneArt. HA sequences were subsequently cloned into the suitable pl.18 vector. The multi-basic cleavage site of VietNam/1194/04 HA was substituted into the other H5 sequences in an effort to generate functionally active pseudotyped vectors and to confer similar entry [6]. Twenty-four hours post infection, 1 U of exogenous neuraminidase (Sigma) was added to allow the release of pseudotypes. Culture supernatants were collected 48 and 72 hrs post-infection, filtered and stored at -80°C. Pseudotypes were then titrated on 293T cells to calculate the TCID50.

Sera. Hyper-immune sheep serum were obtained by immunizing 2 animals subcutaneously with A/VietNam/1194/04 virus (NIBRG-14). Balb/c mice (8 animals per group) were immunized intramuscularly with 0.2 µg/dose of a prime-boost combination of A/VietNam/1194/04 virus (NIBRG-14), A/H9N2/03 or both, adjuvanted with MF59. The mice were bled 2 weeks after the second immunization, and sera were collected and pooled.

Hemagglutination inhibition assay. The HI assay was performed following standard protocol using horse red blood cells [7]. Briefly, pooled serum samples were incubated with strain-specific influenza antigen (whole virus, containing 8 hemagglutinating units) for 60 min at room temperature. The titer was defined as the serum dilution in which the last complete agglutination inhibition occurred.

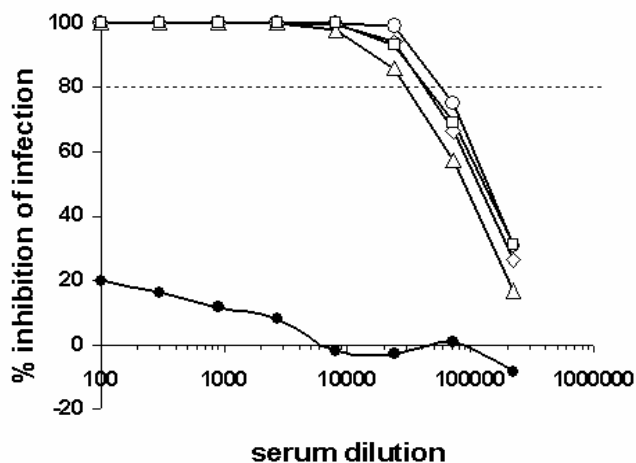
Neutralization assay. Correlation coefficient between HI and neutralizing titers was calculated using Microsoft Excel.

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Results

Evaluation of neutralizing antibodies using a pseudotype-based assay. To evaluate the functionality of this system, pseudotypes expressing HA from VietNam/1194/04 were incubated with serial dilutions of immune and pre-immune sheep serum, and the reduction in luciferase activity in the target cells was measured. The inhibition of infection in the presence of immune sheep serum was observed in a dose-dependent manner, in contrast to the pre-immune sera which had no effect (Figure 1). Moreover, the neutralization was HA-mediated, since no activity was observed using unrelated pseudotypes (data not shown). In order to evaluate the presence of cross-neutralizing antibodies the sheep serum was tested for its ability to neutralize a panel of avian strains, chosen on the basis of WHO recommendation [8]. In particular, HA sequences from Indonesia/5/05, whooping swan/Mongolia/244/05 and Anhui/1/05 H5N1 strains, representatives of subclades 2.1, 2.2 and 2.3 respectively, were used to prepare pseudotypes. Results showed that all the pseudotypes tested were susceptible to neutralization, indicating the presence of cross-protective antibodies among different clades and subclades (Figure 1).

Figure 1. Titration of neutralizing activity against H5N1 pseudotypes neutralization of sheep serum raised against Vietnam/1194/04 versus the homologous pseudotype Vietnam/1194/04 (○) and the heterologous pseudotypes Mongolia/244/05 (◊), Indonesia/5/05 (Δ) and Anhui/1/05 (□). Neutralization of pre-immune serum against Vietnam/1194/04 (●). The dashed lines indicate 80% RLU reduction.

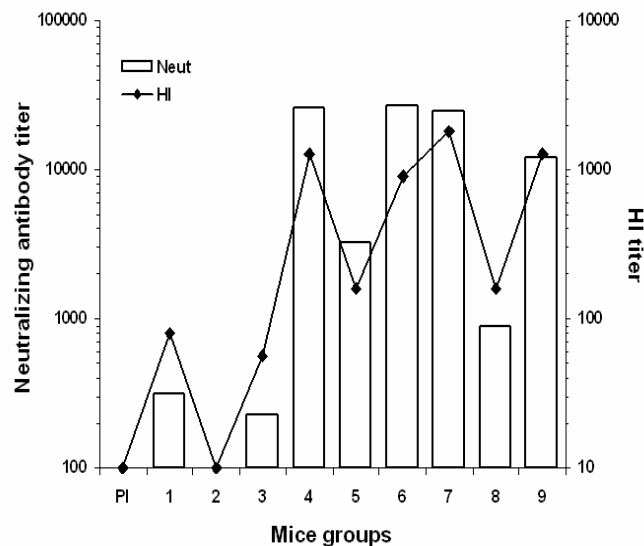


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Pseudotype neutralization and HI titers correlate in mice sera. Sera from mice immunized with a prime-boost combination of H5N1 VietNam/1194/04 and H9N2/03 viruses were collected 2 wks post second dose, and pooled sera were tested for their ability to neutralize pseudotypes expressing HA from the VietNam/1194/04 strain. The neutralizing titers, expressed as ID_{80} , were compared with the titers obtained by HI assay (against H5N1 homologous strain) using horse erythrocytes.

Results showed that sera from mice immunized with 2 doses of H5N1 VietNam/1194/2004 (groups 4, 6, 7 and 9) were able to neutralize the homologous pseudotype with higher titers (ranging from 12000 to 26000) as compared to the groups (1, 3, 5 and 8) that received only one dose of H5N1 (titers below 3000). In contrast, no neutralizing activity was observed in the group 2 immunized with H9N2 as well as in the pre-immune sera. Notably, a similar trend was observed in the HI titers (Figure 2). Statistical tests performed on the logarithmic titers values revealed a correlation coefficient of 0.96 (calculated using Pearson Coefficient) confirming the correlation of neutralizing antibody titers obtained using the pseudotype-based assay with titers obtained by horse erythrocyte HI.

Figure 2. Comparison of neutralizing versus HI titers in mice sera. neutralization titer (White bar, left vertical axis) and HI titers (Diamond symbol, right vertical axis) of pooled sera collected from animals immunized with a prime-boost combination of H5N1 VietNam/1194/04 and H9N2/03 viruses against VietNam/1194/04 pseudotype. Groups 1, 2 and 3 were primed with H9N2 and boosted with H5N1, H9N2 and H5N1/H9N2, respectively; groups 4, 5 and 6 were primed with H5N1 and boosted with H5N1, H9N2 and H5N1/H9N2, respectively; groups 7, 8 and 9 were primed with H5N1/H9N2 and boosted with H5N1, H9N2 and H5N1/H9N2, respectively. The neutralizing titers are expressed as the reciprocal of the serum dilution required to reduce RLU by 80% (ID_{80}).



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Discussion

We have set up a pseudotype-based assay valuable for measuring neutralizing and cross-neutralizing antibodies against H5N1 avian strains in vaccinated animals. This pseudotype system doesn't contain any viral genome and it is useful for a single-round infection making it suitable for testing highly pathogenic strains under Biosafety Level 2 containment. Moreover, a panel of pseudotypes expressing HA from different viral strains could be easily generated and used to evaluate cross-protective antibodies. Neutralizing antibodies

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are measured using a luciferase reporter gene that makes this tool adaptable for high throughput analysis for evaluation of vaccine efficacy. We have shown that pseudotypes bearing HA from Viet Nam/1194/04 strain are susceptible to neutralization using mice and sheep sera raised against the homologous virus strain. Furthermore using a panel of pseudotypes expressing HA from H5N1 subclades 2 we also showed the presence of cross-neutralizing antibodies among the H5N1 clades. These results indicate that the pseudotypes represent safe surrogates of the whole virus to evaluate cross-neutralizing antibodies with high sensitivity. Moreover, we showed a good correlation between pseudotype neutralizing and HI titers in mice sera, suggesting that this assay could be useful for the evaluation of protective antibodies in human sera. Future efforts will be focused to compare results from this assay with HI and micro-neutralization in vaccinated people to validate its ability to predict protection in humans.

Acknowledgements

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Virological Assessment and Usefulness of Rapid Diagnostic Kits for Influenza

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We measured the detection limits of 18 different kits by using virus strains that viral infectivity titers had been measured by plaque assay and performed a comparative assessment with virus isolation and RT-PCR. The detection limit was 1.1 to 8.3 pfu/test for isolation culture and nested RT-PCR and 1.1×10^3 to 5.8×10^5 pfu/test for the rapid diagnostic kits. Detection sensitivity tended to be lower for type B strains than for type A strains. The virus loads in the respective types of clinical specimens varied greatly below 10^8 pfu/ml. Average virus titer of gargle fluid, pharyngeal swabs, nasopharyngeal swabs and nasopharyngeal aspirates were 1.7, 3.5, 3.9 and 5.8 \log_{10} pfu/mL, respectively. Among all the specimen types, nasopharyngeal aspirates had a higher ratio of specimens with larger viral loads than the others. Comparing the results of one rapid diagnostic kit, ESPLINE Influenza A&B-N, and virus loads with clinical specimens collected on the day symptoms developed, detection ratios of the different specimen types, nasopharyngeal aspirate, nasopharyngeal swab, and pharyngeal swab were 82.1%, 75.4% and 56.1% for type A influenza (total 182 specimens) and 81.3%, 47.2% and 31.3% for type B influenza (total 74 specimens), respectively. Virus loads of 10^3 pfu/test or more are needed for rapid diagnostic kits, and when the virus load is low or specimen collection is not adequate, the results become false negative. It is important to collect specimens that contain as much a virus load as possible.

Background

As a result of the widespread adoption of rapid diagnostic tests the accuracy of influenza diagnosis has improved, and treatment with anti-influenza drugs has become routine in Japan. In the 2006/2007 season 15 different types of rapid diagnostic kits were released on the market in Japan, and about 20 million tests were used. On the other hand, epidemic information obtained by using the results of testing with the rapid diagnostic kits has also become useful in providing real-time surveillance information [1]. Objectives: It is difficult to simultaneously evaluate and compare a wide variety of rapid diagnostic kits. We measured the detection limits of 18 different kits by using virus strains that viral infectivity titers had been measured by plaque assay and performed a comparative assessment with virus isolation and RT-PCR. To assess usefulness of the rapid diagnostic kits, we also investigated distribution of viral loads in each clinical specimen.

Materials and Methods

Viruses. Nine different vaccine virus strains, i.e., A/New Caledonia/20/99(H1N1), A/Panama/2007/99(H3N2), A/Wyoming/03/2003(H3N2), A/NewYork/55/2004(H3N2), A/Wisconsin/67/2005(H3N2), A/Hiroshima/52/2005(H3N2), B/Shandong/7/97(Victoria type), B/Shanghai/361/2002(Yamagata type), B/Malaysia/2506/2004(Victoria type) and AH1N2 strain isolated in Yokohama were used. The sensitivities of the rapid diagnostic kits, virus isolation, and RT-PCR [2] were compared in relation to 100 μ l of solutions obtained by serial 10-fold dilutions. The lowest concentration of virus detected is reported. Sensitivity among the rapid diagnostic kits, virus isolation, and RT-PCR were compared.

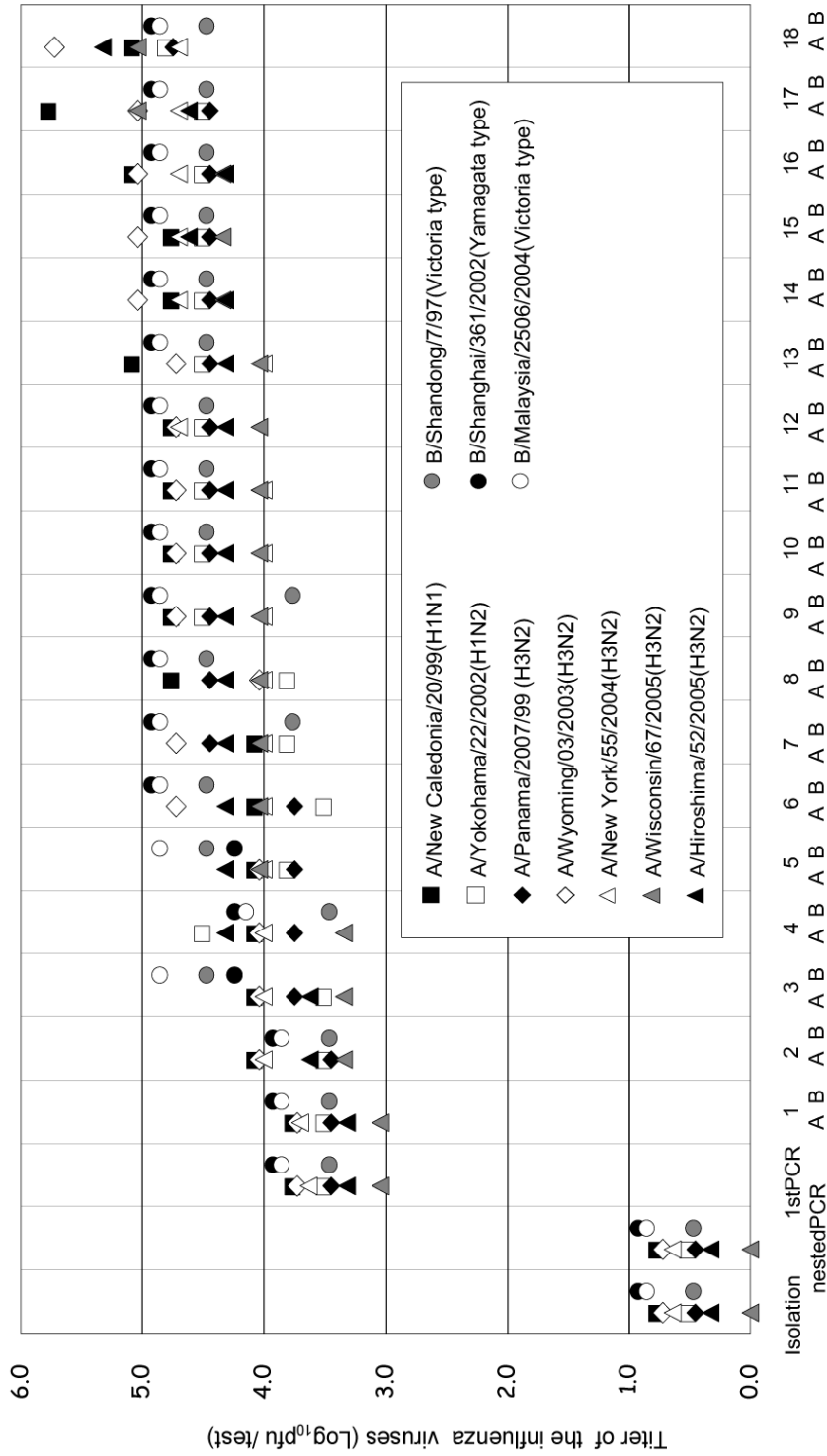
Rapid diagnostic kits. The rapid diagnostic kits evaluated were ESPLINE InfluenzaA&B-N (Fujirebio Inc., Japan), QuickVue Influenza A+B test (Quidel Corporation, USA), Quick-Ex Flu SEIKEN (Denka Seiken Co., Ltd., Japan), Clearview Influenza A/B (Applied Biotech Inc., Japan), RapidTesta FLU Stick (Daiichi Pure Chemicals Co., Ltd., Japan), BD flu exzaman (Becton Dickinson and Company, USA), RapidTesta FLU/Tamitest InfluenzaAB (Daiichi Pure Chemicals Co., Ltd., Japan), Immunotrap Influenza A/B (Shino-Test Corporation, Japan), POCTEM Influenza A/B(Sysmex Corporation, Japan), Capilia Flu A+B (ROHTO Pharmaceutical Co., Ltd., Japan), Protest FLU (Mitsubishi Kagaku Iatron, Inc., Japan), Statmark FLU Stick AB (Nichirei Biosciences Inc., Japan), Quickchaser Flu A, B (MIZUHO MEDY Co., Ltd., Japan), BD Directigen FluA+B (Becton Dickinson and Company, USA), POCTEM S Influenza (Sysmex Corporation, Japan), BinaxNOW Influenza (Binax Inc., USA), CheckFluA/B (ROHTO Pharmaceutical Co., Ltd., Japan), and StatmarkInfluenza A/B (Nichirei Biosciences Inc., Japan).

Clinical specimens. Viral loads in clinical specimens (gargle fluid: 59 samples, pharyngeal swabs: 69 samples, nasopharyngeal swabs: 22 samples, nasopharyngeal aspirates: 60 samples) were plaque assayed. In addition, sensitivity between virus culture and rapid diagnostic kit (ESPLINE Influenza A&B-N) were compared with clinical specimens collected on the day symptoms develop.

Results

The detection limit was 1.1 to 8.3 pfu/test for isolation culture and nested RT-PCR and 1.1×10^3 to 5.8×10^5 pfu/test for the rapid diagnostic kits. There were differences of 10 fold to 100 fold for the same virus strain, and there were differences of up to about 32 fold for the same product due to differences in virus strain. In addition, detection sensitivity tended to be lower for type B strains than for type A strains (Figure 1).

Figure 1. Comparison of detection limit determined by viral load for 18 different rapid diagnostic kits.

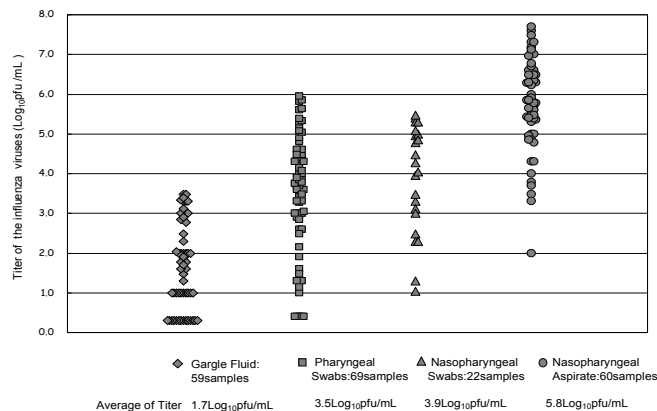


1. ESPLINE Influenza A&B-N, 2. QuickVue Influenza A+B test, 3. Quick-Ex Flu SEIKEN, 4. Clearview Influenza A/B,
5. RapidTesta FLU Stick, 6. BD flu ezaman, 7. RapidTesta FLU II /Tamitest Influenza AB, 8. Immunotrap Influenza A·B,
9. POCTEM Influenza A/B, 10. Capilia Flu A+B, 11. Protest FLU, 12. Statmark FLU Stick AB, 13. Quickchaser Flu A, B,
14. BD Directigen FluA+B, 15. POCTEM S Influenza, 16. BinaxNOW Influenza, 17. CheckFluA·B, 18. Statmark Influenza A/B.

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The virus loads in the respective types of clinical specimens varied greatly below 10^8 pfu/ml. Average virus titers of gargle fluid, pharyngeal swabs, nasopharyngeal swabs and nasopharyngeal aspirates were 1.7, 3.5, 3.9 and 5.8 \log_{10} pfu/mL, respectively. Among all the specimen types, nasopharyngeal aspirates had a higher ratio of specimens with larger viral loads than the others (Figure 2).

Figure 2. Comparison of viral loads for four different clinical specimens.



useful to detect influenza earlier in non-epidemic season or to detect an imported case from abroad and enhance surveillance.

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Comparing the results of one rapid diagnostic kit, ESPLINE Influenza A&B-N, and virus culture with clinical specimens collected on the day symptoms develop, detection ratios of the different specimen types, nasopharyngeal aspirate, nasopharyngeal swab, and pharyngeal swab were 82.1%, 75.4% and 56.1% for type A influenza (total 182 specimens) and 81.3%, 47.2% and 31.3% for type B influenza (total 74 specimens) respectively (data not shown). There was no difference in the detection ratio between type A and type B influenza with the nasopharyngeal aspirate specimens. However, detection ratios of type B were lower than those of type A with nasopharyngeal swab and pharyngeal specimens.

Conclusions

Virus loads of 10^3 pfu/test or more are needed for rapid diagnostic kits, and when the virus load is low or specimen collection is not adequate, the results become false negative. It is important to collect specimens that contain as much a virus load as possible. Conventional definitive diagnostic methods; serum antibody examination, cell culture and RT-PCR; are difficult to use in clinical practice because of their cost, time, or complexity. Therefore their results do not combine well with medical treatments. Under these circumstances, rapid diagnostic kits make a substantial contribution because its spread brought a dramatic advance to diagnosis and treatment of influenza and, furthermore, it led people to pay strong attention to pathologic and epidemic conditions of influenza. Moreover it is thought that the rapid diagnostic kits may be very

Surveillance of Influenza During the Epidemic Season 2006/2007: Comparison of Near Patient Test QuickVue With Immunoperoxidase Staining and Viral Culture for the Detection of Influenza A (H3N2) Viruses

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Objective

Routine virological influenza surveillance is usually based on cell cultures as the “gold standard” in testing; this procedure provides both the most accurate diagnosis and the opportunity for further study of the virus. Nevertheless, it takes at least 2 – 3 days and usually a week or longer in practice. Laboratory diagnosis of influenza using “near patients tests” is suitable for rapid antigen detection (15-20 minutes) and provides very early information on virus circulation and/or a possible influenza outbreak.

Material and Methods

During the epidemic season 2006/2007, the QuickVue Influenza A+B test (Quidel Corporation, USA) was used in parallel with the indirect immunoperoxidase assay (IPA) (1.) and virus isolation. Clinical specimens (nasopharyngeal swabs) from influenza-like illness were collected through the national surveillance network.

QuickVue. The test was carried out according to the kit procedure manual.

Virus isolation. Samples were inoculated in parallel onto Madin–Darby Canine Kidney - MDCK cells (Institut Pasteur, Paris, France) in 96-well multiwell plates (GAMA Group, Czech Republic) and centrifuged 15 min at 3500 rpm. After incubation for 2-3 days at 35.5°C in 5% CO₂ atmosphere, infection was confirmed by IPA and tissue culture fluid was observed for the haemagglutination (with 0.5% suspension of guinea pig red blood cells). Mouse monoclonal antibodies (influenza A – lot 03-003252 and influenza B – lot 020213L, CDC Atlanta, USA, the WHO influenza reagent kit for identification of influenza isolates), swine anti-mouse polyclonal antibodies with conjugated horseradish peroxidase (Sevapharma, Czech Republic) and 3-amino-9-ethylcarbazole substrate (Sigma) were used for IPA.

Results

All of the 454 specimens (collected from October 1, 2006 to February 23, 2007) were tested by the QuickVue, IPA and cell culture. None of 200 samples collected before the influenza epidemic (by January 4, 2007) appeared influenza positive in

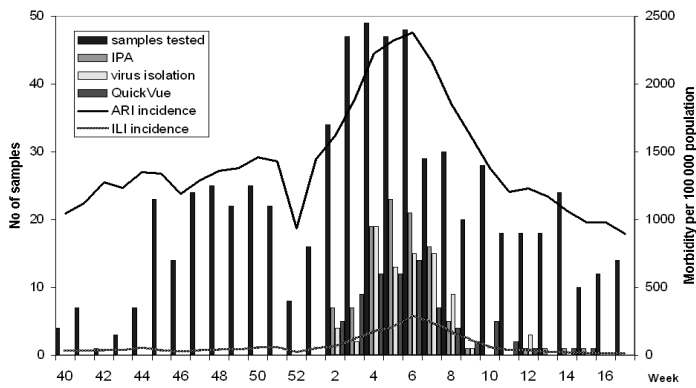
any test. The first unambiguously positive results were detected in the 2nd calendar week, whereas the total ARI/ILI morbidity in this period of time was at the seasonal level, with only a local gentle increase in morbidity being observed. Using the QuickVue test, the first cases of flu A in the Czech Republic were detected as early as 7 days before the rise in morbidity and 10 days before the positive isolation. Altogether 254 samples were tested from the 2nd to 7th calendar week when epidemic activity of influenza A viruses was observed. Eighty-nine (35%) of these specimens were positive for influenza A by IPA, influenza virus A (H3N2) was isolated in 78 (30.7%) specimens and 60 (23.6%) specimens tested positive for influenza A in the QuickVue test. IPA served as a “gold standard” since it allows clear detection of virus-infected cells. Compared with IPA, the QuickVue test showed a sensitivity of 67.4% (60/89) and a specificity of 99.0% (200/202). The positive predictive value (PPV) was 96.8% while the negative predictive value (NPV) achieved 87.3%. Comparison of the three used methods for influenza A/H3N2 virus detection is shown in Figure 1.

Conclusions

Sensitivity of the QuickVue test was lower than that of cell culture or IPA, but the major point of interest is the speed with which information about circulation of influenza viruses was obtained. Confirmation of the QuickVue results by IPA or cell culture was achieved within the next 2 to 10 days. Although not perfect, near patient tests can play an important role in the early identification and ongoing monitoring of influenza viruses and annual epidemics. The rapid detection of influenza virus is important for effective anti-viral therapy, prescription of antibiotics, exclusion of influenza-like pathogens and early institution of measures to limit the spread of infection, particularly in nursing homes, hospitals etc. Furthermore, after the SARS epidemic, rapid diagnosis is more important than ever before. On the other hand, IPA providing results within a reasonable interval is a very sensitive tool for the diagnosis of influenza virus, comparable in sensitivity to “in house” RT - PCR SYBER Green Applied Biosystem based on primers designed by Wright (2.) Combination of the rapid tests with the conventional methods proved highly effective in practice but has the disadvantage of being costly, and posing limitations to the use directly in the general practice.

Options for the Control of Influenza VI

Figure 1. Comparison of the three different methods for influenza A/H3N2 virus detection during 2006/2007 influenza season.



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The Speed and Reliability of the Antigen Detection Kit Capilia Flu A, B When Used in a Clinical Setting

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The diagnostic speed of one antigen detection kit and the relationship between the speed and its diagnostic reliability were investigated in a clinical setting. The appearance of a positive line was visually checked at three and fifteen minutes from the time of specimen application for patients with influenza A. The time to appearance of a positive line was also measured in seconds for 34 patients. The time to positive was ≤ 3 minutes in 76.3%, $3 < \leq 15$ minutes in 20.2% and over 15 minutes in 3.5% of the positive cases. The positive predictive value (PPV) was 96.7% for 90 patients ≥ 3 minutes, 82.1% for 28 patients $3 < \leq 15$ minutes, and 80% for 5 patients over 15 minutes. The mean appearance time of the positive line was 126.5 seconds and the diagnosis was made in 55.9 % of the positive cases within one minute. The appearance time became longer as the antigen concentration decreased. Similar linear relationships between the viral antigen concentration and the time to appearance of a positive line were obtained for the A/H1N1, A/H3N2, and B viruses. The diagnosis by the antigen detection kit was highly reliable, especially for cases diagnosed within three minutes. The time to appearance of a positive line may be a useful clinical indicator of the viral load.

Introduction

High reliability has been widely reported for the commercial antigen detection kits now commonly used in Japan for the diagnosis of influenza infection. That these kits can make a rapid diagnosis is well recognized; however, the speed at which the kits are able to make the diagnosis has not been fully studied. Herein, we determine the diagnostic speed of one antigen detection kit and the relationship between the speed and its diagnostic reliability in a clinical setting. We also did an experimental study of the relationship between the time to appearance of a positive line and the viral concentration.

Methods

Patients. Family doctors, pediatricians, and physicians at 13 clinics belonging to the Influenza Study Group of the Japan Physicians Association participated in the study. Patients with influenza-like symptoms who visited clinics within 48 hours from the onset of fever were enrolled from 10 December 2005 through 30 March 2006. Patients were diagnosed as influenza A, influenza B, or not infected with influenza by the commercial antigen detection kit Capilia FluA+B (Tauns, Japan). This kit

is based on lateral-flow immunochromatography, with test specimens taken from throat swabs, nasal swabs, or nasal aspirates. Because of the low number of patients with influenza B in the 2005/2006 influenza season, they were excluded from study.

Speed of a positive reaction by the antigen detection kit. The appearance of a positive line was visually checked at three and fifteen minutes from the time of specimen application. Patients were classified into three groups according to the results; ≥ 3 min, $3 \text{ min} < \leq 15 \text{ min}$ and over 15min. The time to appearance of a positive line was also measured in seconds for some patients. Virus isolation and PCR. Virus isolation was done by standard methods using Madin-Darby canine kidney cells. PCR was done for samples negative by virus isolation. RNA was extracted from culture supernatant. Using the extracted RNA, first strand cDNA was synthesized using random hexamer primers and reverse transcriptase. One-tenth of the first strand cDNA reaction product was used as a template for PCR. The oligonucleotide primers were as follows: outer primers, IAH1F44 5'-CAGATG CAGACACAATATGT-3', IAH1B1055 5'-AAACCGCAATGGCTCCAAA-3', IAH3F145, 5'-CAG/AATTGAAGTGACTAATGC-3', IAH3B1027 5'-GTTTCTCTGGTACATCCGC-3', IBHF121 5'-GTGACTGGTGTGATACCACT-3', IBHB1023 5'-TGTTTTACCCA TATTGGGC-3'. Inner primers, IAH1F64 5'-ATAGGCTACCATGCGAACA-3', IAH1B1004 5'-CTTAGTCTGTAAACCATCCT-3', IAH3F318 5'-AGCAAAGCTTT/ACAGCAACTG-3', IAH3B909 5'-GCTTCCATTTGG AGTGATGC-3', IBHF143 5'-CATTGCAAATCTCAAAGG-3', IBHB932 5'-TGGAGGCAATCTGCTTACC-3' Amplification was done with 27 cycles for the first PCR and with 28 cycles for the second PCR. Each cycle consisted of a denaturing step at 94°C for 1min, an annealing step at 53°C for 1 min, and an extension step at 72°C for 1 min. The PCR-amplified products were fractionated on a 2 % agarose gel containing 1 μg / mL ethidium bromide. The amplified DNA was visualized by exposure to UV light. **Experimental correlative study of virus concentration and the time to positive.** Serially diluted laboratory prepared influenza A/H1N1, A/H3N2 and B virus antigens were used as test specimens. Each antigen was serially diluted with the diluting solution of the antigen detection kit. Using this solution, a reaction was done with an antigen detection kit. The time to positive was measured for each concentration of viral antigen.

Results

Diagnostic reliability. A total of 192 patients were enrolled; 120 patients with influenza A and 72 patients without influenza, confirmed by virus isolation and PCR. No significant difference was found between patients with influenza A and without influenza for mean age, ratio of females-to-males, vaccination status, or specimen collection method. The overall sensitivity was 95.0% (114/120), specificity was 87.5% (63/72), predictive value for a positive test was 92.7%, predictive value for a negative test was 91.3%(63/69) and accuracy was 92.2%(177/192).

Time to appearance of a positive line. The time to positive was ≥ 3 minutes in 76.3%, $3 < \leq 15$ minutes in 20.2% and over 15

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minutes in 3.5% of the positive cases. The positive predictive value (PPV) was 96.7% for 90 patients ≥ 3 minutes, 82.1% for 28 patients $3 < \leq 15$ minutes, and 80% for 5 patients over 15 minutes. A significant difference ($p < 0.01$) was shown in the PPV between tests taking ≥ 3 minutes (96.7%) and tests taking $3 < \leq 15$ minutes (82.1%). The appearance time of the positive line in seconds was measured for 34 patients with influenza A. The cumulative frequency of positive cases by the time from the start of the test to the recognition of a positive line is depicted in figure 1. The mean appearance time of the positive line was 126.5 seconds and the diagnosis was made in 55.9% of the positive cases within one minute.

Experimental correlative study of virus concentration and the time to positive. The results of the relationship between virus concentration and the time to positive are shown in figure 2. The appearance time became longer as the antigen concentration decreased. Similar linear relationships between the viral antigen concentration and the time to appearance of a positive line were obtained for the A/H1N1, A/H3N2, and B viruses.

Figure 1. Cumulative frequency of positive cases by the time from the start of the test to the recognition of a positive line. Time to positive in 34 cases was 126.6 ± 184.6 sec.

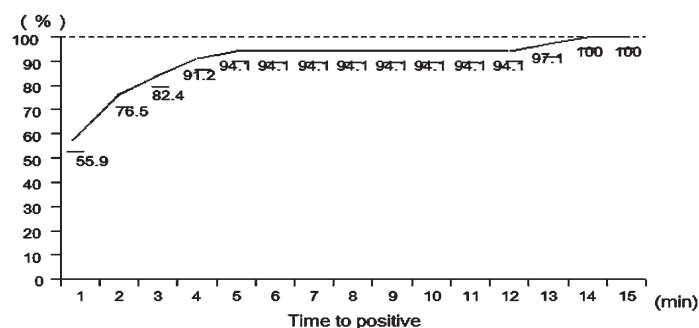
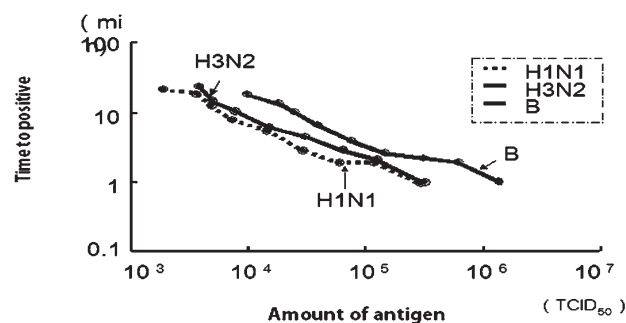


Figure 2. Relationship between virus concentration and the time to positive for clinical specimens of the influenza A/H3N2 and B viruses. The time to positive was measured five times for each virus concentration. The mean value is shown.



Discussion

A precise and rapid diagnosis of influenza is beneficial for patients and clinicians from the standpoint of early administration of the anti-influenza drugs commonly used to shorten the duration of fever and other symptoms (6)-9). Recent developments in commercial antigen detection kits have made it possible to diagnose influenza rapidly and reliably. In this study, the high reliability of antigen detection kits, such as Capilia Flu A+B, was confirmed using clinical specimens. Furthermore, the outstanding speed at which the kit is able to make a diagnosis was shown. A positive line appeared within three minutes after the start of examination in three quarters of the patients with a positive reaction. Over 50% of the patients were diagnosed within one minute. This kit utilizes a colored particle to visualize a positive reaction, and the enzymatic coloring method requires much time to react with the substrate. Colored particles, such as the gold colloid labeled antibody used in Capilia A+B allow the line to appear in far less time because visualization occurs directly. The amount of antigen and the time to positive were reversely correlated. Similar results were obtained for influenza A/H1N1, A/H3N2, and B. The results suggest that a shorter time to positive is associated with a higher antigen concentration. If this is the case, the time to be positive may be useful as an indicator of the virus load of the patients. Further investigation concerning this aspect is necessary and important for establishing the clinical reliability of this hypothesis. Conclusion: The high reliability of the antigen detection kit, Capilia Flu A+B, was confirmed using clinical specimens. Furthermore, the outstanding speed at which the kit is able to make a diagnosis was shown. The diagnosis by the antigen detection kit was highly reliable, especially for cases diagnosed within three minutes. The results showing that the time to appearance of a positive line is shorter for specimens with a high antigen concentration may be important, which would make this parameter a useful clinical indicator of the viral load.

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Evaluation of the Quidel QuickVue® Influenza A+B Kit in Detecting Avian and Swine Influenza Virus

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Background: Pandemic influenza A viruses are often zoonotic. Rapid influenza virus diagnostics can play a role in reducing pandemic virus spread. However, most diagnostic tests have not been evaluated against animal influenza viruses. **Objective:** To evaluate a leading rapid diagnostic test against zoonotic influenza A virus strains. **Study Design:** We evaluated the performance of the QuickVue® Influenza A+B Kit in detecting 19 influenza A viruses of avian, human, and swine origin. Cell culture supernatant solutions were serially diluted (1:10-1:100,000), blanks were added, and the panel was arranged in a random order. A physician ran the 100 assays in a blinded fashion. **Results:** The QuickVue® Influenza A+B Kit detected every influenza A virus with no false positives. Virus detection limits varied from 2.2×10^0 TCID₅₀/ml (AvH6N2) to 1.0×10^5 TCID₅₀/ml (SwH1N2). The average TCID₅₀/ml's for avian, human and swine virus detections were 2.4×10^3 , 1.7×10^3 , and 3.5×10^4 , respectively. **Conclusion:** The QuickVue® Influenza A+B Kit readily detected all influenza A viruses but the kit required a higher viral titer in detecting swine H1N2 virus. When viral titers are relatively high, the QuickVue® Influenza A+B Kit may be useful in rapidly detecting the presence of avian and swine influenza A viruses.

Introduction

Influenza is a highly infectious respiratory virus that causes significant morbidity and mortality worldwide [1]. In developed countries, the disease has been estimated to explain 10–12% of all work absences. It likely contributes to the morbidity of a number of chronic diseases and directly and indirectly escalates medical costs and lost wages each influenza season [2]. In the US alone each influenza season an estimated 36,000 influenza-related deaths occur [3]. In recent years, commercially available, minimally complex, rapid influenza diagnostic tests have become available. While DFA, RT-PCR, and cell culture for influenza viruses have better diagnostic sensitivity, they require significant laboratory resources and significant turnaround time [4]. Hence, minimally complex, rapid influenza tests have frequently been used in outpatient clinical settings where prompt office-based testing may influence treatment decisions [5-7]. France, Switzerland, and certain US states have experimented with integrating such rapid tests into their influenza surveillance systems [2]. Rapid influenza tests' high specificity, simplicity, and increased reporting speed can provide early warning in some influenza

surveillance systems, especially those without advanced medical laboratory support [8]. A number of rapid influenza detection tests are now available that are minimally complex, quick (generate a result in 15 min), and can be performed without sophisticated laboratory support [6]. In controlled laboratory trials and in medical practices, rapid influenza tests have generally demonstrated good sensitivity and excellent specificity in detecting influenza virus [6, 9-11]. One of these tests, the QuickVue® rapid test, a lateral flow immunoassay that detects both influenza virus types A and B nucleoproteins, promises to be useful in clinical outpatient settings. [2, 6, 12-17]. The next pandemic influenza A virus is very likely to be zoonotic in origin. Rapid influenza A diagnostic tests may play roles in detection and the control of the spread of such novel influenza A viruses. However, generally rapid diagnostic tests have been developed to detect human influenza A viruses, and they have not been evaluated against zoonotic strains. In this study we sought to evaluate a leading rapid diagnostic test against a collection of zoonotic influenza A virus strains.

Methods

Nineteen prototypic influenza A viruses were tested with the Quidel QuickVue® Influenza A+B Kit per the manufacturer's instructions. The viruses (13 avian, 3 human, and 3 swine) were grown in MDCK cells or embryonated eggs. Reed-Muench TCID₅₀/ml infectious unit calculations for each virus were determined using trypsin in the neutralization assays. PBS blanks were added as negative controls. Samples were serially diluted from 1:10 to 1:100,000 with PBS. Five negative control specimens were added to the 95 specimens containing influenza A virus. All samples were then ordered by random-number and tested blindly. The 100 assays were run using 250 µl of sample in accordance with the Quidel QuickVue® Influenza A+B Kit package insert instructions.

Results

The Quidel QuickVue® Influenza A+B Kit detected all subtypes of influenza A virus (Table 1). There were no false positives. The detection limits varied from 2.2×10^0 TCID₅₀/ml (AvH2N2) to 1×10^5 TCID₅₀/ml (SwH1N2). The average TCID₅₀/ml's by virus type were 2.4×10^3 , 1.7×10^3 and 3.5×10^4 (avian, human and swine respectively, Table 2).

Table 1. Detection limits of avian, human, and swine influenza A viruses. 19 influenza A viruses tested with the Quidel QuickVue® Influenza A & B Kit 0=negative, +1=VERY light positive, +2=very light positive, +3=light positive, +4=positive.

sample/strain/ TCID50/ml	dilution	Result	sample/strain/ TCID50/ml	dilution	Result	sample/strain/ TCID50/ml	dilution	Result	sample/strain/ TCID50/ml	dilution	Result
AvH1N1 Duck/Alberta/35/76 1.0E+02	1:10	+4	AvH6N2 Turkey/MA/65 2.2E+00	1:10	+4	AvH11N9 Duck/Memphis/546/74 2.2E+03	1:10	+4	SwH1N1 Sw/WI/238/97 5.6E+00	1:10	+4
	1:100	+3		1:100	+4		1:100	+4			
	1:1000	+3		1:1000	+2		1:1000	+3			
	1:10000	0		1:10000	0		1:10000	0			
	1:100000	0		1:100000	0		1:100000	0			
AvH2N2 Mallard/NY/6750/78 7.7E+00	1:10	+4	AvH7N2 Turkey/VA/4529/2002 1.6E+03	1:10	+4	AvH12N5 Duck/Alberta/60/76 1.8E+01	1:10	+4	SwH1N2 Sw/WI/R33F/01 1.0E+05	1:10	+4
	1:100	+4		1:100	+4		1:100	+4			
	1:1000	+2		1:1000	+2		1:1000	+3			
	1:10000	+2		1:10000	0		1:10000	+2			
	1:100000	0		1:100000	0		1:100000	0			
AvH3N8 Duck/Ukraine/1/63 1.8E+04	1:10	+4	AvH8N4 TY/Ontario/68 3.2E+02	1:10	+4	AvH13N2 Gull/MD/704/77 5.6E+01	1:10	+4	SwH3N2 Sw/MN/593/99 3.7E+03	1:10	+4
	1:100	+4		1:100	+4		1:100	+4			
	1:1000	+2		1:1000	+2		1:1000	+2			
	1:10000	0		1:10000	0		1:10000	0			
	1:100000	0		1:100000	0		1:100000	0			
AvH4N6 Duck/Cz/1/56 1.8E+02	1:10	+4	AvH9N2 TY/MN/38391-6/95 1.5E+03	1:10	+4	HuH1N1 NewCaldonia 1.7E+02	1:10	+4	SwH3N2 Sw/MN/593/99 3.7E+03	1:10	+4
	1:100	+4		1:100	+4		1:100	+4			
	1:1000	+3		1:1000	+2		1:1000	+2			
	1:10000	0		1:10000	0		1:10000	0			
	1:100000	0		1:100000	0		1:100000	0			
AvH5N2 Chucker/MN/14591-7/98 4.2E+03	1:10	+4	AvH10N7 Chicken/Germany/49 3.2E+03	1:10	+4	HuH3N2 Nanchang/933/95 1.2E+03	1:10	+4		1:10	+4
	1:100	+3		1:100	+4		1:100	+4			
	1:1000	0		1:1000	+3		1:1000	+2			
	1:10000	0		1:10000	0		1:10000	0			
	1:100000	0		1:100000	0		1:100000	0			

19 influenza A viruses tested with the Quidel QuickVue® Influenza A & B Kit
 0=negative, +1=VERY light positive, +2=very light positive, +3=light positive, +4=positive

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Table 2. Mean limit of detection by virus type.

Virus type	TCID ₅₀ /ml
Avian (n=13)	2.4x10 ³
Human (n=3)	1.7x10 ³
Swine (n=3)	3.5x10 ⁴ *

*SwH1N2 included.

Whe SwH1N1 excluded, swine value=1.9x10³

Discussion

The QuickVue® Influenza A & B Kit has been shown to be effective for detecting human influenza viruses with sensitivities ranging from 67% to 95% [6, 12, 14, 17-19]. None of these studies quantified the amount of virus in the clinical samples. The specificity of the test has often been found to be very close to 100% with one exception where a specificity of 76% was reported [7]. There has been only one report in the medical literature evaluating the test's performance against animal influenza viruses. Woolcock et.al examined 5 commercial immunoassay kits in detecting avian influenza virus (AIV) type A and uniformly concluded the kits were insensitive compared to the gold standard of virus isolation [20]. In Woolcock's evaluation, the QuickVue® Influenza A test did detect purified virus but failed to detect any clinical AIV specimens. However, Woolcock only looked at one avian virus. Following a controlled, blinded experimental designed, the QuickVue® Influenza A & B test detected each purified human, avian, and swine influenza virus we examined. There were no false positive assay results. We looked at the hemagglutinin type, neuraminidase type or species and there was no recognizable distribution pattern of detection limits (2.2x10⁰ to 1x10⁵ TCID₅₀/ml). Since Woolcock's clinical evaluation was limited to AvH6N2, additional testing of the QuickVue® Kit on clinical AIV samples of other subtypes is needed. This was the first evaluation of the QuickVue® assay against swine influenza A viruses. The Quidel QuickVue® Influenza A+B Kit has great potential for field use. Technicians in swine or poultry facilities might employ the QuickVue® Kit as a rapid indicator of influenza infection in accessing ill pigs or birds. If the test is positive, then immediate interventions might be taken to protect the rest of the herd or flock. However, because of imperfect sensitivity and non-validated test on clinical samples, a negative rapid influenza A test should be followed with either culture, DFA, or RT-PCR in further evaluating the ill pig or bird. In conclusion, the QuickVue® Influenza A+B Kit readily detected human, swine, and avian influenza A viruses. Its performance was comparable for human and avian influenza A subtypes. The kit required a higher viral titer for detecting swine H1N2 virus, but its performance against commonly circulating swine H1N1 and H3N2 virus, was similar to that for human and avian viruses. When swine and avian influenza A viral titers are relatively high, the QuickVue® Influenza A+B Kit may be useful in rapidly and accurately detecting influenza A viruses.

Acknowledgements

QuickVue® Influenza A & B test Kits were provided by Quidel, Inc (San Diego, CA). Viruses were kindly provided by Dr. Richard Webby of St. Jude Children's Research Hospital, Memphis, Tennessee, USA; Alexander Klimov from US Centers for Disease Control and Prevention, Atlanta, Georgia, USA, and Dennis Senne of the National Veterinary Services Laboratories, Ames, Iowa, USA. We thank Dwight Ferguson, Gary Heil, Ghazi Kayali and Sharon Setterquist of the Center for Emerging Infectious Diseases for their help with the virus preparations and Whitney S. Baker for her most helpful review of the manuscript. This work was funded in part by NIH / NIAID R01 AI068803-01 (Gray). QuickVue® Influenza A & B Kits were provided by Quidel, Inc (San Diego, CA).

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Development of Competitive ELISA for Determination of Antibody Response to Influenza A (H5) Virus in Human Sera

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The emergence of avian influenza A(H5N1) virus infection in humans and its worldwide spread highlights the necessity of a reliable and simple test for seroepidemiological surveillance to determine the mode of virus transmission and risk factors associated with infection. Routine haemagglutination-inhibition test (HIT) using chicken or turkey erythrocytes appeared to be insensitive in detection of antibody response to avian H5-hemagglutinin after vaccination or infection. Virus neutralization test (VNT) was more sensitive than HIT in detection of H5-specific antibodies (Abs) in humans (3). However, VNT requires infectious virus application, so this method is not convenient for most practical laboratories. The aim of this investigation was to design a new competitive ELISA (c-ELISA) for detection of H5-specific antibodies in serum samples using A(H5) subtype specific monoclonal antibodies (H5-MAbs) developed at the Laboratory of Diagnostic Reagents Biotechnology (LDRB) of Research Institute of Influenza (St. Petersburg, Russia) (5).

Methods

The surface glycoproteins fraction (0.2 µg/ml) of influenza A(H1N1), A(H3N2) or A(H5N1) viruses purified by ultracentrifugation in sucrose gradient was used for adsorption on 96-well microtiter plates to analyse subtype specific Abs in human sera in an indirect ELISA. A modified virus microneutralization (MN) test (with direct detection of virus antigens by peroxidase conjugate of MAb to the conservative site in NP of the influenza A virus) was performed to detect the levels of antibodies to A(H5N1) viruses. The principle of c-ELISA was as follows. Influenza A(H5N1) virus was purified by ultracentrifugation in sucrose gradient, diluted up to concentration of 2-3 µg/ml in 0.05M carbonate buffer (pH 9.5) and adsorbed on plates for 18 h at 4°C. After washing in 0.01M phosphate-saline buffer (PBS), pH 7.2, containing 0.05% tween-20 (PBS-T) and incubation of the plates with PBS-T for 2 h at 37°C, a mixture of H5-MAbs (300 ng/ml) and investigated serum (diluted 1/50) was added in equal volumes (50 µl) into the antigen-coated wells. PBS-T containing 0.5% gelatine (PBS-T-G) was used for H5-MAbs and sera dilutions. After incubation for 18h at 4°C plates were washed three times in PBS-T and peroxidase-conjugated anti-mouse IgG diluted in PBS-T-G (1/10 000) was added for 1h at 37°C. The substrate-chromogen mixture consisted of 0.02% H₂O₂ and 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine in 0.1M acetate-citrate buffer, pH 5.0. Reaction was stopped in 10-15 min

by adding of 2N H₂SO₄. Optical density was determined at a 450-nm wavelength (OD₄₅₀) by photometer. Percent of inhibition (I) of H5-MAbs binding in the presence of serum antibodies was calculated using the following formula:

$$I = \left[1 - \left(\frac{OD_{450 \text{ test}} - OD_{450 K^-}}{OD_{450 K^+} - OD_{450 K^-}} \right) \right] \times 100$$

where: OD_{450 test} is the OD of the tested sample (mixture of serum and H5-MAbs)

OD_{450K⁻} is the OD of the negative control (without H5-MAbs and serum)

OD_{450K⁺} is the OD of the positive control (with H5-MAbs but without serum).

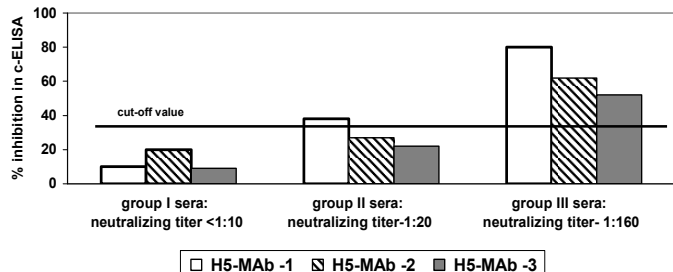
Results and Discussion

Cross-reactivity among Abs to different subtypes of influenza was revealed in indirect ELISA upon investigation of sera in vaccinated people (data not shown). Thus, by indirect ELISA nonspecific increase of antibody to A(H5N1) virus was detected in 13 of 16 (81%) persons immunized by seasonal vaccine "GRIPPOL" or influenza A infected patients. Vice versa nonspecific simultaneous seroconversions against influenza A(H1N1) virus were detected by this method in 46 of 70 (66%) volunteers immunized with inactivated influenza A(H5N1) vaccine. These results could be explained by the presence of shared epitopes in the structure of HAs between subtypes of influenza A viruses. HA molecules of influenza A virus (H1 – H15) share about 40 – 50% sequence identity between subtypes (2, 6). The most close phylogenetic relationship were determined between H5, H1 and H2. Furthermore, it was proved that HAs of the H1 and H5 subtypes contain cross-reactive epitopes recognized by mouse monoclonal antibodies (MAbs) (4). The existence of shared epitope in H1 and H5 molecules was confirmed by use of MAb A2 directed to HA1 of influenza A(H1N1) virus developed at LDRB. High positive signal (OD₄₅₀ 1.1 - 1.5) was registered in ELISA in reaction of MAb A2 (5 µg/ml) with both A(H1N1) and A(H5N1) viruses (data not shown). Taking into account the necessity of a simple and safe test for indication of subtype specific Abs to H5 virus, the idea of competition between subtype specific MAbs to H5 virus and Abs in human sera for binding with virus adsorbed on the plate was investigated. At the first stage of investigation selection of H5-MAbs was done on the basis of efficacy of their binding with H5-virus in indirect ELISA and sensitivity of the test in detection of H5-antibodies in analyzed sera. According to these criteria H5-MAb-1 was chosen for the further development of c-ELISA. The value of OD₄₅₀ was 0.9-1.1 in reaction of H5-MAb-1 (150 ng/ml) with A(H5N1) virus (2 µg/ml) in indirect ELISA. The sensitivity of c-ELISA using H5-MAb-1 was the highest. In contrast to two other H5-MAbs, H5-MAb-1 was capable to determine the low levels of H5-specific antibodies in sera of

Poster Presentations: Developments in Diagnostic and Serologic Techniques

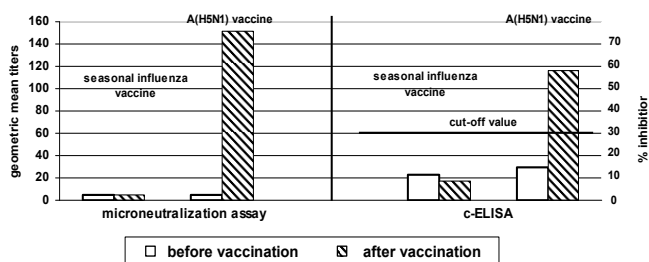
A(H5N1)-vaccinated volunteers without nonspecific reactions with negative sera (taken before vaccination) - Figure 1.

Figure 1. Sensitivity of c-ELISA using three different H5-monoclonal antibodies in detection of H5-specific antibodies in sera of volunteers immunized with A(H5N1) vaccine.



The mean and standard deviation (SD) of percent inhibition values in c-ELISA for H5-negative sera of patients with acute respiratory diseases (n=30) were calculated (data not shown). The threshold (negative cut-off) value of inhibition was estimated as 30% (the mean OD450 for negative sera plus 2.4 SD). Specificity of the new c-ELISA was proved in investigation of paired sera from 37 patients with seasonal influenza including A(H1N1) infection and from 10 volunteers immunized with seasonal influenza vaccine "GRIPPOL". No non-specific reactions with H5-virus in these sera were detected by c-ELISA. Serum samples from A(H5N1) vaccinated volunteers were tested in microneutralization assay and 60 paired sera with H5-specific seroconversions were selected for investigation in c-ELISA. The conversions of H5-specific antibodies were detected by c-ELISA in all vaccinated people (Figure 2). A significant correlation was observed between percents of inhibition registered in c-ELISA and antibody titers in microneutralization assay ($r = 0.5, p < 0.001$). This method appeared to be suitable for detection of Abs in immunized sera. Percent of inhibition in c-ELISA by antibodies contained in sera from H5-immunized rabbits (with HIT-titers 1: 80 – 1: 640) varied from 60% to 99% in c-ELISA ($87.8 \pm 13.2\%$) (data not shown). A significant correlation was observed between percents of inhibition registered in c-ELISA and antibody titers in microneutralization assay ($r = 0.5, p < 0.001$).

Figure 2. Comparative results of detection of H5-specific antibodies in sera of volunteers immunized with seasonal or influenza A(H5N1) vaccine by microneutralization assay and c-ELISA.



Conclusion

Microneutralization assay was evaluated as a sensitive and specific test in detection of antibodies to A(H5N1) virus but needed an application of infectious virus. Newly developed c-ELISA test using neutralizing H5-MABs to HA1 molecule appeared to be a sensitive, specific and more simple method suitable for detection of H5-specific antibodies in practical laboratories. In contrast to indirect ELISA this method allowed to avoid the cross-reactions in detection of H5-specific antibodies because of blocking MABs interaction with unique subtype specific site in H5 - hemagglutinin. c-ELISA could be useful test for investigation of avian virus transmission in human population and evaluation of immune response to infection and vaccination.

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Clinical Evaluation of Rapid Diagnostic Tests for the Detection of Influenza Viruses: Analysis of the Conditions that Influence Their Performance

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We evaluated sensitivity and specificity of four rapid diagnostic tests for detecting influenza viruses, and identified which conditions would influence the performance of tests. Sensitivity of Type A in nasopharyngeal aspirates and swabs ranged from 86- 94%, which was higher than in throat swabs. In Type B, sensitivity was generally lower than in Type A. Sensitivity was possibly low in patients within 12 hours from the onset, with low pyrexia and without nasal discharge, especially in type B. Background: Rapid and accurate diagnosis is very important for the control of influenza, but diagnosis from clinical symptoms may not always be easy. According to an American survey, pediatric influenza is rarely detected. In Japan, commercially available antigen detection test kits for rapid diagnosis of influenza have been increasingly used in general practice, since their launch in 1999. According to an estimate, over ten million tests are conducted every year. Timely diagnosis with these kits makes it possible to appropriately use anti-influenza drugs that must be initiated within 2 days after the onset of influenza, and to reduce other tests and antibacterial treatment. The kits are also useful for prompt implementation of countermeasures against nosocomial infections and the conduct of epidemiological survey. However, as the rapid diagnostic kits are intended for simple visual tests, the results are affected by the amount of viral antigens in specimens. Accordingly, false-positive or false-negative result cannot be avoided. When the kits are to be used, it is important to know diagnostic precision of the reagent and to make a comprehensive judgment. Objectives: In Japan, a great number of rapid diagnostic kits are released or improved every year. Usefulness of the kits is influenced by capability of reagents used. Meanwhile, diverse factors, such as patients and circumstances, are involved in clinical practice for treating influenza, which may affect the diagnostic test results. We conducted clinical evaluation of four kits that were confirmed to have required precision levels by basic investigation of detection limits, using the specimens collected from the patients with influenza-like illness. We then tried to identify which conditions would influence sensitivity and specificity of kits. Study Design: We evaluated the performance of four commercially available rapid diagnostic test

kits: Espline Influenza A&B-N (Fuji Rebio), Rapid Testa FLU stick (Daiich Pure Chemicals), QuickVue Influenza Test(Quidel) and Clearview Exact influenza A&B (Applied Biotech), and examined the conditions that might influence the rate of detection of influenza viruses with these kits. Specimens were collected from the patients with influenza-like illness who visited 6 medical institutions in Kanagawa Prefecture and Sendai City in Japan (2 hospitals and 4 clinics) from January to April 2005. Three types of fresh respiratory specimens: throat swabs, nasopharyngeal swabs and nasopharyngeal aspirates were collected. The results of rapid diagnostic tests were compared with that of viral isolation. When the result was not the same as the result of viral isolation using MDCK and Caco2, the specimen was checked with nested RT-PCR to calculate sensitivity and specificity of each test. The patients were asked to fill out the questionnaire before undergoing the tests. Questionnaire items were age, gender, history of flu vaccination, time of onset, and symptoms (fever, cough, nasal discharge, etc.).

Results

Breakdown of specimens and viral isolation status. A total of 3140 respiratory specimens were collected from the patients, 62% were positive for influenza viruses in cell culture (AH3: 635, AH1: 51, B: 1267, AH3&B: 2). *Sensitivity and specificity compared with viral isolation result (Figure 1):* Sensitivity of Type A in nasal swabs ranged from 86% to 94%, in nasal aspirates ranged from 89% to 94%. Sensitivity of all kits in throat swabs was lower than in nasal specimens, ranging from 67% to 83%. No significant differences were observed between the four kits. Sensitivity of Type B in nasal swabs ranged from 82% to 87%, showing comparability between these kits. Sensitivity in nasal aspirates ranged from 68% to 81%, though no significant differences were observed between the four kits. Excluding Clearview, sensitivity of three kits in throat swabs was about 65% (63~65%, Clearview 85%), which was lower than that in nasal specimens. The sensitivity for type B was lower than type A with these kits. Sensitivity of Clearview could be tested in nasal swabs for AH1, AH3 and B: 82% in AH1, 95% in AH3 and 83% in B. A significant difference was observed between B and AH₃, and between AH1 and AH3. Detection rate by cell culture with throat swabs for type B was lower than nasopharyngeal swabs from the same patients. Therefore, detection rate of throat swabs by kit was actually lower than sensitivity rate. Specificity of all the kits was as high as 96% ~100%. Nonspecificity was hardly shown. Unlike sensitivity, specificity was nearly the same regardless of difference in specimens and types. *Sensitivity and other conditions.* The sensitivity of rapid test was lower when the test was conducted within 12 hours from the onset of the illness than when it was conducted over 12 hours after the onset of the illness especially for type B (Figure 2). The sensitivity was also lower when the patients had low-grade fever than when they had high-grade fever for type B. Sensitivity of nasopharyngeal specimens was higher in patients with nasal discharge than those without nasal discharge for type B, but no difference was observed concerning coughing.

Conclusion

All kits were easy to use, and have high specificity. Detection rates are lower in throat swabs than in nasopharyngeal aspirates and swabs. In Type B, sensitivity was generally lower than in Type A. Sensitivity was possibly low in patients soon after the onset, with low pyrexia and without nasal discharge, especially in type B. Therefore, the false-negative results were likely to be obtained in type B, throat swabs, at the early stage of illness, without high fever and without nasal discharge. The important things for using rapid diagnostic tests are to choice of the appropriate specimens and to collect specimens containing a sufficient amount of antigens. We have to consider the factors that influence the performance of the tests and the accuracy of the tests when attempting to diagnose influenza using these tests.

Figure 1. Sensitivity compared with cell culture.

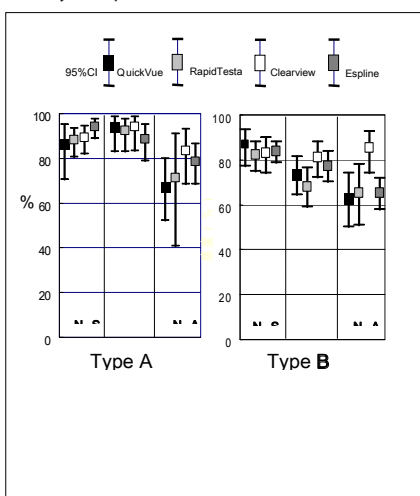
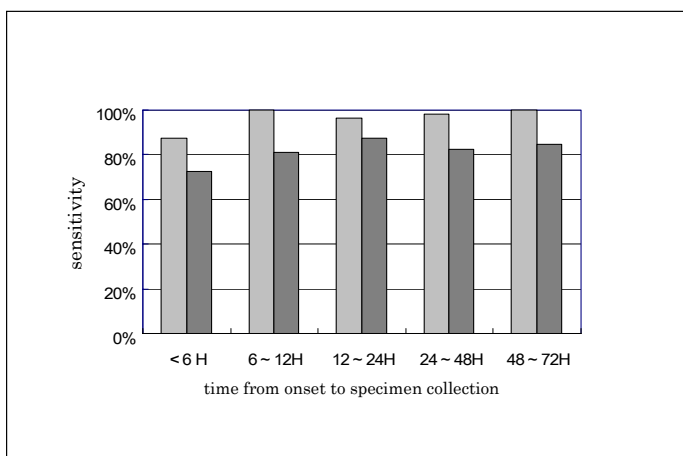


Figure 2. Sensitivity of espline influenza A&B-N and time from onset to specimen collection.



Proceedings Topic #6

Animal Influenza Ecology

**Poster
Presentations**

Avian Influenza in Saudi Arabia: Geographical and Historical Perspectives

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Saudi Arabia is a country that is located in the southwestern corner of Asia. Most of the country's land is desert and is located directly on the Tropic of Cancer. Most of poultry farms are established in the central and western part of the country. Chickens are the main source of poultry meat in the country. Importing of live birds for breeding purposes undergoes a strict examination before bringing these birds into Saudi Arabia. Migratory birds cross the country seasonally, but few of them make direct contact with citizens. The majority of the migratory birds stay in protected areas. Saudi Arabia has 15 different protected areas across the country. Avian influenza is a viral disease that causes respiratory symptoms in birds and other animals and humans. Low pathogenic avian influenza H9N2 was reported in 1998. The H9N2 virus continued to cause mild respiratory disease and also caused slight reduction in eggs' production. In the early 2006 the highly pathogenic avian influenza H5N1, was isolated from smuggled falcons that were brought to a veterinary clinic. Rapid diagnostic tests were conducted, positive samples were confirmed by polymerase chain reaction in the agricultural ministry laboratory. Samples were sent to a reference laboratory for confirmation of the results, and parallel results were obtained. All infected and contacted falcons were killed. H5N1 was not reported in any commercial farms in the country. In conclusion the H9N2 virus is still circulating in the commercial farms. The H5N1 virus has been eradicated and no isolation of this virus has been reported in chickens or in any other birds yet.

Introduction

Saudi Arabia (SA) is a country located in the south west of the Asian continent. This location is called the Arab Peninsula. Most of the country contains desert. There is a little rainfall every year. Different migratory birds use the Arab peninsula route to travel both ways from north to south or from northeast to southwest. Ducks, geese and other bird species fly over SA seasonally as illustrated in Table 1. Avian Influenza (AI) is a contagious disease that infects birds and causes disease in many other animals and human beings. Respiratory symptoms and high mortality rate have been observed in the affected birds. Aquatic birds have been implicated as the reservoir of the AI Virus. Symptoms may not appear in birds even though the virus is being shed from these birds. Different strains of the AI have been recognized and usually they are categorized as low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) (1). The virulence of the AIV is not the same in humans and birds. Some AIV strains are highly pathogenic

to birds but not virulent to human beings. In other countries, AIV is circulating in many domesticated birds including commercial farms and back yard birds' flocks. In addition AIV has transmitted from migratory birds to domestic bird flocks, when these species mix (1). The aim of this study is to focus on the possibility of AIV spreading in SA by comparing the Saudi Arabian situations with neighboring countries and other Asian countries where HPAV is circulating in the bird flocks. The effects of the a geographical location and climate of Saudi Arabia will be studied here to determine how these factors prevent the spreading of AIV in SA.

Results

History. The first official report of AIV disease was in 1998. This report was about the LPAI, H9N2 strain, which probably had circulated in commercial farms for a few years. This virus caused mild respiratory infections and resulted in the reduction of egg production. This virus continued to cause these symptoms in chicken flocks. Luckily, there was no report of this virus in back yard flocks and in any other bird species. The virus strain, H9N2 has not changed since it was isolated and is considered to be stable (3). It has been reported that the Saudi LPAI strain is considered to be of very low virulence as compared to other strains that have been isolated from other countries such as China, Iran, Pakistan, and United Arab Emirates (3). This AIV strain (H9N2) did not cause any influenza like symptoms in humans who have been working in poultry farms and in slaughtering plants (2). This situation continued without any change until 2006, when falcon owners reported falcon deaths with respiratory symptoms at a local avian clinic. The Agriculture Ministry sent a team to investigate. Rapid diagnostic tests were conducted on site. Samples including swabs, serum, parts of the respiratory organs and parts of the digestive track of the dead falcons were collected. Positive results of the rapid diagnostic test were obtained. Virus isolations were attempted and samples were sent to reference laboratories. A decision was made to depopulate all falcons that showed AIV. Contacted falcons that did not show any symptoms of AIV or did not shed virus were also killed (2). Equivalent results were obtained from Agriculture Ministry's laboratory and from reference laboratories. These results confirmed AIV, H5N1 strain in these falcons. The source of these falcons was unknown and they were not bred in Saudi Arabia. This virus, H5N1, has not spread out among commercial chickens, farms, and any other bird species of back yard flocks. There has not been any report of H5N1 in any of the falcon flocks anywhere in SA. This case was an isolated case and the virus was eradicated at that time and has not been reported since (2). *Geographical Location.* Four-Fifths of Arabian Peninsula is occupied by SA. SA is directly located on the Tropic of Cancer in both the northern and the eastern hemisphere. The latitude and longitude of the capital of SA is 24. 39(N) and 46.46(E). Less than 125 mm of rain fall in SA yearly. Most of the water is used for agriculture. When there are flash floods after rain, pools of water are formed especially behind the dumps. This kind

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of water attracts wild birds, especially aquatic birds. Several kinds of migratory birds that cross SA use several routes that pass over the country. Most of these birds fly both ways from north to south or from northeast to southeast. Breeding of most of the migratory birds does not occur in the Arab peninsula. Most of these birds cross SA without resting in the country, but occasionally birds stop in protected areas of SA. In these protected areas there is no contact between the aquatic migratory birds and commercial birds or domestic birds. The climate of SA is mostly dry and hot in the summer and little rain in winter. These conditions are not congenial for the survival of AIV.

Poultry Market in Saudi Arabia. Poultry industry is a huge market in SA. This industry consists of large companies that use a closed system to raise their chickens. Preparation of the poultry and poultry's products are done in such factories where strict hygiene is applied. There are markets for live birds, but they exist on a very limited scale. Most of the population of SA consumes chickens that are processed in licensed factories.

Conclusion

The possibilities of spreading of AIV in SA are very low. Many factors affect the spreading of AIV in SA such as: i) Hot weather, dry air, sunshine and little rain, which limit the spreading of AIV between farms and between birds and humans. ii) Lakes in SA that are small in number and size are found in protected areas. These lakes are used by citizens for agriculture purposes. Serological surveillance is conducted on birds using these lakes to monitor the presence of AIV. iii) Few migratory birds use route that crosses SA compared with other countries. iv) Saudi citizens are used to consume chickens and chicken products that have been processed in factories. Most of the citizens don't handle live birds. These measures limit the spreading of the virus among people. v) Few people keep chickens in their homes and if they do, it is not mainly for consumption. vi) Poultry are raised in complete closed farming system that has little contact with outside conditions.

Table 1. The names of some of the migratory birds that cross over Saudi Arabia.

	Scientific name	English name
1	<i>Porphyrio porphyrio</i>	PURPLE SWAMPHEN
2	<i>Ardea Purpurea</i>	PURPLE HERON
3	<i>Calidris canutus</i>	RED KNOT
4	<i>Phoenicopterus rubber</i>	GREATER FLAMINGO
5	<i>Ardea cinerea</i>	GRAY HERON
6	<i>Merops apiaster</i>	EURPEAN BEE-EATER
7	<i>Lanius schach</i>	SHRIKE
8	<i>Anthus novaeseelandiae</i>	PIPIT
9	<i>Pandion haliaetus</i>	OSPREY
10	<i>Anas platyrhynchos</i>	MALLARD
11	<i>Numenius arguata</i>	WESTERN CURLEW
12	<i>Egretta garzetta</i>	LITTLE EGRET
13	<i>Phalacrocorax capensis</i>	CAPE CORMORANT
14	<i>Anas clypeata</i>	NORTHERN SHOVELER
15	<i>Larus marinus</i>	GREAT BLACK-BACKED GULL
16	<i>Larus argentatus</i>	HERRING GULL
17	<i>Anas acuta</i>	NORTHERN PINTAIL
18	<i>Motacilla alba</i>	PIED WAGTAIL
19	<i>Tadorna tadorna</i>	NORTHERN SHELDUCK
20	<i>Anas americana</i>	AMERICAN WIGEON
21	<i>Eremopterix nigriceps</i>	BLACK-CROWNED FINCH LARK
22	<i>Anas acuta</i>	NORTHERN PINTAIL
23	<i>Falco tinnunculus</i>	EURASIAN KESTREL
24	<i>Vanellus vanellus</i>	NORTHERN LAPWING
25	<i>Upupa epops</i>	HOPOE
26	<i>Corvus corax</i>	COMMON RAVEN
27	<i>A. clarkii</i>	CLARK,S GREBE
28	<i>Calidris alpina</i>	DUNLIN

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Migratory Bird Surveillance Program in Ukraine, Egypt, and Kenya

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Migratory aquatic birds are thought to be natural reservoirs of avian influenza (AI) viruses and play a role in distributing virus throughout their flyways. Monitoring for emergence of AI strains, especially highly pathogenic AI (HPAI) in bird populations, is essential for the prevention of serious socio-economic and public health consequences. NAMRU-3 performed surveillance for influenza in migratory birds through collaborations with Ukraine, Egypt and Kenya. Between July 2005 and January 2007, 16 collection sites were established; five in Dniester Delta in Ukraine, seven in the Nile Delta in Egypt, and four in the Rift Valley in Kenya. By means of mist-netting, birds were humanely caught and cloacal swabs screened by real-time RT-PCR for the influenza A matrix gene with characterization of positive specimens for H5 and N1 genes. In Ukraine, 153 (6.8%) of 2250 birds were positive for influenza A with one mallard duck positive for the H5 gene, characterized as low pathogenic avian influenza (LPAI). In February 2006, an additional 32 samples were collected during a multi-species bird die-off and HPAI H5N1 was detected in 13 birds (40.6%). In Egypt, 354 (11.5 %) of 3,365 birds tested positive for the influenza A matrix gene. Two specimens collected from common teals in October (LPAI) and December 2005 (HPAI) were H5 positive. The December specimen was closely related to H5N1 viruses from subsequent poultry/human cases in Egypt and from the Ukraine bird die-off as judged by phylogenetic analysis. In Kenya, 24 (3.1 %) of 779 birds were influenza A positive with one characterized as LPAI (H5N3). Overall, 531 (8.3%) of 6394 specimens were influenza A positive with three characterized as LPAI H5 and one as HPAI H5N1. Detection of HPAI in the Common Teal implicates migratory birds as possible vectors for virus introduction into new geographical areas. Active surveillance within migratory flyways may serve as an early warning system to detect novel viral isolates having the potential to spread into domestic flocks. These data support the need for ongoing surveillance to define other major reservoir species and their individual migratory patterns. The lack of H5N1 introduction into Eastern Africa clearly illustrates that broad, generalized, multi-species "migratory flyways" may not be useful in predicting spread.

Introduction

Influenza A viruses are orthomyxoviruses and have a segmented, linear, negative sense, single-stranded RNA

genome. Influenza A viruses are further classified into subtypes based on the antigenic characteristics of their hemagglutinin (HA) and neuraminidase (NA) molecules. Currently 16 HA and 9 NA subtypes have been identified (1). Influenza A viruses are the etiologic agents of Avian Influenza (AI) and infect a wide variety of domestic and wild bird species. Migratory birds and waterfowl, especially of the orders Anseriformes and Charadriiformes, are thought to be the main reservoirs of these viruses in nature and play a key role in virus distribution over wide geographical areas throughout their flyways (2). Influenza A viruses, infecting poultry can be classified as low (LPAI) or high (HPAI) pathogenicity strains, according to the severity of the disease. HPAI viruses seem to originate from low pathogenic precursors by mechanisms such as mutation or insertion at the hemagglutinin cleavage site (3). New influenza viruses can also emerge in an immunologically naïve human population by reassortment or direct transmission across the species barrier and have the potential of initiating catastrophic pandemics. The large genetic pool of influenza viruses maintained in waterfowl serves as the origin for new genes or viruses. Since 1996, a marked increase in HPAI poultry outbreaks, especially caused by H5N1 strains, has been reported. Outbreaks in wild birds demonstrate that HPAI, especially H5N1, constitute an important threat to avian biodiversity. The rising number of HPAI outbreaks and the zoonotic characteristics of these viruses constitute a major threat to animal and human health and are becoming a great concern to veterinary and public health authorities worldwide. Surveillance for influenza in wild birds plays an important role and allows for the development of regional control strategies by facilitating early recognition of the virus. Global surveillance for avian influenza may demonstrate temporal and spatial variations in circulating influenza viruses relating to their epidemiology, ecology, and evolution (4). Objectives: To investigate the role of migratory birds in HPAI H5N1 transmission by developing a surveillance network covering flyways from Eastern Europe (Ukraine) to Africa (Egypt and Kenya).

Materials and Methods

The Naval Medical Research Unit No. 3 (NAMRU-3) has established collaborations with the Anti-Plague Institute, Ministry of Health of Ukraine, Ministry of Environment of Egypt, National Museum of Natural History and Centers for Disease Control in Kenya to develop an active surveillance network for H5N1 in birds migrating through these countries from the Northern to the Southern Hemisphere. Collection sites were selected in areas where birds congregate during their annual migration cycle in each country. A total of 16 collection sites were established: five in the Northwestern coast of the Black Sea in Ukraine, seven in the Delta Region in Egypt, and four in lakes along the Rift Valley in Kenya (Figure 1). Birds were humanely trapped using mist nets with collection of two cloacal swabs from each animal (Table 1). Egyptian specimens were obtained from birds hunted by licensed sport hunters, strictly controlled by national authorities. Specimens were screened

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for the presence of AI using real-time RT-PCR to detect the influenza A virus matrix gene with positive specimens tested for H5 and N1 genes using specific primers (5). Characterization of the H5 gene by sequencing with phylogenetic comparative analysis of the obtained isolates with H5 genes of viruses from Asia, Europe and Middle East obtained from Gene Bank was performed to establish patterns of relatedness among isolates using Clustal, Phylip and Treeview programs.

Table 1. A total of 6,394 samples were collected and screened.

Country	Total number of samples	Positive AI	H5N1
Egypt	3365	354	1
Ukraine	2250	153	13
Kenya	779	24	0

Results

In Ukraine, 2,250 samples were collected with 153 (6.8%) positive for AI. One H5 positive specimen from a mallard (*Anas platyrhynchos*) was characterized as LPAI. In February 2006, a multi-species bird die-off reported in the Skhoy liman region resulted in 32 additional specimens. H5N1 was identified in 13: four grebes (*Podiceps spp*), five mute swans (*Cygnus olor*), and four red-breasted Merganser (*Mergus serrator*). Phylogenetic analysis of these samples showed high homogeneity (average intrinsic genetic distance 0.34%) and were closely related to African, European, and Russian isolates (average genetic distance 0.6%) (Figure 1). In Egypt, 3,365 specimens were collected with 354 (15.7%) AI positive. Two specimens from common teals (*Anas crecca*) in Damietta, in October and December 2005 were positive for H5 gene. The October sample was characterized as a LPAI strain. The December isolate collected from a bird trapped and caged by a fisherman was characterized as HPAI H5N1. Phylogenetic comparison revealed that the December HPAI virus segregated with other HPAI isolates obtained from human and poultry cases in Egypt as well as isolates from Afghanistan, Croatia, Italy, Nigeria, Russia, Slovenia, Sudan, and Ukraine forming a highly homogeneous group with an intrinsic average genetic distance of 0.8% and a high bootstrap value (100%). Isolates from the bird die-off in Ukraine also segregated within this group with an average genetic distance of 0.5% from the December common teal HPAI strain H5 gene (Figure 1). In Kenya, 779 specimens were collected with 24 (3%) AI positive and four identified as H5 low pathogenic strains.

Discussion

It has been proposed that introduction of H5N1 in new geographical areas is due to several factors such as poultry and poultry products trade, wild bird trade, and movement of migratory birds (6). The common teal is an abundant and widespread duck, which breeds in the northernmost areas of Europe and Asia. This dabbling duck is strongly

migratory and winters in Southern Africa and Asia. It is highly gregarious outside of the breeding season forming large flocks. Experimental data suggest that ducks may be infected with HPAI without clinical signs and may harbor and transport the viruses across wide geographical regions, thus increasing the risk of distribution of these viruses (7). The genetic homogeneity of the Egyptian strains, strongly suggests a single source introduction for H5N1 influenza in this country. In Egypt, a HPAI H5N1 virus was detected in a common teal before poultry outbreaks and human cases were reported in this country. Phylogenetic comparison of the H5 gene revealed high homogeneity with isolates from Eastern Europe and Africa. These findings suggest migratory birds could have been responsible for the introduction of HPAI in Egypt and support migratory birds as possible vectors for these viruses. However, the possibility that a HPAI positive animal could acquire the infection by contact with domestic poultry cannot be ruled out. For the Egyptian common teal identified with H5N1 that was trapped and caged by a fisherman, exposure to infected domestic poultry should also be considered. Despite active surveillance efforts, additional H5N1 isolates have not been detected in wild bird species in Egypt. These results suggest that other mechanisms, such as movement of poultry and poultry products related to human activities may also be implicated in virus maintenance and distribution in the region. While Kenya is connected by migratory flyways from areas where HPAI H5N1 outbreaks in wild birds and poultry are reported and has several migratory species common to these countries, HPAI H5N1 has not been reported or detected by active surveillance in Kenya. To optimize the efficiency of HPAI active surveillance, it is essential to improve our knowledge regarding the temporal and spatial characteristics of regional flyways of each species, the identification of mating and resting sites, and to determine the most appropriate time of the year and site for sample collection as each region has its own ecological characteristics.

Disclaimer

The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or as reflecting the views of the Department of the Navy, Department of the Army, Department of Defense, the United States Government, or the Ministry of Environment of Egypt, Ministry of Health, Ukraine, the National Museum of Natural History, Kenya.

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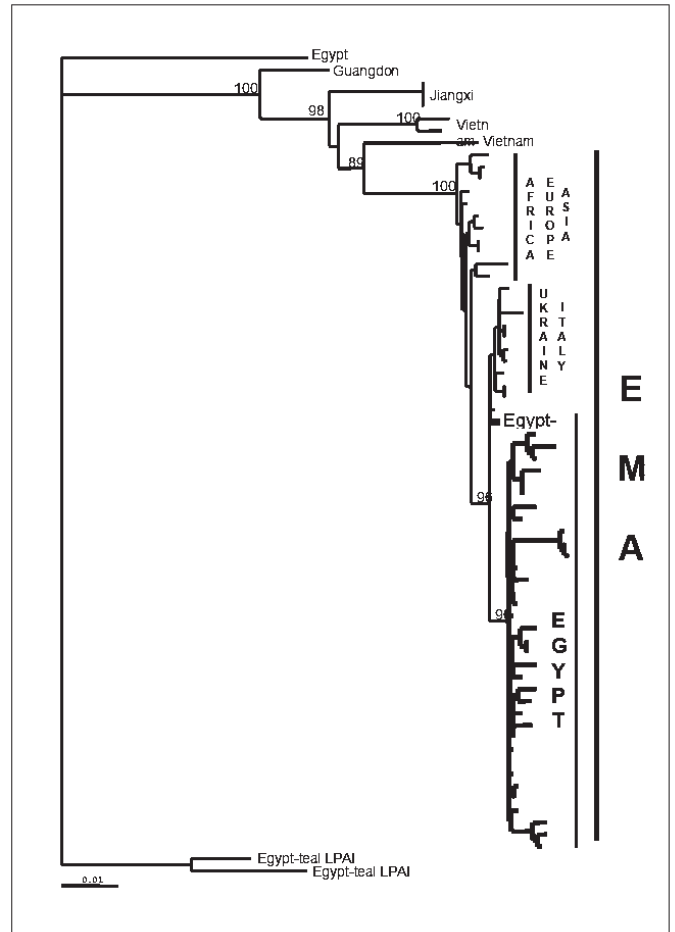
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Figure 1. Phylogenetic neighbor-joining tree of AI isolates obtained from Egypt, other African countries, Middle East and Asia. Bootstrap values were obtained from 100 resamplings of the data using distance matrix methods are shown at the nodes corresponding to major clades found in this collection of samples. The bar at the left corner indicates 0.01 nucleotide substitutions per site. EMA: Europe-Middle East-Africa clade.



Clinical Evaluation of Conventional and Reverse Genetic Vaccines for Chickens Against H5N1-HPAI Viruses

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Highly pathogenic H5N1 avian influenza viruses have been infected many chickens, pigs, cats and humans in Indonesia since 2003. To control H5N1 infection in chickens in Indonesia, inactivated oil-emulsion vaccines, have been used since 2004, based on avian influenza field isolate H5N1, H5N2, and H5N9 subtypes. In 2006, an inactivated oil-emulsion H5N1 reverse genetic vaccine was licensed in Indonesia, which includes modified HA and wild-type NA genes from influenza A/chicken/Indonesia/Legok/2004 (H5N1) and remaining genes from A/PR/8/34 (H1N1) viruses. The aim of this study is to investigate the clinical efficacy of these vaccines in chickens. The reverse genetic vaccine showed the best antigenic performance, and the H5N2 and H5N9 vaccines showed the worst efficacy. Chickens vaccinated with all conventional vaccines, H5N1, H5N2, and H5N9, showed virus shedding at least six days after challenge with recent Indonesian HPAI H5N1 virus, and could not prevent virus transmission to unimmunized chickens. On the other hand, chickens vaccinated with the reverse genetic vaccine showed shedding only one day at the maximum, and prevented the virus transmission to unimmunized chickens.

Introduction

Outbreaks of H5N1-HPAI in chickens began in Indonesia in 2003, since then the viruses have infected pigs, cats and humans. Vaccines have been used in Indonesia to assist the control of Avian Influenza, since 2004. The purpose of vaccination is to reduce morbidity and mortality; eliminate shedding, and environmental contamination with the viruses and also to prevent infection by field viruses. Various vaccines have been used since 2004 in Indonesia; however, the control of the HPAI was unsuccessful. The various vaccines are commercially available, and the efficacy of these vaccines have not been systemically studied. Two kinds of seed viruses are derived from homologous H5N1 virus, i.e conventional H5N1 and reverse genetic H5N1 vaccines. A conventional H5N1 seed virus was obtained by attenuation through multiple passage in embryonated eggs, but is currently not under production. The reverse genetic vaccine was licensed in 2006 in Indonesia, but has not been widely used because of limited production. Other seed viruses are heterologous H5N2 and H5N9 subtypes, which have been widely in Indonesia and elsewhere. Currently, all

vaccines are inactivated oil-emulsion vaccines prepared using embryonated eggs. In this study, we investigate the efficacy of these vaccines, including efficacy in reducing virus shedding and transmission to unimmunized chickens, which is essential for the vaccine strategy.

Materials and Methods

Eight inactivated oil-emulsion commercially available vaccines were used (2 vaccines of H5N1, 4 vaccines of H5N2, 1 vaccine of H5N9, and 1 vaccine of reverse genetic H5N1). Each vaccine (0.5 ml) was injected intra-muscularly into ten one-month-old native chickens, according to standard protocol. Three chickens were used as controls. The HPAI A/Chicken/Banten/2004 (H5N1) virus was used for challenge experiment in three vaccinated and one control chicken. The chickens were then used for shedding and transmission test. Transmission test was done by placing unvaccinated chickens in the same cage together with challenged chickens. Shedding viruses from cloaca and trachea were tested for H5 and N1 gene by real-time RT-PCR. Antibody levels to the H5 HPAI virus in chicken sera were tested by the standard OIE assay (Alexander 2000). HI test procedures used A/chicken/Banten/2004 virus antigen (Nidom, 2005).

Results and Discussion

The reverse genetic H5N1 vaccine showed the highest antibody response to A/chicken/Bantem/2004 (Figure 1 and Figure 2), reduced virus shedding and prevented virus transmission to uninfected chickens (Table 1).

Figure 1. Antibody response after immunization using various vaccines.

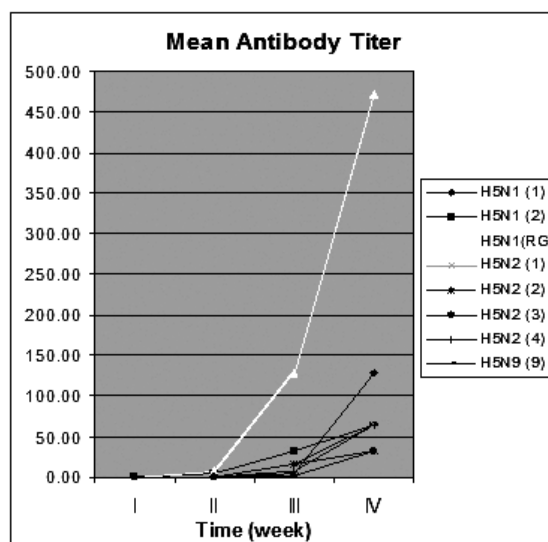


Figure 2. Antibody response after 1st week 2nd vaccination (Booster).

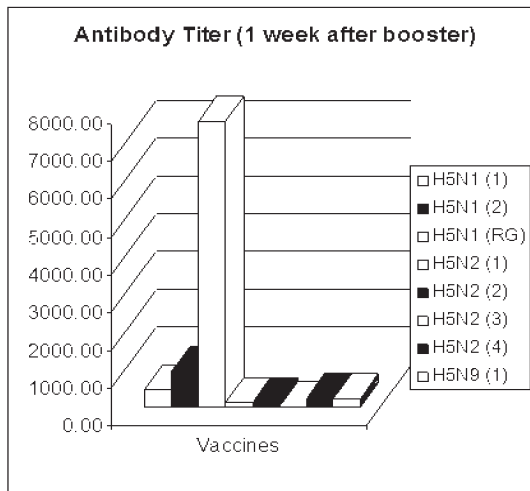


Table 1. Virus shedding and transmission after immunization using various vaccines and following HPAI-virus-challenge.

Vaccines	Ab Titer	1 st day	2 nd day	3 rd days	4 th days	5 th days	6 th days	Transmission Test	
		p.c	s p.c	p.c	p.c	p.c	p.c*)	Trachea	Cloaca
H5N1 RG	2 ^{13.6}	+	-	-	-	-	-	-	-
H5N1 (1)	2 ^{9.4}	+	+	+	+	+	+	2 ²	-
H5N1 (2)	2 ^{9.5}	+	+	+	+	+	+	2 ²	-
H5N2 (1)	2 ^{7.2}	+	+	+	+	+	+	2 ³	2 ²
H5N2 (2)	2 ^{7.4}	+	+	+	+	+	+	2 ³	2 ²
H5N2 (3)	2 ^{5.2}	+	+	+	+	+	+	2 ³	2 ²
H5N2 (4)	2 ^{7.5}	+	+	+	+	+	+	2 ²	2 ²
H5N9 (1)	2 ^{7.3}	+	+	+	+	+	+	2 ³	2 ¹

On the other hand, the conventional H5N2 and H5N9 vaccines showed lower immunological responses (Figure 2) against A/chicken/Bantem/2004 virus but could not limit virus shedding and transmission to unimmunized chickens (Table 1.). The H5N1 conventional vaccines showed lower efficacy than that of reverse genetic vaccine. This could be explained by the quality and quantity of antigens in the product vaccines. Lowest efficacy of the H5N2 and H5N9 vaccines can be explained by lower HA homology (less than 90%), which indicates importance of antigenic homology of seed virus with circulating virus. Using the reverse genetic vaccine, virus shedding was positive at 1 day post challenge, however, no transmission was detected. Because we detected viruses by real-time RT-PCR, which is highly sensitive, we may detect negligible amount of viruses or remaining challenged virus itself. In any case, they may not be enough for transmission to healthy chickens. Although we may have to confirm the present data by multiple experiments, our data suggest the importance of selecting seed virus and efficacy of reverse genetic technology. Use of the conventional vaccines may be a reason for the unsuccessful

control of HPAI in Indonesia. In addition, the reverse genetic vaccine may be essential for the vaccine strategy and such strategy could control current HPAI epidemics.

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Application of DNA Barcoding Techniques to Avian Influenza Virus Surveillance in Migratory Bird Habitats in Korea

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The role of wild birds in the spread of H5N1 remains speculative, although wild waterfowls are considered the natural reservoir of influenza A viruses. During wild bird surveillance for 5 years, we isolated 95 AIVs from 7,093 fecal droppings collected in migratory bird habitats in South Korea. To identify host species correctly, we optimized DNA barcoding techniques, which have been used to identify species or sex of animals using species specific mtDNA from various tissues. The sequence of COI genes of mtDNA extracted from feces was identical with that of muscle tissues of various birds. Further, experimental challenge with influenza A virus revealed that feces excreted from AIV infected birds could be useful sources to isolate influenza virus and to extract mtDNA for 7 days, when they were transported or stored below -20°C. To identify species, sequence similarity of the COI genes of AIV positive feces were analyzed with sequence database of COI genes. Our result showed that the most prevalent bird species shed AIV in feces could be identified as *Anseriformes* including *Anser albifrons* (white-fronted goose) and *Anser fabalis* (bean goose), which are representative winter birds moved from Russia. Identifying bird species using their feces will give us more extensive information about the role of migratory birds for spreading of AIV as well as continuing evolution of AIV.

Introduction

Beginning in late 2003, H5N1 highly pathogenic avian influenza outbreaks occurred in several southeast Asian countries and areas, with the viruses being transmitted into humans in several countries [1]. In recent years, the virus has undergone a number of mutational changes and enlarged its host range, which now includes migratory birds that have moved widely along the major flyways of the world. Wild birds are considered to be the natural reservoirs of AIV [2] and the *Anatidae*, many of which are long distance migrants, generally have a higher incidence of infection than other birds. Therefore, extensive sampling of live wild waterfowl has been practiced in many countries. However, the ecology of AIV in nature and the role of migratory birds in the spread of influenza virus are still unclear. To isolate influenza virus from birds, cloacal swabs from captured birds [3]

or collection of feces from the ground can be used because the birds shed virus in their feces at high concentration. In the case of wild birds, the cloacal swab methods are useful to identify bird species correctly, but catching individual birds is difficult. On the other hands, the fecal collection method is easy and convenient, but it is difficult to identify the bird species. Both methods are not best tools for large scale AIV surveillance. DNA barcoding technique is an animal species or sex typing method performed by analyzing DNA sequences from a standardized genomic region. To identify species, mitochondrial DNA has been widely employed in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species. Among mitochondrial DNA, the gene of cytochrome C oxidase I (COI) is generally used to identify bird species using tissues [4]. From late 2004, we have isolated 95 avian influenza viruses from wild bird feces collected in several migratory bird habitats in South Korea. Without capture of wild birds, however, identification of bird species were rarely possible. In this study, we validated and optimized from DNA barcoding technique for bird feces to identify their species correctly. In addition, we aimed to apply these techniques to feces collected during surveillance to obtain information of bird species shedding virus in feces and to understand the role of migratory birds in the spreading of AIV.

Material and Methods

Specimens. Fresh feces samples of domestic poultry and wild birds were collected from Seoul Grand Park in Korea. Fresh feces samples of migratory bird were also collected from migratory bird habitats, Cheonsu-man, Upo swamp, Eulsukdo, Geum River and Mankyung River in Korea.

Virus isolation. Feces samples were collected and dissolved in phosphate based saline (PBS) with 400ug/ml gentamicin. The suspensions were centrifuged and the clear liquid supernatant was collected. All specimens were inoculated into the allantoic cavity of 9-11 days old specific pathogen-free (SPF) embryonated chicken eggs. After 72 hrs of incubation at 37°C, the eggs were chilled and allantoic fluids were harvested and the undiluted allantoic fluids were tested for hemagglutinin activity. RNA was extracted by using the RNeasy kit (Qiagen, CA) according to the manufacturer's instructions. RNA was tested for AIV by real-time reverse transcription-PCR (RRT-PCR) using the matrix (M) gene primers. Subsequently, AIV positive specimens by RRT-PCR were further characterized for HA subtype by RT-PCR as described previously [5].

DNA extraction, PCR amplification and sequencing for species identification. In order to establish a method for species identification using fecal DNA, universal primers (BirdF1, 5'- TTC TCC AAC CAC AAA GAC ATT GGC AC-3', BirdF2 5'- ACG TGG GAG ATA ATT CCA AAT CCT G-3', and BirdR2 5'- ACT ACA TGT GAG ATG ATT CCG AAT CCA G -3') were designed based on mtDNA COI gene sequences of birds [4]. Modified AnserF1 (5'- GTG ACC TTC ATC AAC CGA TG-3') were newly designed to amplify COI gene of *Anseriformes*, which was not amplified

with universal primers. DNA was extracted from feces using DNA stool kit (Qiagen, CA) and from tissues (*Quadriceps femoris*) using DNeasy mini kit (Qiagen, CA) according to manufacturer's recommendations. PCR was performed on thermocycler using the following cycle parameters [6]: The 1 min denaturation step at 94°C; 5 cycles of 1 min at 94°C, 90 s at 45°C, and 90s at 72°C, followed in turn by 30 cycles of 1 min at 4°C, 90 s at 51°C, and 90 s at 72°C and a final 5 min at 72°C. PCR products (about 750-bp size) were visualized in a 1.5% agarose gel. The generated PCR products were sequenced and evaluated sequence identity with sequence database of COI genes offered by the BARCODE OF LIFE DATA SYSTEMS [7].

Optimization of DNA barcoding technique. Experiment 1: Feces of Canadian goose and Spot-billed duck were stored at different temperature conditions independently. After 2, 3, 5, and 7 days storage, each feces were examined to confirm stability of DNA in feces. Stability of extracted DNA was evaluated by measurement of DNA concentration and by PCR amplification of COI gene.

Experiment 2: Chickens, turkeys, and mallard ducks, three birds of each species were challenged experimentally with A/WB/5-80 (H7N8) at dose of $10^{6.0} \text{EID}_{50}/0.1\text{ml/bird}$ intranasally. On day 6 after challenge, fresh fecal dropping were collected and stored at different temperatures (-70°C, -20°C, 4°C, 20°C, and 37°C). After 3, 5 and 7 days post-storage, stored feces were used to isolate challenge virus and to extract mtDNA for amplification of the COI gene of the host.

Results

Optimization of DNA barcoding technique. Using DNA barcoding techniques, most bird species including *Galliformes* (chickens, quail, turkey), *Anseriformes* (mallard duck, mute swan, domestic duck, and moscovy duck), *Falconiformes* (vulture), *Columbiformes* (pigeon), *Gruiformes* (sandhill crane and whooping crane), and *Ciconiiformes* (pelican) were correctly identified using universal primers, BirdF1 and BirdR1 as previously described [4, 8]. On the other hand, some bird species including *Anseriformes* (white-fronted goose, bean goose, and domestic goose) were identified only using BirdR1 and newly designed AnserF1. Species identified from the sequence of COI genes originated from muscle tissues were identical to those identified from feces of chickens, Japanese quails, turkeys, domestic ducks, mallard ducks, muscovy ducks, and pigeons.

Effect of storage temperature on isolation of influenza virus and amplification of COI gene from feces samples. In Experiment 1, DNA extracted from feces stored at all temperatures (37°C, 20°C, 4°C, -20°C, and -70) was suitable to amplify COI gene for up to 7 days (Table 1). In Experiment 2, amplification of COI gene of birds from feces excreted from birds after challenge with influenza A/WB/5-80 (H7N8) virus was successful at all different storage conditions tested. AIV was isolated up to 3 days from feces stored at 20°C, 4°C, -20°C, and -70°C, respectively, but not from feces after 5 days of storage at above 4°C (Table 1). Although viability of AIV in feces decreased in a time-dependent manners, mtDNA in feces were highly stable for 7 days post-storage.

Table 1. Effect of storage temperature and duration on amplification of COI gene of birds and isolation of influenza A virus from feces.

Birds		Storage condition (°C)														
		3 days				5 days				7 days						
		37	20	4	-20	-70	37	20	4	-20	-70	37	20	4	-20	-70
<i>Experiment 1</i>																
Canada goose	COI genes ^B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spot-billed duck	COI genes ^B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Experiment 2</i>																
Chicken	Influenza virus ^A	NT ^C	+	+	+	+	NT	-	-	+	+	NT	-	-	+	+
	COI genes ^B	NT	+	+	+	+	NT	+	+	+	+	NT	+	+	+	+
Turkey	Influenza virus	NT	+	+	+	+	NT	-	-	+	+	NT	-	-	+	+
	COI genes	NT	+	+	+	+	NT	+	+	+	+	NT	+	+	+	+
Mallard duck	Influenza virus	NT	+	+	+	+	NT	-	-	+	+	NT	-	-	+	+
	COI genes	NT	+	+	+	+	NT	+	+	+	+	NT	+	+	+	+

Identification of host species of AIV positive feces samples collected during 2003-2007. During winter seasons in 2003-2007, we have collected 7093 feces samples in migratory bird habitats including Cheonsu-man, Upo swamp, Eulsukdo, Geum River and Mankyung River in Korea and isolated 95 avian influenza viruses. Although all AIV isolates were low pathogenic AIV, we demonstrated that the subtypes of H5, H7, and H9 were present in migratory birds (data not shown). As shown in Table 2, we identified host species of AIV positive feces collected during surveillance by analysis of COI gene. The most prevalent bird species shedding AIV in feces could be identified as *Anseriformes* including *Anser albifrons* and *Anser fabalis*.

Table 2. Identification of migratory bird species excreting the feces containing avian influenza A virus.

Treatment	Conc.	Excretion of challenge virus				
		Virus isolation ^A		Virus titer (log ₁₀ EID ₅₀ /g)		
		Trachea	Cecal tonsils	Trachea	Cecal tonsils	
Byproduct	Feed additive (% v/v)	1	3/10	1/10 ^δ	0.75 ±1.30	0.35 ±1.10 ^{***}
		0.4	5/10	6/10	1.35 ±1.67	2.10 ±1.87 ^{***}
		0.1	5/10	4/10	1.25 ±1.40	2.30 ±2.98 [*]
	Drinking water (mg/l)	10	3/10	4/10	0.55 ±0.93	1.30 ±2.06 ^{***}
		1	3/10	3/10 ^{δδ}	0.65 ±1.18	1.15 ±1.93 ^{***}
		0.1	6/10	6/10	1.80 ±1.60	3.80 ±3.29
Control ^B		5/10	9/10	1.25 ±1.48	4.95 ±2.43	

Discussions

Among migrating birds, the prevalence of influenza virus is the highest in *Anseriformes*, which are distributed globally including in Korea, except for the most arid regions of the world [9]. Therefore, newly designed AnserF1 might be valuable to detect migratory *Anseriformes*. Further, the results of species identification of AIV positive feces showed that the most prevalent bird species shedding AIV in feces might be an *Anseriformes* among winter bird in Korea. The identified species, white-fronted goose and bean goose, are known as winter bird in Korea coming from Russia and passing through China. These results will be helpful to understand avian influenza ecology. Extraction of mtDNA from animal feces seems to be

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unreliable, because feces contain food materials and various enzymes, which could inhibit PCR reactions, as well as intestinal epitheliums. However, fecal DNA analyses are generally used for species identification in wildlife animals like brown bears (*Ursus arctos*), wild bonobos (*Pan paniscus*), and Japanese mustelids [11]. Further, fecal collection methods are non-invasive and easy-to-use. Therefore, feces are valuable samples to identify host species. As shown in Table 1, both fecal DNA and AIVs are stable for long periods when stored at or below -20°C. Therefore, low temperature transportation and storage of feces or cloacal swabs are needed to isolate AIV and obtain stable mtDNA effectively during influenza surveillance. In this study, we optimized the DNA barcoding techniques to apply as a surveillance tools for studying the role of migratory birds in the evolution and spreading of AIV. DNA barcoding techniques used in this study are easy to apply and non-invasive to protect wild bird as well as samplers. In conclusion, identifying species by sequence data of mtDNA could be useful for large scale national based annual AIV surveillance especially in seasonal migratory bird habitats near epicenters of H5N1 HPAI.

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Influenza Subtype H3 Circulation in Fattening Pigs of Argentina

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Introduction

Pigs are important hosts in the ecology of the influenza viruses because they are susceptible to the infection by avian and mammalian influenza strains, including the human ones. For that reason, they have been proposed to be a possible "mixing vessel" for events of reassortment between human and avian viruses. In the United States for more than 80 years, the classic subtype H1N1 was the only one associated with influenza respiratory disease in pigs and since 1998 the subtype H3N2 has been associated with respiratory symptoms and/or reproductive disorders, whereas in East Europe this subtype was detected since 1984.

Objective

The objective of this work was to investigate if A (H3) viruses are circulating in fattening pigs in some argentine farms and its seroprevalence.

Material and Methods

Samples. From 2000 to 2002, 490 serum samples of fattening pigs, with 13 to 23 weeks of age were collected from 19 farms. **Sampled area:** The farms were located in 3 provinces: Buenos Aires (10), Cordoba (4) and Santa Fe (5). **Hemagglutination Inhibition Test.** The classical microtechnique with 4 hemagglutination units per 25 µl of antigen was used. Sera with titres > 40 were considered positive.

Antigen. The A/Sydney/5/97(H3N2) strain was propagated in MDCK cells. The culture was centrifuged at 1500 rpm for 10 minutes. The supernatant was treated with tween 80(0.125% v/v final concentration) and Diethyl ether (33.3 % v/v final concentration). It was placed in an ice bath to allow for the separation into hydrophobic and hydrophilic phases. The glycoproteins were retained in the aqueous phase.

Antiserum. Ferret reference antiserum prepared with A/Sydney/5/97(H3N2) was provided by Centers for Disease Control, USA.

Serum Treatment. One volume of serum was treated with 3 volumes of RDE, incubated over night at 37°C, and inactivated for 1 hour at 56°C; 6 volumes of physiological solution were added. Finally, 1 ml of the diluted serum was adsorbed with the pellet of 10 ml of a 0.5 % Turkey red blood cell suspension.

Results

Antibodies against influenza A (H3) virus were detected in the studied population. Eighty positive sera were detected in 8/19

(42%) farms, giving an overall positivity of 16.5 % (80/483). The % of positivity, the titre geometric mean and location of each farm is presented in Table 1.

Table 1. Distribution of locations, number of taken samples, number of positives samples, percentages of positivity, titre geometric means, titre range of each sampled farm.

Farm n°	Province	n	n positives	Titre geometric means	Titre Range	% positives
1	Buenos Aires	27	21	160	40-640	77.8
12	Buenos Aires	26	12	160	40-320	46.1
10	Córdoba	22	7	160	40-1280	31.8
9	Buenos Aires	42	13	80	40-320	30.9
2	Buenos Aires	52	12	80	40-320	23.1
5	Córdoba	27	6	80	40-320	22.2
16	Buenos Aires	27	5	60	40-80	18.5
11	Santa Fe	33	4	190	80-1280	12.1
3	Buenos Aires	12	0	0	<40	0
4	Santa Fe	23	0	0	<40	0
6	Buenos Aires	6	0	0	<40	0
7	Buenos Aires	28	0	0	<40	0
8	Buenos Aires	31	0	0	<40	0
13	Córdoba	31	0	0	<40	0
14	Santa Fe	13	0	0	<40	0
15	Córdoba	25	0	0	<40	0
17	Buenos Aires	10	0	0	<40	0
18	Santa Fe	23	0	0	<40	0
19	Santa Fe	25	0	0	<40	0
Total		483	80			16.5

Discussion

Antibodies against influenza A virus were detected in the studied population, showing the contact of the argentine swine population with influenza A viruses. Serological A (H3) activity was found in 50 % of the sampled farms located in Buenos Aires and Córdoba provinces but 20% of the farms of Santa Fe Province. At the same time, the farms with the highest % of positivity correspond to Buenos Aires and Córdoba Provinces, see table 1. A possible explanation of these results may be the higher economical development of this farming activity in those provinces. These results would indicate an interaction with A (H3) strains, therefore viruses of this subtype are circulating in pigs in the sampled provinces of Argentina. Due to the age of the animals it is not possible to infer if the antibodies were developed by the studied population or if they are maternal antibodies passively acquired. It becomes essential to conduct further studies. They would have to include viral isolation to attempt the antigenic and genomic characterization of the circulating strains among pigs, and to establish the relationships with the circulating strains in human population and other animal species.

Serological Evidence for the Avian Influenza Subtype H9 Virus in Domestic Birds in Nepal

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Surveillance for the Influenza A virus was conducted in Nepal from January 2006 to December 2006. Passive surveillance was done by observation for the presence of clinical signs. Active surveillance was done by collecting 930 specimens from domestic and wild birds in 26 districts of the country. Of 930 specimens, 558 were serum and 372 were cloacal swabs, tracheal swab or tissues. Serum samples were tested for antibodies against influenza A viruses by C-ELISA and by HI to detect antibody against Influenza A subtype H5, H7 and H9 viruses. All sera tested were negative for antibody to the H5 and H7 subtypes, however, 3.94% of tested sera were positive for antibody to the H9 subtype of avian influenza virus. Meanwhile 372 swabs and tissue samples were tested by rapid antigen detection tests, virus isolation or RT/PCR. All 372 samples were found negative for the presence of Influenza A virus, viral antigen or viral RNA. These results suggest that Nepal is free of H5 and H7 avian influenza viruses at the time of testing.

Introduction

The commercial poultry industry in Nepal has been developing for 30 years and contributes 4% to GDP. Currently there are 31,557,808 broilers, 306,000 layers, 394,459 broiler parents and 36,000 layer parents that produce 41,813,189 kg chicken meat, 545,292,000 table eggs and 35,153,205 chicks annually. The poultry industry in Nepal employs 65,000 people with an estimated investment of 1,600 million Rupees [1]. Poultry are scattered all over the country, however, the greatest density of poultry is in commercial poultry areas of Chitawan and Kathmandu. In Nepal, backyard poultry are estimated to be about 55% of the total population, which also reflects the cultural value of backyard poultry. The poultry sector has been challenged by outbreaks of H5N1 bird flu in Asian countries, especially in both countries neighboring Nepal [2]. There are several risk factors, which can facilitate the outbreak of HPAI in Nepal. These include the high density of poultry in commercial poultry areas, wild birds in national parks, free range poultry, back yard poultry, the routes of migrating birds, inappropriate bio-security measures on poultry farms, close contact between wild birds and domestic poultry, illegal trade, open and closed national borders, live poultry markets, mixed farming systems and a complex epidemiology. Realizing the economic importance and threat of HPAI, the Central Veterinary Laboratory (CVL) under the Department of Livestock Services, Government of Nepal has been doing surveillance for avian influenza since 2004. This paper describes the research

work performed at CVL in 2006 in collaboration with three OIE Reference laboratories for avian influenza in Australia, UK and Italy.

Materials and Methods

Samples were collected from domestic and wild birds in 26 districts of Nepal (Figure 1). The total number of samples collected from January to December 2006 was 930 (793 chicken, 53 ducks, 26 pigeon and 58 wild birds). Of the 930 samples, 372 were cloacal and tracheal swabs and tissue samples collected from either sick or recently dead (not decomposed) birds and stored at 4°C until tested. Meanwhile, 558 serum samples were collected either from sick or healthy birds and stored at -20°C until use. Collected samples were tested by following method either in Central Veterinary Laboratory, Kathmandu or in OIE References laboratories. Rapid antigen detection tests were done on 372 swabs or tissue samples collected from birds showing clinical signs suggestive of avian influenza by using two commercially available avian influenza test kits. These were the Anigen AIV Test Kit (Animal Genetics Inc., Korea) and the Influenza Type A Antigen Test Kit (Synbiotic Corporation, USA). Virus isolation in eggs was done according to protocol of World Organization for Animal Health, 2004 [3] on 38 swabs or tissues collected from the farms located in Nuwakot, Kanchanpur, Chitin Kathmandu, Morang and Sharlahi, where higher mortalities in domestic birds was reported. RT/PCR tests for the H5 and M gene of avian influenza viruses was done on 38 samples collected from farms where a high mortality occurred [4]. Serum samples were tested for antibodies to the nucleoprotein of influenza A viruses by competitive ELISA (C-ELISA) according to protocol of Australian Animal Health Laboratory, Australia [5]. HI tests were done using H5, H7 and H9 virus reagents provided by Veterinary Laboratory Agency, UK by the protocol described by World Organization for Animal Health [3].

Results

All swabs and tissues tested for influenza A virus antigen were negative by the rapid antigen detection tests. No hemagglutinating viruses were isolated from 38 swabs or tissues samples after inoculation into eggs. All samples collected from birds in 6 districts were negative for avian influenza viral RNA by RT/PCR. All the serum samples were negative for the presence of antibodies against Avian Influenza subtypes H5 and H7 by HI. However 22 serum samples collected from domestic chickens were positive for antibodies by C-ELISA and for subtype H9 virus (Table 1). All ducks, pigeons and wild birds tested were negative for antibodies in all tests.

Figure 1. Sampling locations for avian influenza surveillance in Nepal, 2006 (From www.mapsofworld.com).



Table 1. Test result of samples from different districts.

S. No.	Name of the districts	Number of tested samples			Test result	
		Serum	Swabs & tissue	Total	Negative	Positive
1	Makwanpur	17	4	21	21	0
2	Chitawn	168	12	180	166	14 sera only
3	Nawalparasi	17	30	47	47	0
4	Kapilbastu	4	11	15	15	0
5	Rupendahi	1	0	1	1	0
6	Surketh	40	0	40	40	0
7	Morang	40	84	124	124	0
8	Jhapa	31	5	36	36	0
9	Dhankuta	3	0	3	3	0
10	Banke	93	11	104	101	3 sera only
11	Kanchanpur	35	70	105	103	2 sera only
12	Nuwakot	30	26	56	56	0
13	Rasuwa	15	0	15	15	0
14	Kailahi	32	13	45	44	1 sera only
15	Bara	16	5	21	21	0
16	Parsa	11	5	16	14	2 sera only
17	Bardiya	5	0	5	5	0
18	Kathmandu	0	57	57	57	0
19	Dahnusa	0	6	6	6	0
20	Lalitpur	0	2	2	2	0
21	Sharlahi	0	9	9	9	0
22	Dadeldhura	0	3	3	3	0
23	Shyanja	0	1	1	1	0
24	Gorakha	0	8	8	8	0
25	Kavre	0	6	6	6	0
26	Dolakha	0	4	4	4	0
Total		558	372	930	908	22

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Discussion

This study was done to further test domestic poultry flocks in Nepal for freedom from infection with avian influenza viruses. The first finding in October 2005 of birds with antibodies to influenza H9 virus but no positive birds prior to this time suggests that the H9 virus is a recent introduction into Nepal [5]. In the study reported in Pant and Selleck [5] antibodies were detected in chickens in two districts, Kathmandu and Chitawn. In this study antibody positive chickens were again identified Chitawn and in a further four districts. The Parsa district borders Chitawn but the Banke, Kanchanpur and Kailali districts border each other in the south west of Nepal, along the border with India (Figure 1). This raises the possibility that the H9 virus responsible for the antibodies has spread further through the poultry flocks of Nepal since the initial identification in 2005. No evidence of antibodies to H5 or H7 viruses was found, indicating that Nepal is free of these viruses. The absence of antibodies to notifiable H5 and H7 subtypes of influenza A virus provides additional evidence of freedom from these viruses. Further surveillance will be done on the flocks identified as antibody positive and on other flocks to determine the prevalence and distribution of antibodies to H9 virus. As the origin if the H9 virus in Nepal is not clear, further surveillance of migratory and wild water birds will be done in order to determine the subtypes of influenza viruses circulating in these species. Attempts will be made to isolate the virus from infected chicken flocks to permit further characterization. The absence of clinical disease in birds with antibody to H9 virus may indicate that the birds were vaccinated against H9 virus rather than infected with a naturally circulating virus. As vaccination against influenza viruses is not practiced in Nepal vaccinated birds may have been illegally imported into Nepal. Negative rapid test kit results also support the conclusion that the H9N2 virus is not currently circulating in domestic poultry, however the sensitivity of this test for detecting LPAI viruses has not been established. The finding of antibody to H9 influenza A virus in commercial poultry raises the possibility that other influenza A viruses may also enter these populations. Large, commercial poultry farms in Nepal will be advised to monitor biosecurity and to make improvements where inadequacies are identified.

Conclusions

Nepal is free from infection with notifiable H5 and H7 viruses at present. During this surveillance antibodies to avian influenza subtype H9 were found in 5 districts (Kanchanpur, Kailali, Banke, Chitawn and Parsa) of Nepal. The antibody positive sera were collected in March, 2006. Only domesticated chicken were found sero-positive for H9 virus, whereas all ducks, pigeon and wild birds tested were negative. The OIE has reported outbreaks of H5N1 in China, Pakistan, Bangladesh and India in 2007 and 2006 respectively. Nepal is at high risk as it is bordered by China and India.

Acknowledgements

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Evolutionary Dynamics of Avian Influenza A Virus in the Natural Reservoir

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Avian influenza viruses were thought to exist in a form of evolutionary stasis within their natural reservoirs, i.e. waterfowls. However, a recent study demonstrated very high evolutionary rates, with epidemic-like population growth, for individual influenza subtypes in both aquatic birds and poultry, suggesting the stasis theory may be incorrect. Yet the evolutionary dynamics of the influenza gene pool within one species of migratory waterfowl remains unclear. We therefore tested influenza virus population behavior by estimating rates of nucleotide substitution of the internal genes from different subtypes of influenza viruses exclusively from mallard ducks in North America. This dataset represents a co-circulating influenza gene pool in the natural reservoir from, importantly, a region without long-term prevalence of influenza in poultry populations. Our results showed high substitution rates for each internal gene examined ($>10^{-3}$ substitutions/site/year), in line with the results of previous studies. Furthermore, the majority of these substitutions were neutral with little evidence of positive selection. To evaluate the evolutionary stasis hypothesis, we compared the evolutionary dynamics of influenza hemagglutinin genes from the gene pool (mallards) that are not associated with disease in poultry, human or other animal populations (H4 subtypes) or with those that may be subsequently introduced and linked with disease outbreaks in poultry (H6 subtypes). Even though both subtypes share the same host species, analyses revealed that the mean evolutionary rate of the HA of H6 viruses was almost two-fold greater than that seen in H4. Although there was little site-specific positive selection in both H4 and H6 subtypes, the ratio of d_N/d_S was much greater for H6 isolates than those of H4 isolates, indicating that a higher proportion of mutations in the H6 subtype were nonsynonymous. These findings suggest that H4 isolates are under strong purifying selection, while H6 isolates are under Darwinian selection. Thus, high substitution rates seen in influenza viruses in mallard likely represent the neutral rate of evolution of the influenza gene pool in its natural host and that evolutionary stasis does not describe the dynamics of all influenza viruses in their natural reservoirs.

Introduction

Avian influenza A viruses (AIV) infect a variety of host species including humans, pigs, poultry, shorebirds, whales and seals. Migratory waterfowl are believed to be the natural hosts and AIV were thought to exist in a form of evolutionary stasis within this natural reservoir [1,2]. However, a recent study demonstrated very high evolutionary rates, with epidemic-like

population behavior, for individual influenza subtypes from multiple hosts, suggesting the stasis theory is incorrect [3]. Yet the evolutionary dynamics of the influenza gene pool in the natural reservoir remains unclear. In the present study we took two approaches to examine the stasis hypothesis. First we examined the gene pool behavior by comparing the population dynamics of AIV in its natural gene pool with that of disease associated poultry populations. We then investigated the evolutionary dynamics of the hemagglutinin sequences from H4 and H6 subtype viruses isolated from mallards. H4 subtype viruses are rarely isolated from poultry, humans or other animal species and have not been linked to disease outbreaks, while it is commonly isolated from the natural reservoir. In contrast, H6 subtypes are commonly isolated from mallard hosts but they have also been linked to disease outbreaks in poultry.

Methods

All data analyzed for this study was downloaded from GenBank. More than 2,000 sequences were analyzed for this study. For the analysis of the population dynamics of the influenza gene pool in its natural hosts, datasets for each internal gene (PB1, PB2, PA, M, NP and NS) isolated from all wild waterfowl were compared with results from datasets consisting of viral sequences from each host. In the current study the results of population dynamics of wild waterfowl and mallards are discussed, and compared with the population dynamics of disease associated AIV. To test the evolutionary dynamics of the surface hemagglutinin protein in its natural hosts, datasets of the H4 and H6 hemagglutinin subtypes were also analyzed. Dated phylogenies, Bayesian skyline demographic plots and substitution rates with associated highest posterior density (HPD) were determined with BEAST 1.4.2 [5,6]. Demographic growth models were tested using the dated phylogenies generated in BEAST with GENIE [6]. Growth models tested were constant, exponential, expansion, logistic, and piecewise constant. The best-fit model was determined using the Akaike information criterion. DATAMONKEY [7] and HYPHY [8] were used to test for site-specific positive selection and determine the global d_N/d_S based on an appropriate nucleotide substitution model for each dataset analyzed.

Results

Population dynamics in natural hosts vs. poultry. Utilizing data from wild birds isolated from North America [4], we estimated and compared the population dynamics and substitution rates of all AIV isolated from mallard hosts. Analysis of the population dynamics for the internal genes from all waterfowl is similar to those isolated only from mallards. This indicates that AIV isolated from mallards are part of the same natural gene pool found in all aquatic waterfowl and provides a suitable model for the entire natural gene pool (data not shown). However, the populations of AIV from poultry hosts over the thirty-year time period sampled, are not stable and show high degrees of fluctuation (data not shown). These results show that the AIV population in domestic poultry is not stable and likely

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results from repeated introductions from the natural gene pool and therefore can be excluded from subsequent analyses. *Population growth models.* Our results show that the internal genes of AIV have high substitution rates ($>10^{-3}$ substitutions/site/year) in its natural reservoir hosts (Table 1). This result is similar to previous studies for influenza viruses [3], and for all RNA viruses [9]. However, most of these mutations are neutral; as indicated by the low number of nonsynonymous changes (d_N) over the thirty-year sampling period. The population growth models show that, while the population is stable, there are still changes over time.

Table 1. Population dynamics of AIV in Mallard.

Gene Segment	Population growth model	Mean substitution rate (10^{-3} substitutions/site/year)	HPD range	d_N/d_S
H4	Constant	5.268	2.627-8.083	0.079
H6	Constant	9.508	6.604-12.68	0.144
PB2	Logistic	2.526	1.712-3.383	0.03
PB1	Logistic	3.986	3.012-4.946	0.02
PA	Constant	3.088	2.207-3.975	0.02
NP	Piecewise constant	3.104	2.181-3.967	0.02
M	Logistic	2.076	1.475-2.682	0.02
NS	Logistic	3.791	2.489-5.162	N/A*

Population dynamics of the H4 and H6 hemagglutinin genes isolated from mallard show the same population growth patterns. However, the mean substitution rate is much higher for H6 subtypes and the d_N/d_S is nearly twice as high as compared to H4 subtypes. Positive selection analysis of the H4 dataset failed to detect any sites under positive selection and only one site (aa 154) for the H6 dataset ($p = 0.1$).

Dated phylogeny. Figure 1 shows a dated phylogeny reconstructed from the analysis of the hemagglutinin (HA) of H4 and H6 subtype viruses isolated from mallards. H6 viruses show periods of rapid diversification and high evolutionary rates (Fig. 2A). The tree topology and the constant population growth model suggest that H6 viruses are subjected to continual Darwinian evolution with high rates of lineage extinction and that surviving lineages are regularly selected for. This result is contrary to that seen in the dated phylogeny of H4 subtype viruses where substitution rates along lineages are low (Fig. 2B), suggesting H4 viruses have been adapted to mallard hosts for a longer period.

Discussion

The dataset analyzed represents a co-circulating influenza gene pool in the natural reservoir from, importantly, a region with no long-term endemicity of influenza viruses in terrestrial poultry populations. Our results showed high substitution rates for each gene examined ($>10^{-3}$ substitutions/site/year), in line with the results of previous studies [3,4]. These substitutions were neutral and mutations along a lineage showed little evidence of positive selection. Results indicate that populations of H4 and H6 subtypes of influenza viruses in mallard duck were under strong purifying selection pressure ($d_N/d_S < 0.2$); in contrast to the high background mutation rate. Variation in tree topologies,

d_N/d_S and evolutionary rate suggests that antigenic drift may explain the evolution of the faster evolving H6 subtypes, but is inadequate to explain the changes of H4 viral population within its natural host. The naturally high substitution rate of H6 subtypes in mallard results in a rapid accumulation of genetic changes and therefore accumulation of many more nonsynonymous changes over time. These results indicate that the high substitution rates seen in influenza viruses in mallard, which are consistent with other RNA viruses, likely represent the neutral rate of evolution of the influenza gene pool in its natural host.

Acknowledgements

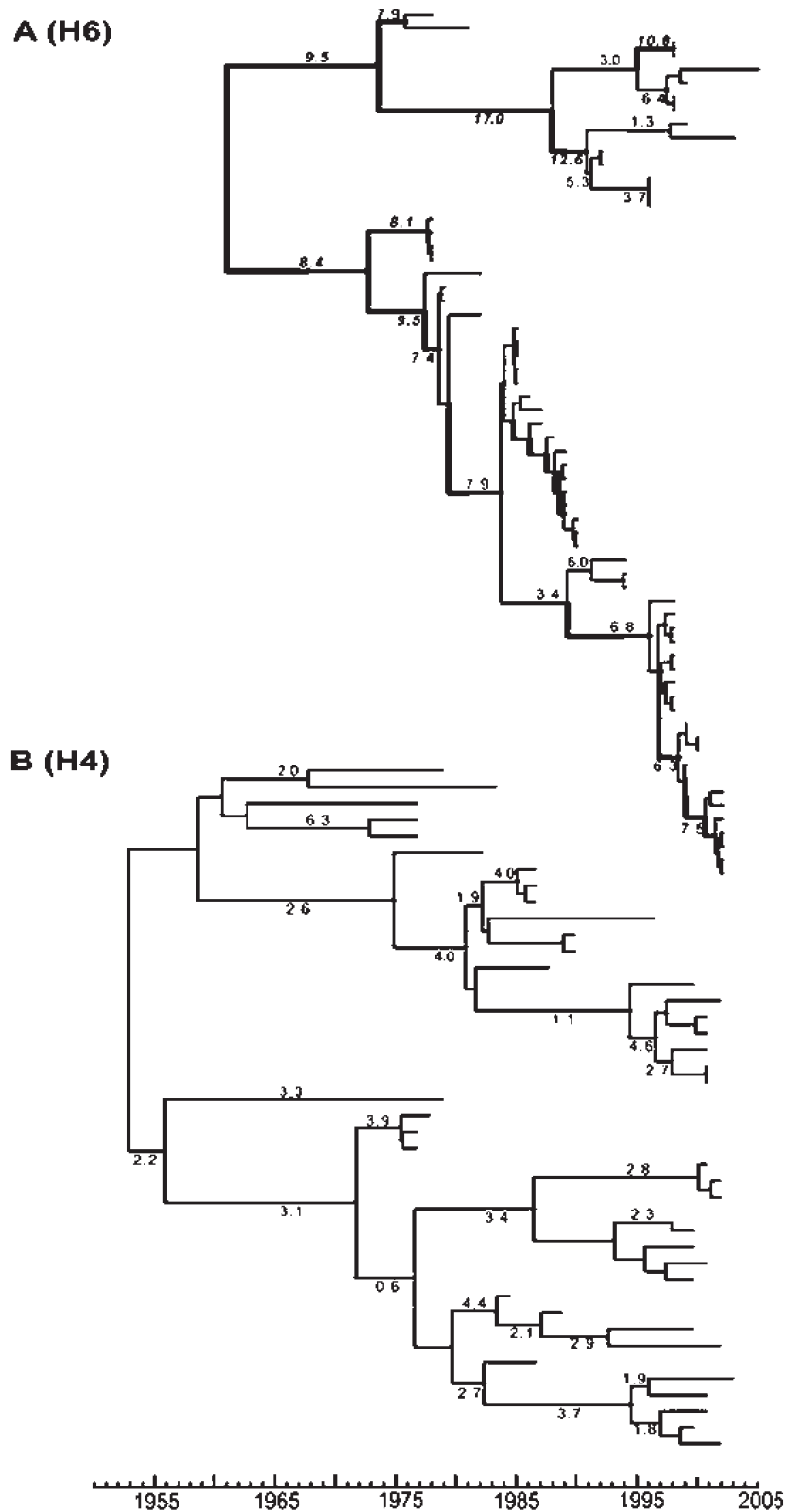
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Figure 1. Dated phylogeny of the HA gene of 1A) H6 and 1B) H4 subtype viruses. grey bars represent 95% HPDs. Numbers shown on branches represent the evolutionary rates along each lineage (10^{-3} Substitutions/Site/Year). Thickened branches shown in black indicate evolutionary rates along diversifying branches of H6 that are much greater than evolutionary rates seen in H4 during similar time periods. Numbers in bold and italicized are evolutionary rates significantly greater than the mean evolutionary rates for H4 shown in Table 1.



Characterization of H3N2 Influenza Viruses Isolated From Pigs in Southern China

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Human-like H3N2 influenza viruses have repeatedly transmitted to domestic pigs in different regions of the world, but it is still not certain whether any of those variants have become established in pig populations. The detection of different subtypes of avian influenza viruses from pigs makes it an ideal candidate for the genesis of a possible reassortant virus with both human and avian gene segments. However, whether pigs could act as a "mixing vessel" for a possible pandemic virus remains unanswered. Long-term influenza surveillance in pigs in southern China revealed that H3N2 influenza viruses were regularly detected from domestic pigs from 1998 to 2003. Antigenic analysis of representative strains revealed that two distinguishable groups of H3N2 influenza viruses were present in pigs during this period: a contemporary human-like viruses (represented by Sydney/5/97), and Port Chalmers/1/73-like (PC-like) viruses. Phylogenetic analysis of the representative strains confirmed those two groups. In general, the PC-like viruses were most closely related to those H3N2 reassortants recognized from European pigs since the mid-1980s, while the remaining isolates were most closely related to those contemporary human H3N2 viruses. It is interesting to note that one PC-like isolate contained a classical swine H1N1-like NP gene, Sw/HK/1197/02, suggesting that after introduction to pigs in southern China the European swine H3N2 virus further reassorted with local swine virus. The contemporary human-like H3N2 viruses isolated from pig appeared to have resulted from repeated introduction from humans to pigs. Interestingly, one isolate (Sw/HK/NS1128/03) clustered with those human isolates detected in the early 1990s. These findings suggesting that some recent human H3N2 variants may be maintained long-term in pig populations in southern China. The present study provides updated information on the role of pigs in the interspecies transmission and genetic reassortment of influenza viruses in this region.

Introduction

Pigs are considered an important host of influenza A virus as they might be associated with the generation of human pandemic influenza strains [1]. Historically, two human pandemic viruses, H1N1 in 1918 and H3N2 in 1968, were detected almost simultaneously in humans and pigs [2, 3]. Even though pandemic H3N2 virus was initially detected in humans and the full genome of the 1918 H1N1 pandemic virus was recently decoded [4], it is still unknown whether this virus

was first introduced into humans or pigs before it became a human pandemic strain. Currently, H1N1, H1N2 and H3N2 influenza viruses co-circulate in pigs in different regions. All of these viruses resulted from either interspecies transmission or reassortment events [5-7]. The Sydney-like H3N2 variants from pigs in the USA in 1998 were double and triple reassortants containing viral genes of avian, human and swine origins [8], highlighting the complex and dynamic influenza ecology in pig populations. Our long-term surveillance also revealed that H1N1, H1N2 and H3N2 subtypes viruses also co-circulated in pigs in southern China [9]. Here we present the findings of genetic and antigenic characterization of swine H3N2 influenza viruses isolated from 1998 to 2003.

Results

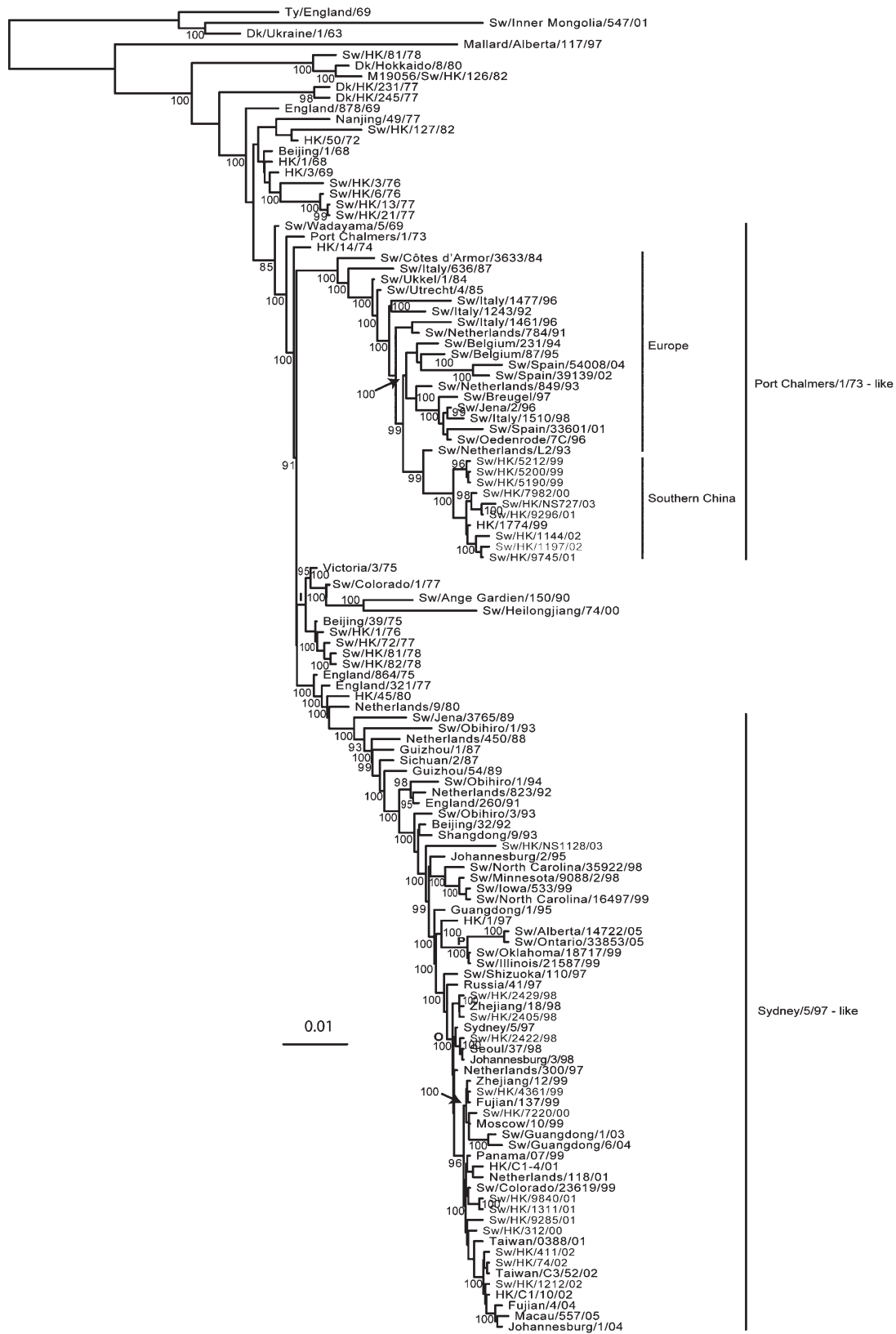
Genetic analysis showed that two sublineages of H3N2 influenza viruses were co-circulating in pigs in southern China. One sublineage of viruses, PC-like viruses, was closely related to those viruses prevailing in European pigs, while the other sublineage consisted of contemporary human-like variants resulting from multiple interspecies transmissions from humans to pigs. Epidemiological and genetic results also suggest that the PC-like H3N2 variants may have been introduced into pigs in this region via imported pigs from Europe in the mid-1990s.

Prevalence of swine influenza viruses in southern China. During the 8-year period of virological surveillance, 535 influenza viruses were isolated from 22,562 nasal and tracheal swabs (isolation rate 2.4%) from January 1999 to September 2006. Influenza virus in pigs was prevalent year-round but an increased isolation rate of H1 subtype virus was usually observed during the winter season. Ninety-nine of those isolates were H3N2 subtype, and 288 were H1N1 subtype viruses. The remaining 157 isolates were identified to be H1N2 viruses that were also detected each year during the surveillance period. In general, H1 subtype virus accounted for an increasing proportion of the overall number of isolates each year.

Phylogenetic analysis of the surface genes. Phylogenetic analysis of the H3N2 swine hemagglutinin (HA) genes revealed two distinct groups. The PC-like swine viruses formed a monophyletic clade with viruses isolated from swine in Europe since 1984 (Fig. 1). The PC-like viruses in southern China show high genetic similarity, forming a sister group to the European swine viruses isolated between 1994 and 2004, Sw/Netherlands/L2/93 in particular. Therefore, these viruses appear to have been introduced into pig populations in southern China from a Sw/Netherlands/L2/93-like virus (Fig 1). After their introduction into this region during 1997 these viruses appear to have persisted in pigs in southern China and have been detected in HK from 1999 to 2003. Phylogenetic analyses of the HA gene also shows that the Sydney-like swine H3N2 viruses that were isolated between 1998 and 2002 are closely related to several human viruses as well as the annual vaccine strains, indicating that these viruses were introduced into pigs several times from the human population. These results provide evidence for multiple introductions of H3N2

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Figure 1. Phylogenetic relationships of the HA genes of representative influenza A viruses isolated in pigs from southern China. Trees were generated by neighbor-joining method in the PAUP* program (Bayesian analysis revealed similar relationships.) Numbers at branches indicate bootstrap values. The tree was constructed based on nucleotides 161-952 of the HA gene and rooted to A/Duck/Alberta/28/1976 (H4N6). Scale bar, 0.1 substitution.



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viruses from humans to pigs. However Sw/HK/NS1128/03 was most closely related to the earlier human strains (e.g. Shandong/9/93), indicating that some human H3N2 variants could be maintained in pig populations for a long period. These phylogenetic relationships are consistent with the antigenic reaction pattern. A similar phylogenetic relationship was observed in the neuraminidase (NA) gene tree, except for Sw/HK/NS1128/03, which clustered with recent swine H1N2 isolates (Sw/Zhejiang/1/04) in China.

Phylogenetic analysis of the internal genes. Phylogenetic analysis of the six internal genes showed that the H3N2 viruses from pigs in southern China have undergone reassortment. In the PB2 gene tree, 11 representative H3N2 isolates clustered into three different lineages. The PB2 gene of representative PC-like swine viruses clustered with European swine viruses that appear to have originated from an avian source. Except for one virus, all Sydney-like viruses clustered with recent human H3N2 viruses, indicating a human origin. The PB2 gene of one of the Sydney-like viruses, Sw/HK/NS1128/03, belonged to the classic swine H1N1 lineage. Similar phylogenetic relationships was observed in the PB1, PA and M gene trees. However, in the NP gene tree, PC-like viruses belonged to a recent human lineage, while Sydney-like viruses belonged to an avian lineage, except for the virus Sw/HK/1197/02. This virus and Sw/HK/NS1128/03 belonged to the classic swine H1N1 lineage. In the NS gene tree all PC-like viruses grouped with the European swine lineage, while all Sydney-like viruses joined a recent human lineage. Taken together, four different reassortants, or genotypes, of H3N2 viruses were recognized. Two of them were the European swine (PC-like) virus, and PC-like virus but with a NP gene from classical swine H1N1 virus. The other two genotypes included contemporary human-like, and a triple reassortant (Sw/HK/NS1128/03) that contained surface genes from an early 1990's human-like H3N2 virus, but with five internal genes from classical H1N1 swine virus and an NS gene that of avian origin.

Table 1. Gene origins of swine H3N2 influenza viruses from southern China from 1998 to 2003^a.

Viruses	Subtype	Lineage	Gene									Genotype
			PB2	PB1	PA	HA	NP	NA	M	NS		
Sw/Germany/2/81	H1N1	Av	Av	Av	-	-	Av	-	Av	Av	Av	Av
Sw/HK/168/93	H1N1	Av	Av	Av	Av	Av	Av	Av	Av	Av	Av	Av
Sw/HK/5190/99	H3N2	PC	Av	Av	Av	Hu	Av	Hu	Av	Av	Av	Double(Hu-Av)
Sw/HK/7982/00	H3N2	PC	Av	Av	Av	Hu	Av	Hu	Av	Av	Av	Double(Hu-Av)
Sw/HK/9296/01	H3N2	PC	Av	Av	Av	Hu	Av	Hu	Av	Av	Av	Double(Hu-Av)
Sw/HK/1197/02	H3N2	PC	Av	Av	Av	Hu	Csw	Hu	Av	Av	Av	Triple(Hu-Csw-Av)
Sw/HK/NS727/03	H3N2	PC	Av	Av	Av	Hu	Av	Hu	Av	Av	Av	Double(Hu-Av)
Sw/HK/2405/98	H3N2	SY	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu
Sw/HK/4361/99	H3N2	SY	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu
Sw/HK/7220/00	H3N2	SY	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu
Sw/HK/9285/01	H3N2	SY	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu
Sw/HK/1212/02	H3N2	SY	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu	Hu
Sw/HK/NS1128/03	H3N2	SY	Csw	Csw	Csw	Hu	Csw	Hu	Csw	Av	Av	Triple(Hu-Csw-Av)

Conclusions

Antigenic and phylogenetic analysis of swine H3N2 influenza viruses in southern China from 1998 to 2003 provided evidence of persistence of both early and contemporary H3N2 variants in pig populations. Results also revealed multiple interspecies transmissions of influenza viruses from human to pig and subsequent reassortment events, particularly with internal genes from avian lineages in this region. In the present study, PC-like viruses consistently formed their own clades for each segment except the NP gene of Sw/HK/1197/02. This indicates that most of the PC-like viruses may be derived from a single reassortant progenitor virus from European pigs imported into China as breeding stock, which subsequently spread among pigs, rather than as the products of multiple, independent reassortment events. Sydney-like viruses were from the contemporary human lineage and probably arose from multiple introduction events into pig, rather than being established in this host. Our findings demonstrate continuing interspecies transmission and reassortment events in pigs in southern China that naturally increase the possibility for pigs to be an important host for generating potential pandemic strains.

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Genetic and Pathogenic Evolution of Influenza Viruses Isolated From Korean Chicken Farms

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Introduction

H9N2 influenza viruses have become panzootic in Eurasia during the past decade and have been isolated from terrestrial poultry worldwide (2,4,7). Detailed antigenic and molecular analyses have identified three groups of H9N2 influenza virus in avian species in southeastern China: the G1 group, represented by A/quail/Hong Kong/G1/97; the Y280 group, represented by A/chicken/Hong Kong/Y280/97; and the Korea group, represented by A/duck/Hong Kong/Y439/97 (6). However, the genetics and pathogenicity of the Korea group-like H9N2 viruses are not characterized as well. In late 2003 to 2004, several Korean chicken farms suffered serious economic damage due to avian influenza viruses. Most of the affected birds showed clinical signs typical of influenza infection, such as a pronounced drop in egg production, diarrhea, and moderate to high mortality rates, compared with those infected in 2002. Antigenic and genetic analyses show that two antigenically distinguishable H9 viruses are circulating in Korea. Whereas the H9N2 viruses isolated from 1996 to mid-2003 caused mild disease signs, those isolated in late 2003 and 2004 associated with relatively high mortality in experimentally inoculated chickens. Therefore, the H9N2 viruses circulating in Korea appear to have undergone antigenic and pathogenic changes.

Materials and Methods

Antigenic analysis. We investigated the cross-reactivity of the isolated viruses by hemagglutinin-inhibition (HI) assay using polyclonal antibodies to seven H9N2 viruses that recently circulated in southeastern China and Korea.

DNA sequencing and phylogenetic analysis. Nucleotide sequences were analyzed by directly sequencing the PCR products on an automated 3700 DNA sequencer (Applied Biosystems, Foster City, CA). The sequences were resolved using the ABI PRISM collection program (Perkin-Elmer, Foster City, CA). The DNA sequences were compiled and edited using Lasergene sequence analysis software package (DNASTAR, Madison, WI).

Measurement of pathogenicity in chickens and mice. Chickens (specific pathogen-free [SPF] White Plymouth Rock chickens [*Gallus gallus domesticus*]) and mice (BALB/c, *Mus musculus*) were inoculated intranasally with allantoic fluid containing 10^{5.5} 50% egg-infective doses [EID₅₀] of each genotype of virus.

Tracheal and cloacal swabs, and lung tissue specimens were collected from chickens on days 3, 5, and 7 post-inoculation (p.i.), and virus was titrated in 10-day-old embryonated chicken eggs. Eight 5-week-old chickens and twelve 5-week-old mice were inoculated with each virus.

Results

All of the 12 H9 viruses tested in this study have a glutamine at position 226, which is associated with high binding affinity for 2,3-linked sialic acid (SA) moieties but a low binding affinity for the 2,6-linked SA moieties found in mammals, as did the earlier Korea group viruses and avian H9 viruses isolated from ducks in Asia in the 1970s. Although the relative importance of this receptor trait and the potential for transmission are unknown, some Korean H9N2 viruses replicated well in mice (Table 1). These observations suggest that the H9 viruses of Korea have not acquired human virus-like receptor specificity, as did Hong Kong H9N2 viruses. Interestingly, one isolate that we tested, Ck/Kor/164/04, was of the H9N8 subtype, which is a novel subtype in domestic chickens. Genetic characterization revealed that the HA and PB1 genes of A/Ck/Kor/164/04 belong to the Korea group-like H9N2 viruses, whereas its other genes are closely related to those of an H3N8 avian influenza virus (A/aquatic bird/Kor/W04/04) isolated in 2004 from a wild aquatic bird. Our previous genetic study of influenza viruses isolated from live-bird markets in Korea showed the H3N2 subtype to be one of the main subtypes in chickens and ducks (5). Although avian H3N2 viruses consistently are isolated from live-bird markets and can infect experimentally inoculated chickens, no H3N2 virus was detected in chicken farms during this study. Although infection of chickens with various avian influenza viruses has been documented, this report is the first (to our knowledge) of H9N8 infection in this host. Most of the Korean genotype A viruses isolated in 2004 replicate in mice without pre-adaptation (4 out of 5 isolates), except the Ck/Kor/Q19/04 (H9N2) virus (data not shown). All isolates of genotype C and B were not recovered from mice lungs during 7 days of post inoculation. The NS and M genes of genotype A isolates are of the lineages from unknown aquatic bird viruses. The factors that control host range restriction of influenza viruses remain unknown. However, the NP, NS, and M genes are responsible for the replication of avian influenza viruses in mammalian hosts and of mammalian influenza viruses in avian hosts (1,8,11)

Options for the Control of Influenza VI

Table 1. Replication of the Korean influenza viruses in 4-week-old chickens and 5-week-old mice^a.

Virus	Chickens					Disease signs	Mice	
	Trachea	Lung	Cecal tonsil	Kidney	Spleen		Lung	Disease signs
H9N2								
Ck/Kor/MS/96	1/8 ^b (4.3/0.5) ^c	1/8 (2.7/0.5)	2/8 (4.7/2.5)	1/8 (0.0/3)	0/8	None	0/8	None
Ck/Kor/150/03	4/8 (3.7/1.7)	3/8 (3.3/1.0)	4/8 (4.3/2.7)	0/8	0/8	None	0/8	None
Ck/Kor/136/04	5/8 (4.7/4.2)	5/8 (3.7/1.0)	8/8 (6.0/6.0)	3/8 (1.3/0.7)	7/8 (1.7/0.7)	None	0/8	None
Ck/Kor/116/04	6/8 (5.0/4.5)	5/8 (4.5/2.3)	5/8 (5.5/5.7)	3/8 (2.3/1.7)	4/8 (2.0/0.5)	Depression	8/8	Weight loss
Ck/Kor/Q30/04	8/8 (4.7/3.5)	7/8 (3.3/2.0)	8/8 (5.7/5.7)	3/8 (1.7/0.5)	7/8 (1.3/0.7)	Depression	0/8	None
AB/Kor/W02/04	0/8	0/8	1/8 (1.3/0.7)	0/8	0/8	None	0/8	None
Control chickens								
H9N8								
Ck/Kor/164/04	4/4 (4.3/3.0)	3/4 (4.7/2.2)	3/4 (5.3/1.8)	2/4 (1.5/1.0)	2/4 (1.3/0.7)	Depression	7/8	Weight loss
H5N1								
AB/Kor/W04/04	2/4 (1.7/0.7)	0/4	2/4 (1.7/0.7)	0/4	0/4	None	6/8	None

Ck, chicken; AB, aquatic bird; Kor, Korea.

^a The dose of inoculum for chickens and mice was 105.5 EID₅₀/0.1 ml

^b No. of animals infected/no. inoculated by intranasal and tracheal route and

^c (in parentheses) the mean virus titer (log₁₀ EID₅₀/0.1 ml) in samples taken on day 3/5 post-inoculation

Therefore, the NS and M genes of genotype A may contribute to the replication of these viruses in mice. However, further studies are needed to understand the role of these genes in restricting the host ranges of these viruses to chickens and mice. In chickens, the genotype A isolates (Ck/Kor/116/04 and Ck/Kor/Q19/04), including Ck/Kor/164/04 (H9N8), were recovered from all organs tested and at higher titers at 5 days p.i. than those of Ck/Kor/MS/96. However, Ck/Kor/MS/96 was recovered from the kidneys at a low titer (10^{0.5} EID₅₀/ml) in only 1 of 8 infected chickens and was not recovered from the spleen. Furthermore, the Ck/Kor/116/04 and Ck/Kor/Q30/04 isolates caused more severe clinical signs than did the genotype C isolate (A/Ck/Kor/150/03) in mature breeder chickens. Specifically, the Ck/Kor/Q30/04 isolate showed higher viral titer and mortality than did other isolates in tissues of Hy-Line white (43.7%) and brown (18.7%) breeder chickens at 7 days p.i.

Discussion

At least 2 subtypes of avian influenza virus, including the novel reassortant H9N8 subtype, were co-circulating in Korean chicken farms during 2002–2004. These H9 viruses had evolved continuously by antigenic drift and had undergone reassortant with aquatic avian influenza A viruses, and their pathogenicity in animals had changed. New influenza virus genes can easily be introduced into this area by migrating birds, as occurred nearby in China (10). In light of this situation, we would expect to isolate additional reassortant viruses with unique combinations of genes. Therefore, continued monitoring of the domestic and wild bird populations is needed to better understand interspecies transmission, including that which has resulted in infection of humans with H5N1 (3,12,14,15), H9N2 (13), and H7N7 (9) avian influenza viruses, and to clarify the importance of avian hosts in the ecology of influenza viruses.

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Effect of an Inactivated Avian Influenza Vaccine Prepared From an Apathogenic H5N1 Reassortant Virus Generated Between H5N2 and H7N1 Isolates From Migratory Ducks in Asia on Protection of Chickens Against Challenge With Highly Pathogenic Avian Influenza Virus Strains

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A stamping-out policy is the base for the control of highly pathogenic avian influenza (HPAI). Vaccination may be a part of optional control measures in cases when the outbreak is widespread (OIE). In the present study, we developed an inactivated avian influenza vaccine prepared from apathogenic avian influenza virus strains isolated from migratory ducks in Mongolia. The vaccine was adjuvanted with mineral oil to form a water-in-oil emulsion. The test vaccine was injected into chickens intramuscularly and evaluated by protection tests of the birds against the challenge with HPAI virus strains. Of the library of influenza virus strains (Kida and Sakoda, 2006), 12 H5 subtype viruses were investigated for their phylogenicity and antigenicity. The vaccine strain was a reassortant virus generated from A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1). The resulting reassortant virus, A/duck/Hokkaido/Vac-1/04 (H5N1) was grown in chicken embryos. The resulting virus was inactivated with formalin and adjuvanted. A single dose of test vaccine was injected into chickens intramuscularly. Three weeks post vaccination (p.v.), either A/chicken/Yamaguchi/7/04 (H5N1) or A/whooper swan/Mongolia/3/05 (H5N1) were inoculated intranasally into the birds. Clinical signs of birds and virus recovery from the birds were examined. Antibody responses were investigated by hemagglutination-inhibition (HI) tests. In addition, the duration of effective protection provided by the test vaccine in chickens was investigated. A reassortant virus, A/duck/Hokkaido/Vac-1/04 (H5N1) was obtained by mixed infection of chicken embryos with A/duck/Mogolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1). Although infectious viruses were recovered from the vaccinated chickens, the test vaccine protected chickens from each of the two HPAI viruses without showing any disease signs for 14 days. All chickens challenged with A/chicken/Yamaguchi/7/04 (H5N1) on 6 days p.v. died, and all the chickens challenged with A/chicken/Yamaguchi/7/04 (H5N1) on 8 days p.v. survived, indicating that duration of protection provided by vaccination was 8 days. The present test vaccine was confirmed to prevent manifestations of disease signs in chickens but not shedding of infectious virus, although high titers of serum antibody were induced.

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have occurred worldwide, especially in Asia, since 1996 [1]. More than 300 million poultry including chickens, quails, ducks, and geese have died or been culled, impacting not only the poultry industry but public health, since 18 people were infected with HPAI viruses and 6 died in 1997, Hong Kong [2]. Since 2003, more than 290 cases of human infection have been reported with 60 % death rate in 12 countries [3]. "Stamping-out" is the basic measure for control of HPAI. Vaccination may be an optional measures in cases where the disease is wide spread [4]. Since commercial vaccines have been prepared from viruses of North American lineage in the USA and Mexico, they may be less effective for control of current HPAI outbreaks in Asia. Inactivated influenza vaccines should therefore be prepared from an H5N1 virus strain belonging to the Eurasian lineage. Inactivated influenza vaccines with oil adjuvant conferred immunity to protect chickens from manifestation of disease signs, but did not prevent virus shedding from the birds after challenge with an HPAI virus, leading to silent spread of the infection in the poultry population [5]. It has been established that influenza viruses circulate between natural reservoirs, ducks and the water of lakes where they nest in summer [6,7]. Viruses of 16 hemagglutinin (HA) and 9 neuraminidase subtype have been identified in avian species [8]. Phylogenetic analysis revealed that each of the influenza A virus of birds and mammalian hosts including humans originated from water birds reservoirs [7]. Thus, continuous surveillance of avian influenza is essential for preparedness against the emergence of pandemics. To provide information on the precursor genes of future pandemic influenza viruses, global surveillance of avian influenza has continued since 1997. During 1996-2004, twelve H5 influenza virus strains were isolated from fecal samples of water birds in Mongolia and Japan [6, 9-11]. In the present study, an apathogenic H5N1 influenza virus was generated from two influenza viruses isolated from the natural host, migratory ducks in Asia. The resulting reassortant virus A/duck/Hokkaido/Vac-1/04 (H5N1) was propagated in embryonated chicken eggs. The yielded virus was inactivated with formalin and adjuvanted with oil. Potency of the vaccine was evaluated by challenge tests with H5 HPAI virus strains belonging to the Eurasian lineage.

Results and Discussion

Isolation of H5 influenza virus. A total of 5,633 fecal samples were collected from water birds in 1996-2004 in Mongolia and Japan. Each sample was inoculated into the allantoic fluid of 10-day-old embryonated chicken eggs. Subtypes of all hemagglutinating agents were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests using antisera specific to the reference strains of influenza viruses. Of these, twelve strains were identified as H5 influenza viruses. To evaluate the genetic relationship between H5 isolates and H5 influenza virus strains studied previously, nucleotide sequences of the HA genes (position 65-1637) were analyzed and compared with other H5 viruses from existing databases. The amino acid sequence at the cleavage site of the HA was deduced from the nucleotide

sequence of the corresponding gene of each of the isolates. The phylogenetic tree indicates that all the isolates were of the Eurasian lineage except for Duck/Mongolia/84/02 (H5N3), which is North American (Figure 1). The 11 isolates of Eurasian lineage formed a different cluster from that of HPAI viruses isolated in Asia. HA genes of Eurasian isolates showed more than 90% amino acid identity with those of HPAI viruses.

Generation of H5N1 vaccine strain. One of the H5 isolates, A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) were mixed and inoculated into 10-day-old embryonated chicken eggs and incubated for 48 hours at 35°C. Viruses in allantoic fluids were plaque-purified in MDCK cells. From these virus clones, an H5N1 subtype influenza virus was selected by HI and NI tests and designated as A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/Vac-1/04) to use as a vaccine strain. The origin of the internal protein genes (PB2, PB1, PA, NP, M, NS) of Dk/Vac-1/04 (H5N1) was determined by sequencing. The NA and NS genes of Dk/Vac-1/04 (H5N1) were derived from A/duck/Mongolia/47/01 (H7N1), and the other genes were from A/duck/Mongolia/54/01 (H5N2). Nucleotide sequences of eight genes of Dk/Vac-1/04 (H5N1) were submitted to the DNA Data Bank of Japan under accession numbers; AB253760 (PB2), AB253761 (PB1), AB257726 (PA), AB263192 (HA), AB263193 (NP), AB263194 (NA), AB263195 (M), and AB263196 (NS).

Vaccine and Potency of the test vaccine in chickens. Dk/Vac-1/04 (H5N1) was inoculated into the 10-day-old embryonated chicken eggs and propagated for 48 hours. The virus was inactivated with 0.2% formalin for 3 days. The inactivated virus suspension containing 512 HA units was homogenized with the mineral oil adjuvant to form a water-in-oil emulsion. Thirty-four four-week-old chickens were vaccinated. The vaccine (0.5 ml) was injected into the lower thigh muscle of the chickens. Three weeks later, 24 vaccinated chickens and 12 seven-week-old non-immunized chickens were challenged intranasally with 100 50% chicken lethal dose (CLD₅₀) of A/chicken/Yamaguchi/7/04 (Ck/Yamaguchi/04) (H5N1). On the same day, the other 10 vaccinated chickens and 3 seven-week-old non-immunized chickens were challenged intranasally with 10^{4.5} CLD₅₀ of A/whooper swan/Mongolia/3/05 (Swan/Mongolia/05) (H5N1). All vaccinated chickens challenged either with Ck/Yamaguchi/04 or with Swan/Mongolia/05 survived without showing any disease signs, whereas all control chickens died within 2 days post challenge (p.c.). To investigate virus shedding from vaccinated chickens, 12 four-week-old chickens were vaccinated. Three weeks later, 6 vaccinated chickens and 6 seven-week-old non-immunized chickens were challenged intranasally with Ck/Yamaguchi/04 (H5N1), and the other 6 vaccinated chickens and 6 seven-week-old non-immunized chickens were challenged intranasally with Swan/Mongolia/03 (H5N1). Six chickens in each group were sacrificed on day 2 and 4 p.c. From non-immunized chickens, viruses with high titers were recovered from each of the tracheal and cloacal swabs and tissues tested (Table 1).

Table 1. Virus in vaccinated chickens.

	Challenge virus	No. of chickens	HI titer ^a (log ₂)	Virus recovery ^b						
				Days p.c. ^c	No. of the chickens each virus was recovered [GM value of the virus titer (log ₁₀)]					
					Swab (logEID ₅₀ /ml)		Organs (logEID ₅₀ /g)			
Vaccinated chickens	Ck/Yamaguchi/04 (H5N1)	6	5.7 ± 1.1	2	0	0	0	2 (2.0)	0	
		6	5.7 ± 1.8	4	0	0	0	0	0	
	Swan/Mongolia/05 (H5N1)	6	6.7 ± 0.7	2	2 (1.3)	0	2 (1.8)	0	0	
		6	5.2 ± 0.4	4	2 (0.8)	0	2 (1.8)	0	0	
	No immunized chickens	Ck/Yamaguchi/04 (H5N1)	6	< 2	2 ⁺	6 (5.7)	6 (5.3)	6 (6.9)	6 (7.3)	6 (8.4)
			4	< 2	2 ⁺	4 (6.0)	4 (5.7)	4 (6.6)	4 (7.2)	4 (8.2)
Swan/Mongolia/05 (H5N1)		2	< 2	3 ⁺	2 (7.0)	2 (5.4)	2 (5.2)	2 (5.4)	2 (7.3)	

^a Challenge virus strain was used as the HI antigens. Mean HI titer ± SE.

^b Digit: number of chickens in which each virus was isolated. Parenthesis: average of virus titers (logEID₅₀/ml) and (logEID₅₀/g). 0 indicates no virus was isolated from chickens.

^c Number indicates the sampling day at p.c., N-0 indicates that chickens were dead at that day.

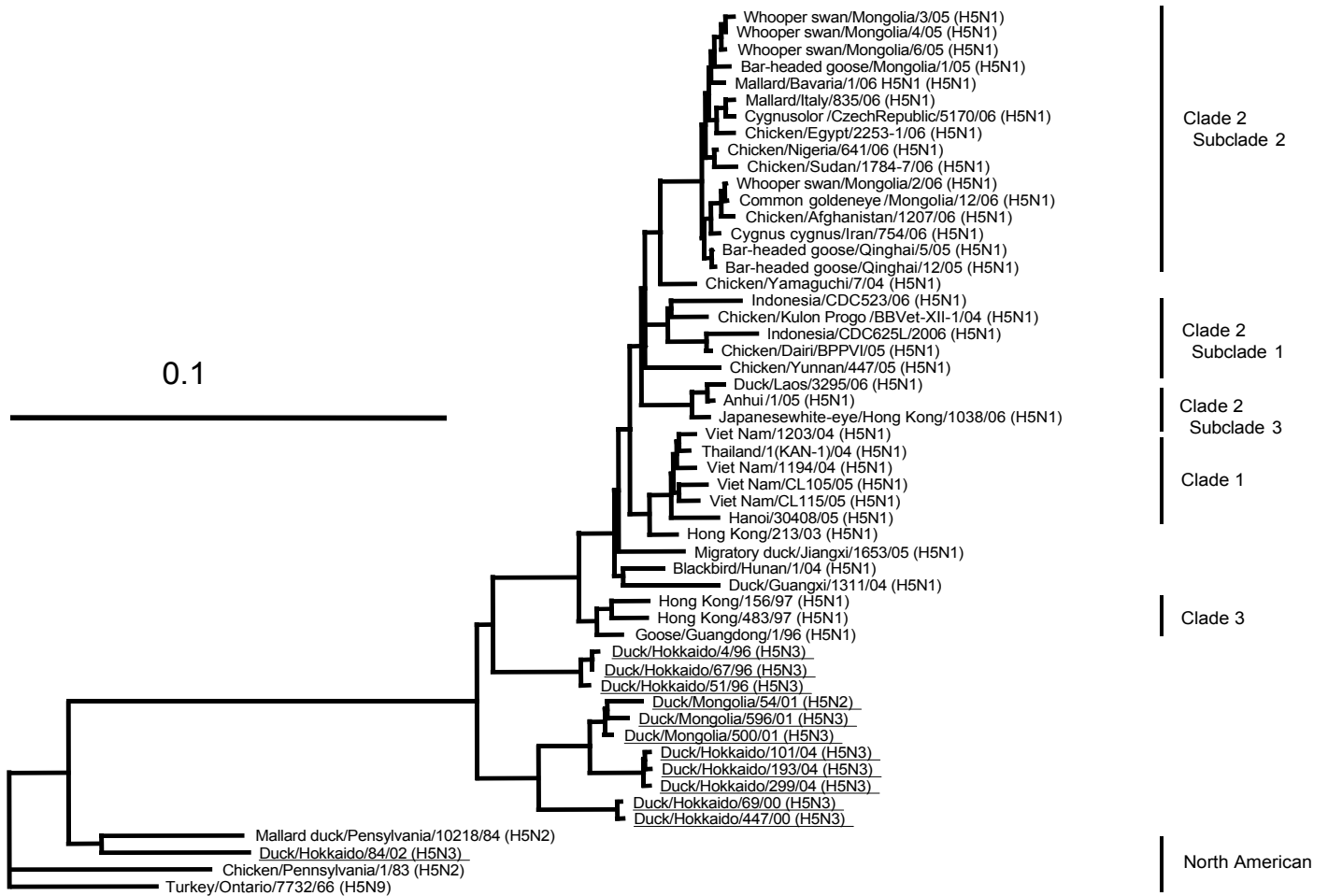
Viruses were not recovered from the swabs of any of the vaccinated chickens after challenge with Ck/Yamaguchi/04 (H5N1). However, infectious viruses were recovered from the colons of two vaccinated chickens after challenge with Ck/Yamaguchi/04 (H5N1) on day 2 p.c., although titers of viruses recovered from these birds were lower than those from non-immunized chickens. Viruses were recovered from tracheal swabs and tissues of vaccinated chickens after challenge with swan/Mongolia/05 (H5N1) at both 2 and 4 days p.c. Therefore, although our test vaccine induced high titers of HI antibodies in chickens in a short time, infectious viruses were recovered from vaccinated chickens after challenge with two HPAI viruses. **Onset of protective immunity in chickens.** Six groups of 3 vaccinated chickens and 3 non-immunized chickens were challenged intranasally with Ck/Yamaguchi/04 (H5N1) at 14, 10, 8, 6, 4, and 2 days p.v. Serum HI antibodies to Ck/Yamaguchi/04 (H5N1) were not detected in any chickens challenged at 6, 4, and 2 days p.v., and all of these birds died by day 3 post challenge (p.c.) (Fig. 2). On the other hand, all chickens challenged with Ck/Yamaguchi/04 (H5N1) at 14, 10, and 8 days p.v. survived without showing any disease signs, while serum HI antibodies were not detected in two of the three chickens challenged at 8 days p.v. These results indicated that our test vaccine required 8 days to confer effective immunity to chickens.

Acknowledgements

We extend special thanks to the National Institute of Animal Health for providing the A/chicken/Yamaguchi/7/04 (H5N1) influenza virus strain.

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Figure 1. Phylogenetic tree of the HA genes of H5 influenza viruses. Nucleotides 65-1637(1572 bases) of the H5 HA genes were analyzed.



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Isolation of Swine H1N1 Influenza Viruses Possessing Classical Swine H1 and Avian-Like N1 Genes in Thailand

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Swine husbandry is one of the significant livestock industries in Thailand. Swine influenza viruses (SIVs) of H1N1, H1N2 and H3N2 subtypes have been detected in Thailand by serological and virological surveillance conducted by National Institute of Animal Health, Thailand since 2000. Five strains of the H1N1 and one of the H1N2 subtypes, isolated from pigs with respiratory diseases, were characterized in this study. Nucleotide sequencing and phylogenetic analysis of both HA and NA genes were carried out. All of the H1 genes examined belong to the classical SIVs lineage. However, independent introductions to Thai pigs were shown as H1 genes of Thai isolates have at least two distributions within classical the SIVs lineage. In N1 phylogeny, all of the isolates examined formed a single cluster within the avian-like H1N1 SIVs lineage, suggesting that the N1 genes of Thai isolates share a common ancestor. Thus, our results demonstrated that the H1N1 SIVs used in this study have a genome contributed by both classical and avian-like H1N1 SIVs.

Introduction

Swine influenza viruses (SIVs) have been recognized throughout the world since 1918 when the Spanish flu occurred in humans [1]. Three subtypes of SIVs, H1N1, H1N2 and H3N2, have been predominantly disseminated among pig populations. For H1N1, phylogenetic analysis of H1 and N1 genes reveals three distinct lineages; a human lineage originating from human epidemic strains, and classical swine and avian lineages, respectively. The classical swine lineage has established mainly in North America and Asia; on the other hand, H1N1 avian influenza viruses entered the swine population in 1979 in Europe and have established an avian-like swine lineage since then [2]. In addition, an H3N2 virus whose internal genes are closely related to avian-like SIV circulating in Europe was also isolated from a child in Hong Kong in 1999 [3]. Swine husbandry is one of the major elements of livestock industry in Thailand, although economical impact of SIVs on swine industry has not been fully understood. SIVs cause not only economical losses, but may also cause public health concerns because pigs are considered to have the potential to act as mixing vessels of avian and human influenza viruses. Highly pathogenic avian influenza H5N1 viruses in Thailand

have caused severe economical damage on poultry industry as well as public health concerns since 2004. Since those viruses may have a potential to infect pigs, sufficient care should also be taken to prevent the emergence of new pandemics from pigs. Continuous surveillance of SIV would be effective not only from an animal hygiene point of view but also as an early detection system for SIVs with public health concern. As a part of effort for early detection of newly emerging virus among pig populations, National Institute of Animal Health in Thailand has been conducting virological and serological surveillance for SIV. Existence of SIVs of H1N1, H1N2 and H3N2 subtypes were serologically and virologically confirmed by this surveillance since 2000. However, the genetic evolution of these SIVs has not been investigated. The aim of this study is to elucidate the following: i) what is the origin of Thai SIVs? ii) what is the evolutionary relationship between Thai and other SIVs in the world? iii) what is the genetic constellation of Thai SIVs? We have sequenced the entire hemagglutinin (HA) and neuraminidase (NA) of SIVs (5 strains of H1N1 and 1 strain of H1N2 subtypes) isolated in Thailand between 2000 and 2005. Phylogenetic analysis was applied to those sequences and the evolutionary pathway was elucidated.

Materials and Methods

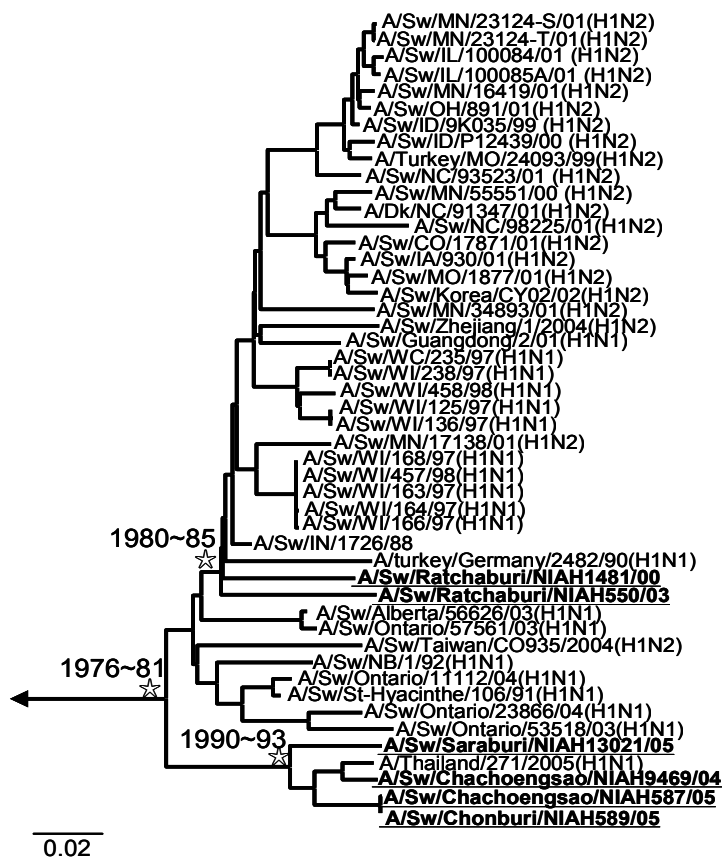
SIVs of H1N1 subtype, designated A/Sw/Ratchaburi/NIAH1481/00, A /Sw/Ratchaburi/NIAH550/03, A/Sw/Chonburi/NIAH9469/04, A/Sw/Chonburi/NIAH589/05, A/SW/Chachoengsao/NIAH587/05 and H1N2 subtype, designated A/Sw/Saraburi/NIAH13021/05 were isolated from nasal swab by inoculation into embryonated eggs. Swab samples were collected from pigs which showed clinical signs of the respiratory diseases with nasal discharge. All of the pig farms were located in the central region of Thailand. RNA was extracted from infectious allantoic fluid, followed by cDNA synthesis and PCR with H1 and N1 specific primers. PCR products were directly sequenced. Entire sequence data for HA and NA gene of Thai isolates were analyzed to identify the closest virus with sequence in the GenBank by BLAST search. Phylogenetic trees were also constructed by neighbor-joining method with the nucleotide sequences obtained in this study along with those available in the GenBank. To estimate the year of branch node for H1 and N1 genes of Thai isolates, we carried out a regression line analysis by plotting the nucleotide changes from branch point against year of isolation.

Results and Discussion

The H1 HA genes of Thai isolates were most closely related to the genes of classical swine influenza virus. H1 genes of A/Sw/Ratchaburi/NIAH1481/00 and A/Sw/Ratchaburi/NIAH550/03 showed the highest identity by BLAST search with A/Wisconsin/4755/94 (94% identity) and A/Sw/Indiana/1726/88 (93% identity), respectively [4,5]. The other four Thai isolates have the highest identity with a human isolate, A/Thailand/271/05 (93-97% identity), which also has H1 gene related to classical swine viruses [6]. Phylogenetic

analysis of entire H1 HA gene confirmed that all of the six Thai isolates including five H1N1 and one H1N2 belonged to classical H1 lineage, as indicated by BLAST analysis (Figure 1). It is apparent that two distinct evolutionary pathway exist among Thai isolates. *A/Sw/Chonburi/NIAH9469/04*, *A/Sw/Chonburi/NIAH589/05*, *A/Sw/Chachoengsao/NIAH587/05* and *A/Sw/Saraburi/NIAH13021/05* formed one cluster in classical SIVs lineage. The common ancestor of this cluster was estimated to diverge in early 1990's. On the other hand, *A/Sw/Ratchaburi/NIAH1481/00* and *A/Sw/Ratchaburi/NIAH550/03* belonged to different branches within the classical SIV lineage. Putative ancestor of this cluster was estimated to diverge in early 1980's. Hypothetical common ancestor of those six isolates diverged between 1976 and 1981, estimated by regression line analysis. Thus, existence of two distinct sublineages among Thai isolates within the classical swine H1 was demonstrated.

Figure 1. Phylogenetic Tree for H1 HA gene of classical swine lineage. Thai isolates are underlined and numbers with ★ indicate estimated years when the common ancestor diverged.



The N1 genes of Thai isolates were shown to be highly related to avian-like swine viruses circulating in European swine population by BLAST analysis. N1 genes of Thai isolates except for *A/Sw/Chonburi/NIAH9469/04* have 93-95% identity with *A/Sw/England/195852/92* [7]. The N1 gene of *A/Sw/Chonburi/*

NIAH9469/04 showed higher identity (98%) with the human isolate *A/Thailand/271/05*, which has an N1 gene related to avian-like swine viruses [6]. Phylogenetic analysis of N1 genes also revealed that all the five Thai isolates (H1N1) belonged to avian-like swine lineage currently circulating in Europe (Figure 2). In contrast to H1 genes of Thai isolates, N1 genes formed single cluster. Hypothetical common ancestor of Thai isolates possessing such N1 gene was estimated to diverge between 1988 and 1993. Phylogenetic analysis showed that the N2 gene of *A/Sw/Saraburi/NIAH13021/05* shares common a ancestor with H1N2 virus which circulated in European swine in the 1990's (Data not show). It has the highest identity (93%) with *A/Sw/UnitedKingdom/119404/91(H3N2)* [8] by Blast analysis. Thus, SIVs of H1N1 subtype currently circulating in Thai pig population possess classical swine derived H1 gene and the N1 gene of avian-like SIVs origin. Such combination of surface antigens is seldom seen in SIVs of H1N1 subtype found in other part of the world. It was suggested from our study that avian-like swine H1N1 viruses may have been introduced in to the Thai swine population around 1990. Although isolation of avian-like swine viruses in Thailand has not been reported, classical H1N1 and H3N2 subtypes were isolated from Thai swine population as early as in 1970's and 1980's [9-11]. In recent years, SIVs with a variety of genetic constellations have been reported in the region where more than two distinct SIVs are co-circulating. In Canada, swine H1N1 viruses which have PB1 polymerase gene derived from human lineage and the other genes derived from classical swine lineage were isolated between 2003 and 2004 [12]. Olsen et al. reported that triple reassortant H3N2 SIVs whose genes were derived from human (HA, NA, PB1), classical swine (NP, M, NS) and avian (PA, PB2) lineages, respectively, were isolated from Canadian swine in 2005 [13]. Identification of H1N1 SIVs with the unique combination of surface antigens in Thai pig populations adds another significant example of increasing genetic diversity of SIVs. SIVs in such regions may be in a transitional state until a highly adapted virus establishes a new stable lineage.

Conclusion

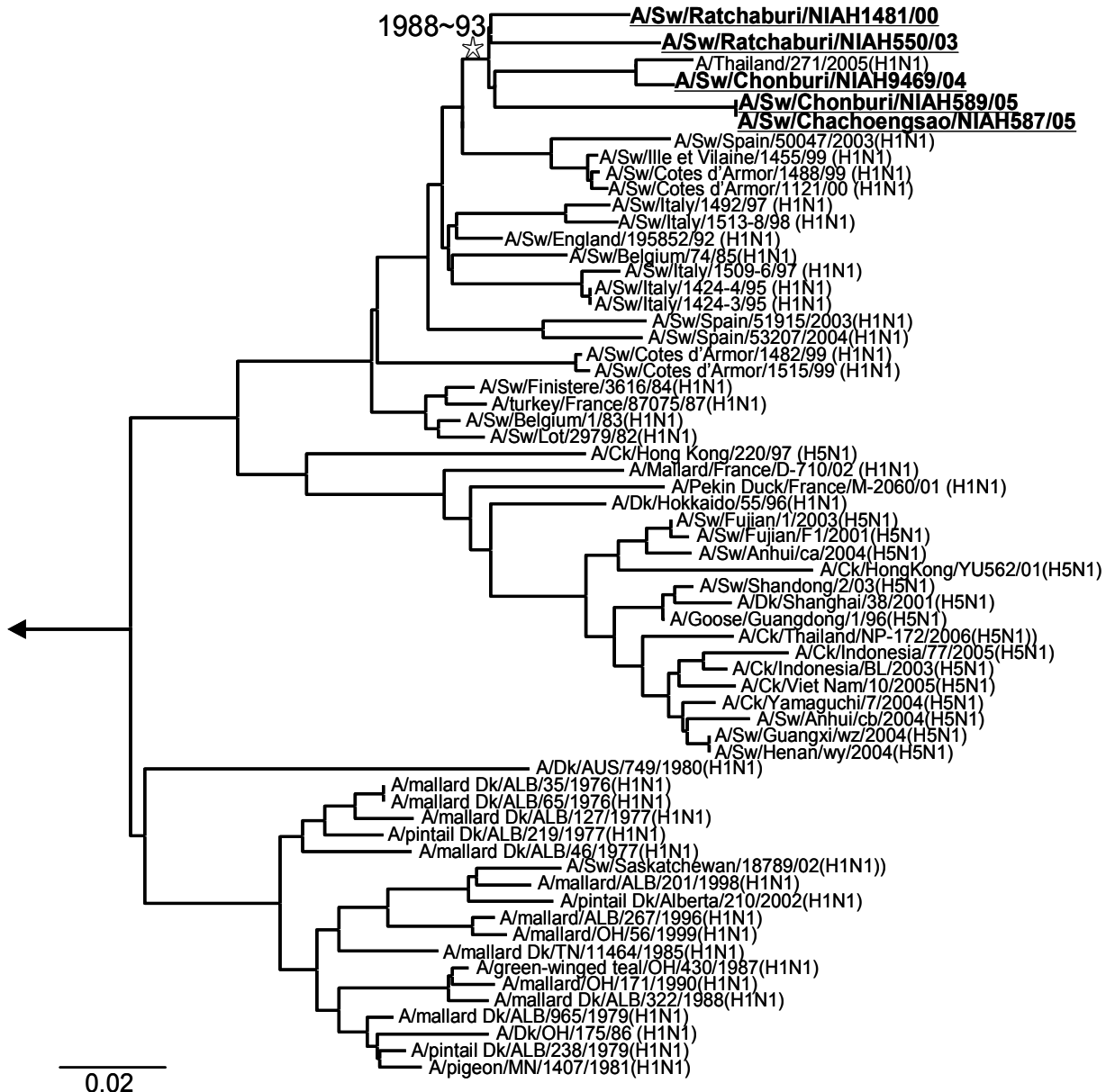
The present study firstly demonstrated that the SIVs possessing the classical swine H1 HA gene and avian-like swine N1 NA gene have circulated in Thailand. Further analysis of not only H1N1, and H1N2 subtypes but also H3N2 subtypes isolated from Thai pig populations should determine the complete genetic background of those viruses, as well as the ecology of SIVs in Thailand.

Acknowledgements

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Figure 2. Phylogenetic tree for N1 NA gene of avian virus lineage. Thai isolates are underlined and numbers with ★ indicate estimated years when the common ancestor diverged.



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New HPAI Viruses H5N1 in Russia (2006-2007 Years)

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Introduction

The first outbreak of highly pathogenic avian influenza H5N1 viruses in the Russian Federation occurred in summer 2005 in the western part of Siberia. With the beginning of the autumn migration period, the epizootic spread to the West and South-West. It spread to the Southern part of Russia, to a number of European countries, Azerbaijan, Turkey, Egypt and Nigeria, encompassing almost all territories of Eurasia. [1]. The phylogenetic analysis of the HA gene showed that viruses isolated in Russia in 2005 and in all the above-listed countries formed a cluster with H5N1 viruses isolated from wild birds during the spring outbreak in Qinghai Lake, China in 2005 [2]. Because of the high possibility and risk of a second outbreak in spring of 2006, we continued to monitor the circulation of HPAI viruses in Russia to understand the evolution of these viruses. At the beginning of the spring migration in May, 2006, deaths among domestic chickens in the western part of Siberia were reported. In the middle of June of 2006, we detected high mortality among wild birds near the border with the Mongolian Republic. H5N1 viruses were isolated also from clinical healthy wild birds in the territory of South Western Siberia in summer 2006. In the winter of 2007, there was sporadic mortality among poultry in the western part of Russia, where the highly pathogenic H5N1 influenza viruses were isolated from dead domestic birds. These viruses were also phylogenetically similar to Qinghai group of viruses (clade 2.2).

Materials and Methods

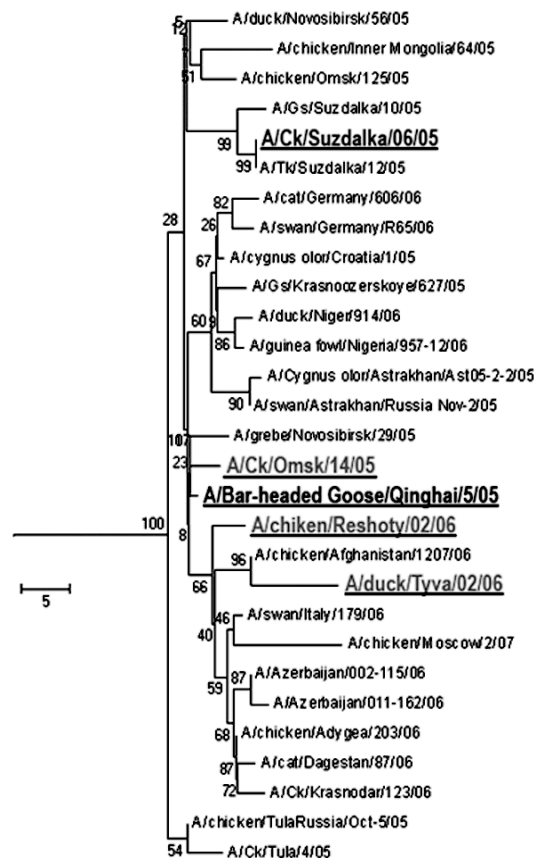
Cloacal swabs were collected from dead and sick poultry and from clinically healthy wild birds. Specimen processing and virus isolation were performed at the FSRI "Vector," a certified Biosafety Level 3 laboratory. Aliquots of field samples (0.1 mL of swab media) were injected into the allantoic cavity of 10-day-old, specific pathogen-free embryonated chicken eggs. After incubation at 35°C for 48 h, the allantoic fluid was harvested, and virus was titrated by hemagglutination test with a 0.5% suspension of chicken red blood cells. Virus-containing allantoic fluid was stored at -80°C. The subtype of the hemagglutinin (HA) was determined by hemagglutination-inhibition test with 0.5% chicken red blood cells and a panel of antiserum against avian HAs [3]. The neuraminidase (NA) subtype was determined by NA inhibition assay with a panel of anti-NA serum [3]. Diagnostic sera were kindly provided by Dr. R.G. Webster (St Jude Children's Research Hospital, Memphis,

Tennessee, USA). PCR amplification and sequencing and phylogenetic analysis were performed as described previously [4].

Results

The surveillance identified mortality among domestic chickens in the western part of Siberia at the beginning of spring migration in May of 2006. We isolated two H5N1 influenza viruses that were phylogenetically similar to viruses isolated in Qinghai Lake in 2005. The analysis of flyways and timing of poultry deaths suggested that these viruses did not spread from China but were introduced from the Near East or southern part of Russia. Despite analysis of more than 800 samples taken from wild birds of different species during the spring migration 2006, no other subtypes other than the H5N1 subtype were isolated. In the middle of June of 2006 we detected high mortality among wild birds on Ubsu-Nnur Lake which is situated near the border with the Mongolian Republic. Ornithologists determined that about 4000 birds died; the majority of deaths occurred in the species *Podiceps cristanus*. We also isolated H5N1 highly pathogenic influenza viruses from dead birds that were phylogenetically similar to viruses from Qinghai group. The analysis of birds' migration flyways and timing of bird deaths suggested that this virus spread from China.

Figure 1. Phylogenetic analysis of HA genes of H5N1 viruses isolated in spring-summer 2006.



The surveillance for influenza viruses in wild birds that was carried out in the territory of South Western Siberia in summer 2006 uncovered further interesting features of the HPAI H5N1 epizootic in Russia. We isolated some H5N1 viruses from clinically healthy wild birds belonging to different species; all the isolated viruses were highly pathogenic for chickens (Table 1; data not shown).

Table 1. Isolation of HPAI H5N1 viruses from clinically healthy wild birds (Chany Lake, Novosibirsk Region, Russia, June 2006).

Bird species	Number of HPAI H5N1 isolates
<i>Larus ridibundus</i> (Common gull)	3
<i>Sterna hirundo</i>	3
<i>Aythya ferina</i>	3
<i>Ardea cinerea</i>	1
Total number of HPAI H5N1 isolates	10
Number of samples taken	84

Comparative antigenic analysis performed by HI assay determined that the H5N1 viruses isolated from the Novosibirsk Region were antigenically similar to HPAI H5N1 viruses that circulate in southeastern countries. Genetic characterization of the viruses determined that they belonged to the Qinghai genotype circulating in the world since 2005. The isolation of HPAI H5N1 viruses from clinically healthy birds suggests that these wild birds may act as asymptomatic carriers of these viruses. During the autumn migration there were no deaths among wild or domestic birds in Russia detected. However in the winter of 2007, there was sporadic mortality among poultry in the western part of Russia, specifically in Moscow Region, Krasnodar and Adygea Republic. Highly pathogenic H5N1 influenza viruses were isolated from dead domestic birds; these viruses were also phylogenetically similar to the Qinghai group of H5N1 viruses. The epizootic situation in territories where poultry outbreaks took place suggest that the bird deaths in south western regions of Russia (Krasnodar territory, Adygea Republic) may have been caused by virus spread by migrating birds that have over-wintered in these territories. The Moscow Region, epizootic was probably caused by importation of birds from epizootic regions.

Conclusion

Outbreaks of H5N1 disease in wild and domestic birds in Russia are the result of the spread of H5N1 viruses to the Russian Federation by migratory birds from China in 2005. Preliminary findings from our analysis of samples from humans exposed to ill or dead birds has not found serological evidence for H5N1 influenza virus infection among people.

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Detection of Influenza Virus in Wild Birds by RT-Nested PCR in West and East Slovakia

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Introduction

In April 2006, a total of 33 wild birds were caught in National Park (NP) - Senianske ponds in east Slovakia. In June 2006, a total of 42 wild birds of 11 free living species were caught in NP - Parizske mociare Marsh, one of the largest wetlands in west Slovakia, located near to the villages Gbelce and Nova Vieska. Throat and cloacal swabs were examined for the presence of avian influenza virus (AIV) by RT-nested PCR. In east Slovakia, 13 samples obtained from throat and 11 samples obtained from cloaca were found positive for influenza virus. Total 18 birds, what represents 54%, were found positive for influenza virus in east Slovakia. The influenza virus was detected in 8 individuals in cloacal swabs, and in six causes in throat swabs obtained in Jun. Total 11 birds, what represents 26%, was found positive for influenza virus in west Slovakia. Introduction: In Europe, the migratory routes of birds are more diverse than in North America. Two of dominant European north-south and east-west birds' migratory routes pass across Slovakia. In Europe, several studies have been done concerning the role of birds as distributors of several pathogenic agents. In most cases, the routine testing of samples obtained from wild birds will find some influenza viruses. The vast majority of these viruses cause no harm to their carriers. Only after transmission of the virus from wild birds to domestic poultry and subsequent circulation in the poultry populations, the low pathogenic avian influenza viruses may mutate into high pathogenic avian influenza viruses. The minor genetic and antigenic diversity between the viruses recovered from wild birds and those causing high pathogenic avian influenza outbreaks indicate that influenza A virus surveillance studies in wild birds are indeed useful to design and evaluate diagnosis tests and to generate the prototypic vaccine candidates, prior to the occurrence of outbreaks in animals and humans [1].

Materials and Methods

Collection of samples. Birds were captured in the National Park - Senianske ponds in Eastern Slovakia and in National Park Parizske Mociare, one of the largest wetlands in West Slovakia, located near the villages of Gbelce and Nová Vieska. Captured birds were recognized, ringed, the cloacal and throat samples were collected. The birds were not harmed or killed. **RNA isolation.** Cloacal and throat swabs

and fecal samples were thoroughly blended and extracted in 3 ml of phosphate-buffered saline, pH 7.2. 100 µl of the extract was used for purification of RNA, using the RNeasy Mini kit (Qiagen) following manufacturer's recommended protocol and extracted RNAs were stored at -20°C. **RT-nested-PCR.** cDNAs of viruses were synthesized by reverse transcription of viral RNA with random oligonucleotide primers. The first PCR was done by using the primers against conserve region of M gene as described by Betakova et al. [2]. The second PCR was done as described by Gronosova et al. [3].

Results and Discussion

Throat and cloacal samples were collected from wild birds and analyzed for the presence of AIV. All water or near water living birds belonged to the orders *Charadriiformes* and *Passeriformes* (*Motacilla flava*). The other species represented forest dwelling birds or birds in other terrestrial habitats and belonged to the orders *Passeriformes* and *Cuculiformes*. In Table 1 are listed species belonging to the order of *Passeriformes*, *Charadriiformes*, and *Cuculiformes*, which were caught in east Slovakia. 13 samples obtained from throat and 11 samples obtained from cloaca were found positive for influenza virus. Only in 5 causes were the samples positive in throat and cloaca. Total 18 birds, what represents 54%, were found positive for AIV. In the table 2 are listed species of birds caught in west Slovakia. All species belong to the order *Passeriformes*. The AIV was detected in 8 individuals in cloacal swabs, and in six causes in throat swabs. Throat and cloacal swabs were positive only in three birds. Total 11 birds, what represents 26%, was found positive for AIV in west Slovakia. The results (Table 1 and 2) showed that the virus was rarely detected in both throat and cloacal samples. The majority of positive samples were obtained from cloaca. These results do not correspond with previous findings, where the throat swabs were more suitable for detection of AIV in the chicken infected through the intranasal route of inoculation [4, 5]. The presence of AIV in chicken infected through the intranasal route of inoculation was possible to prove in throat swabs one day after inoculation [5]. In the case of chicken infected through the oral and contact-exposed routes, the virus was detected only after 2 or 3 days post infection, respectively [5]. It was shown that AIV can be detected from throat swabs for at least 6 days p.i. [6]. However, the data from cloacal swabs are missing. Moreover, only a little is known about replication and spreading of AIV in wild birds. Our results show that the optimal results are obtained when throat and cloacal samples are tested simultaneously. Use of a nested PCR increased the sensitivity of the virus detection by simple PCR reported previously [1, 7]. Previous studies of other authors have reported 9.9–10.5% of PCR positive samples collected from wild birds [8, 9] and considerable variation of positivity among different species [9]. It is obvious that the percentage of positive samples depends on i) sensitivity of the methods used for detection of the virus, ii) from the birds species, and iii) from the season. Monitoring AIV is a good way to anticipate human influenza outbreaks. Coordinated surveillance of influenza in

humans and animals is needed, and the human and veterinary surveillance systems should be linked to exchange information, diagnostic tools and antigens. The surveillance of AIV can play a key role in the early identification and ongoing monitoring of a pandemic influenza virus as well as the annual epidemics.

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Table 1. Detection of AIV in the samples collected in April 2006.

Order	Species	No. of tested birds	No. of positive samples from		No of positive birds
			Throat	Cloaca	
Passeriformes	<i>Sternus vulgaris</i> (common starling)	1	0	0	0
	<i>Motacilla flava</i> (citrine wagtail)	1	0	0	0
Charadriiformes	<i>Actitis hypoleucos</i> (common sandpiper)	1	0	0	0
	<i>Charadrius dubius</i> (little ringed plover)	1	0	1	1
			2	2	
	<i>Larus ridibundus</i> (black-headed gull)	11	3	0	5
	<i>Numenius arquata</i> (european curlew)	2	2	0	2
			0	3	
	<i>Phlomis pugnax</i> (ruff)	9	2	2	7
			2	0	
	<i>Tringa erythrorus</i>	1	1	1	1
	<i>Tringa nebularia</i> (greenshank)	1	1	0	1
	<i>Tringa totanus</i> (common redshank)	1	0	0	0
	<i>Gallinago gallinago</i> (common snipe)	2	0	0	0
	<i>Lymnocyrtus minutus</i> (jack snipe)	1	0	1	1
Cuculiformes	<i>Cuculus canorus</i> (common cuckoo)	1	0	1	1
Total		33	13	11	19
%		100	39,4	33,3	57,6

Table 2. Detection of AIV in the samples collected in June 2006.

Order	Species	No. of tested birds	No. of positive samples from		No of positive birds	
			Throat	Cloaca		
Passeriformes	<i>Hirundo rustica</i> (swallow)	3	0	1	1	
	<i>Locustella luscinioides</i> (Savi's warbler)	3	0	0	0	
	<i>Acrocephalus melanopogon</i> (moustached warbler)	2	1	1	1	
	<i>Acrocephalus schoenobaenus</i> (sedge warbler)	2	0	0	0	
	<i>Acrocephalus scirpaceus</i> (reed warbler)		10	1	1	3
				1	0	
				0	1	
				0	0	
	<i>Acrocephalus arundinaceus</i> (great reed warbler)	2	1	0	1	
	<i>Pyrrhuloxia collybita</i> (chiffchaff)	1	0	0	0	
	<i>Parus biarmicus</i> (bearded tit)	7	1	0	1	
	<i>Parus caeruleus</i> (blue tit)	3	0	1	1	
	<i>Sternus vulgaris</i> (starling)	3	0	0	0	
				0	2	
<i>Emberiza schoeniclus</i> (reed bunting)	6	1	1	3		
Total		42	6	8	11	
%		100	14,2	19	26,2	

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Surveillance for Avian Influenza in Wild Birds in New Zealand

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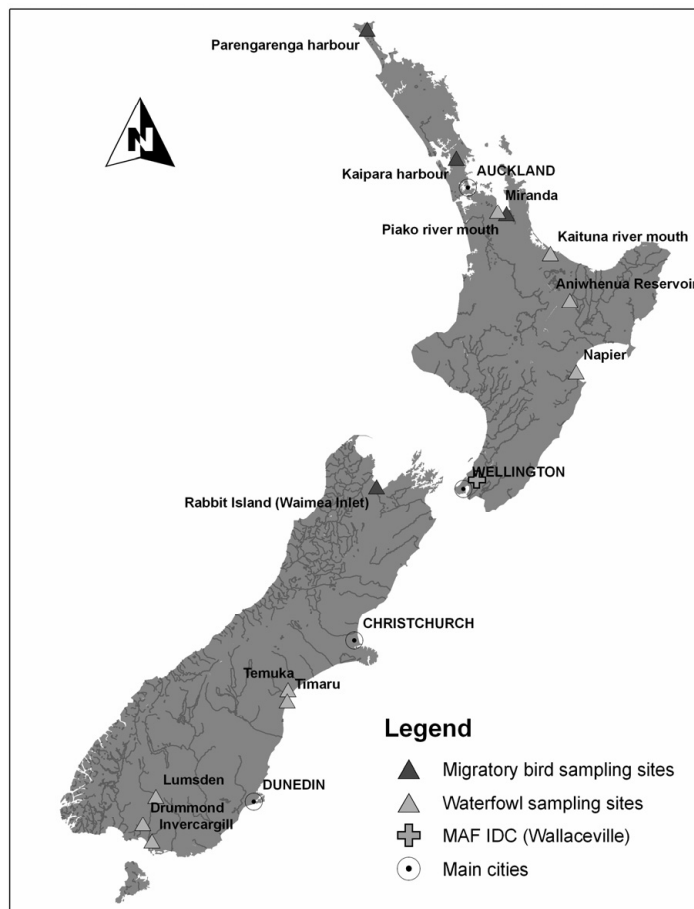
Introduction

The spread of strains of highly pathogenic avian influenza (HPAI) H5N1 around the world has prompted Biosecurity New Zealand to expand its surveillance programmes to include the avian influenza (AI) status of resident waterfowl and migratory shorebirds arriving in New Zealand. New Zealand lies at the southeastern extremity of the East Asian-Australasian Flyway, and because of its geographical isolation relatively few Arctic-breeding migratory shorebirds reach the country. In total, about 200,000 birds representing 47 species arrive but the vast majority of these comprise only three species: Bar-tailed Godwits (*Limosa lapponica*), Red Knots (*Calidris canutus*), and Ruddy Turnstones (*Arenaria interpres*) [10]. Godwits are believed to fly directly to New Zealand [6], knots have stopovers in East Asia [12], and turnstones have a number of stops in the Pacific [19] before they reach New Zealand. For these birds to introduce avian viruses to New Zealand, they would have to be infected before or during migration. On arrival, they could then shed the virus whilst co-habiting with endemic shorebirds, gulls, waterfowl, and other species and thereby introduce infection. Wild birds, particularly waterfowl such as ducks – Mallard (*Anas platyrhynchos*), Grey Duck (*Anas superciliosa*), Paradise Duck (*Tadorna variegata*), geese and swans are the natural reservoir for avian influenza viruses (AIV). However, waterfowl do not migrate to New Zealand (except singly or in very small numbers) [22] so they present little threat.

Materials and Methods

Sample collection. Between 2004 and 2006, 834 cloacal swabs were collected from migratory knots (661), godwits (159), and turnstones (14), upon arrival in New Zealand. Most of these samples were collected at Miranda and the Firth of Thames in the North Island, during late September through November, but samples were also collected from birds in Northland, and at Rabbit Island, Nelson in the South Island (Fig. 1). Birds were trapped using cannon and mist nets by Ornithological Society personnel. An additional 1754 cloacal swabs were collected from waterfowl caught by Regional Fish and Game Council personnel using wire mesh cage traps in coastal areas in the summer (December–February) (Fig. 1; Table 1), and another 583 blood samples were collected from mallard ducks. Cloacal swabs and blood samples were collected as described in Stanislawek et al., 2002 [16].

Figure 1. New Zealand sites where birds were captured for sample collection.



Virus isolation. Cloacal swab samples were placed into transport medium and then stored at -70°C . Swabs were then thawed, pooled into samples of three, and tested for the presence of haemagglutinating viruses by inoculation into the allantoic sac of embryonated specific-pathogen-free fowl eggs using established procedures [1]. All allantoic fluid samples with haemagglutinating activity (HA) were tested using Influenza A real time RT-PCR TaqMan [14] to confirm the presence of avian influenza virus. The haemagglutinin glycoprotein subtypes of all AIV isolated were determined using the haemagglutination inhibition test [1] with reference antisera to all 16 known subtypes.

Influenza A real time RT-PCR TaqMan, matrix H5 and H7 RT-PCR. RNA from cloacal swab samples or from infectious allantoic fluid were extracted after clarification at 1000g for 10 min using Qiagen (QIAamp Viral RNA Mini Kit) using the manufacturer's procedure. Influenza A real time RT-PCR (RRT-PCR) TaqMan was performed using primers and probe developed by Spackman et al. [14]. Primers for conventional influenza A PCR for matrix gene and H5 and H7 were used as described by Ellis et al. [4] and Senne et al. [13] respectively. Sequencing and sequencing analysis were performed as described in Stanislawek et al. 2002, using PCR primers [16].

Haemagglutination inhibition test (HI) for H5 and H7 antibodies.

An HI test was performed on duck sera using the method described by Alexander (2004) [1] using inactivated H5N3 and H7N3 antigens. Results were recorded for all samples with titres $\geq 1:4$.

Results

Migratory shorebirds. No AIVs were isolated from cloacal swabs from migratory shorebirds. However, in 2006, AI viral RNA was detected by influenza A RRT-PCR, and confirmed using nested matrix PCR, in two samples collected from Lesser knots. When the sample matrix genome (740bp) sequences obtained from the above were compared to sequences deposited in GenBank, they had 96.5% identity to the matrix protein genome sequences of AI A/chicken/Hubei/wk/1997, an H5N2 virus. In this study, no H5 viruses were identified, and subtypes H5 and H7 were excluded in any matrix AI PCR positive sample using specific RT-PCRs. **Waterfowl.** Thirty (30) AIVs (H1, H2, H4, H10, and H11 subtypes) was isolated from resident waterfowl (Table 1). A total of 103/584 sera reacted with H5 HI antigen with titres between 1:4 and 1:128 and 24/584 reacted with H7 HI antigen with titres between 1:4 and 1:32.

Table 1. Avian influenza surveillance results for Waterfowl in New Zealand (2004-2006).

Year	Species	Location (Island)	No. of cloacal swabs collected	No. of Avian influenza viruses isolated (subtypes)	No. of blood samples collected	No. of samples with HI titres between $\geq 1/4$ and $1/128$	
						H5	H7
2004	Mallard and Paradise ducks	North	159	13 (H1, H2, H4, H11)	86	1	0
		South	222	1 (H2)	130	15	4
2005	Mallard	North	360	5 (H1, H4, H10)	90	4	2
		South	445	3 (H1, H4)	178	68	16
2006	Mallard	North	568	8 ^a (subtypes to be determined)	100	15	2
		South	0	0	0	0	0
Total			1754	30	584	103^b	24^b

a = Avian influenza subtypes H5 and H7 were excluded by subtype specific RT-PCR.

b = Further testing with H5 and H7 antigens with different neuraminidase is required to determine the specific haemagglutinin reaction.

Discussion

To date, no pathogenic AIVs have been isolated from New Zealand birds though pathways for potential entry such as in illegal imports or migratory wild birds do exist. The epidemiology of AIV in shorebirds is poorly understood. Regular isolation of AIV from shorebirds in the USA, especially in spring [9], stands in marked contrast with a general absence of AIV in Europe [5, 7, but see 17]. AIVs have rarely been isolated from shorebirds in the East Asian-Australasian Flyway [11], but Chen et al. [3] reported seropositive shorebirds on southbound migration near Shanghai, and Hurt et al. [8]

reported low pathogenic AI (LPAI) from shorebirds in Australia. LPAI has been found in three species in Alaska, including bar-tailed godwits [20], but even if godwits were infected with LPAI before departing Alaska it remains uncertain whether infected birds could complete an 11,000 km non-stop flight to New Zealand – LPAI infection of swans significantly reduced their migratory performance [21]. Knots and turnstones undertake shorter distance flights than godwits and they could potentially become infected with exotic influenza viruses during stopovers on their trip to New Zealand. No viruses have been found in these birds upon arrival in New Zealand, but the sample size has been small and possibly below an acceptable survey design prevalence. Only two samples collected from migratory shorebirds contained viral RNA consistent with influenza A. However, no AIV was isolated indicating that, currently, these species are not a likely means of introducing AIVs to New Zealand. The significance of the influenza A RNA detected in samples collected from knots should be investigated further by analysing sequences of other genes. Sequencing currently circulating AIVs in New Zealand bird populations will build up information on these viruses and provide us with greater confidence that any viruses isolated from or detected in migratory birds is likely to be a new introduction. Although all viruses isolated from waterfowl were non-notifiable LPAI, previous studies have reported low pathogenic strains of H5 and H7 in New Zealand [16, 18]. The HI titres obtained in sera when tested with H5 and H7 antigens are difficult to interpret because they could represent a cross-reaction with the neuraminidase antigens. Therefore, further work using H5 and H7 antigens with different neuraminidase is required to confirm these results. The risk of HPAI viruses entering and becoming established in New Zealand via migratory birds is considered to be very low. However, monitoring of shorebirds and waterfowl will continue.

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Influenza Viruses in Healthy Wild Birds in Hong Kong

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Wild waterfowl are considered the natural reservoir of influenza A viruses. The recent outbreak of highly pathogenic avian influenza (HPAI) H5N1 in Asia which has now spread as far as Africa highlights the importance of defining the influenza virus gene pool in these birds and understanding the potential role played by migratory waterfowl in such HPAI outbreaks. Seventy-three influenza viruses were isolated from 16,724 samples collected from feral waterfowls or their fecal droppings during 2003-7 at Mai Po Marshes and Lok Ma Chau in Hong Kong. A diversity of influenza viruses representing hemagglutinin subtypes of H1, H2, H4, H5, H6, H7, H8, H9, H10 and H11; neuraminidase subtypes of N1, N2, N3, N4, N6, N7, N8 and N9, were isolated. Seventy-two of these 73 positive isolates were collected during the winter period coinciding with the southern migration of waterfowl along the East Asian flyway. No HPAI H5N1 viruses were isolated from healthy birds sampled in this study, though H5N1 viruses have been isolated from dead wild birds found in Hong Kong. Phylogenetic analyses of the HA gene of the H5 viruses isolated in the study showed that they clustered with other LP H5 viruses isolated from Hokkaido, Mongolia and Siberia but they seemed not to be very closely related to the HP H5N1. Six of 150 blood sample collected from wild ducks and one of 43 from shorebirds were tested to have antibody by neutralization tests for H5 subtype hemagglutinin.

Introduction

Influenza viruses from wild aquatic birds contributed to the genesis of the pandemics of the last century which then led to significantly global mortality. For example the "Spanish flu" pandemic in 1918 is estimated to have killed over 25 millions of people [1]. It is suggested that the 1918 pandemic virus was a purely avian virus that adapted to efficient human-to-human transmission [2]. The pandemic viruses of 1957 and 1968 also acquired three or two gene segments from wild birds, respectively [3]. Reconstruction of the sequence of the hypothetical ancestral strain at the avian-human transition indicated that only six amino acids in the mature hemagglutinin molecule had been changed during the transition between the avian virus strain and the human pandemic strain [4]. More recently, a highly pathogenic avian influenza H5N1 virus has spread to affect poultry in a number of countries in Asia and beyond. This virus transmits zoonotically to humans, albeit inefficiently [5]. However, when it does, it often causes a fulminant viral pneumonia with high case-fatality. In some instances, wild birds and waterfowls are implicated in the spread of this infection to poultry. Studies in Siberia and Hokkaido

(Japan)[6] have indicated that the NP gene of H11N9 viruses isolated from ducks in Kobayasky area in 1996 forms a cluster with that of the highly virulent H5N1 influenza viruses isolated from chickens and humans from 1997. This supports the contention that wild birds are the ultimate reservoir of the gene pool of influenza viruses. Influenza virus surveillance of wild birds, especially migratory birds, is therefore of vital importance for both animal and human health.

Materials and Methods

Routine sampling of the fecal droppings were carried out 2-3 times per week at designated areas in Mai Po Nature Reserve and Lok Ma Chau of Hong Kong during the waterfowl migratory months (October – March). The Mai Po Nature Reserve is one of the sites along the East Asia-Australian flyway where migratory waterfowls stop over and congregate during autumn – spring (Oct –Mar) each year. The largest group of wild waterfowl consist of ducks and grebes and a peak of around 30,000 of this species were counted each year in Hong Kong [7]. Trapping of birds was also done twice a month during the same period, depending on the number of birds present at the trapping site and the weather. Tracheal and cloacal swabs together with blood samples were collected from the trapped birds. Samples were inoculated into 9-10 day old embryonated eggs and positive isolates were subtyped using standard antisera by the hemagglutination inhibition test and one-step RT-PCR (8). Sequencing was performed by BigDye Terminator V3.1 cycle sequencing kit on ABI PRISM 3700 DNA analyzer (Applied Biosystems). All sequence segments were assembled and aligned by BioEdit version 7. A phylogenetic tree was generated by neighbor-joining bootstrap analysis (1,000 replicates) using the Kimura 2-parameter model in MEGA, version 3.1. The sera collected from trapped birds were tested for H5 antibodies using the microneutralisation test.

Results

During the 4-year surveillance period from Sep 03 to Feb 07, influenza viruses were isolated from 73 out of 16,724 (0.43%) fecal dropping samples collected from wild migratory waterfowls. These viruses were of hemagglutinin subtypes H1, H2, H4, H5, H6, H7, H8, H9, H10 and H11; neuraminidase subtypes N1, N2, N3, N4, N6, N7, N8 and N9. (Table 1). Seventy-two of these 73 positive isolates were collected during the winter period coinciding with the southern migration of waterfowl along the East Asian flyway. Nevertheless, none of the water samples collected from the ponds where these birds congregate was found to be positive for the virus. For non-waterfowl, none of the 6499 samples collected was found to be positive for avian influenza during the same study period in 2003-06. The H5 and H7 subtype isolated from these fecal droppings of apparently healthy birds were low pathogenic influenza viruses. No HPAI H5 viruses were isolated in this study. Phylogenetic analyses of the HA gene of the LPAI H5 viruses isolated in the study revealed that these viruses clustered with other LPAI H5 viruses isolated from Hokkaido, Mongolia

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and Siberia. They were phylogenetically distinct from HPAI H5 viruses with amino acid homology of only 90%. Six out of 150 blood samples collected from wild ducks and one out of 43 blood samples from shorebirds, revealed neutralisation of LPAI H5N3 in microneutralisation assay providing evidence of circulation of these viruses in shore birds as well as in wild ducks.

Table 1. The number of different hemagglutinin and neuraminidase subtypes isolated from wild migratory birds in Hong Kong Sep 03-Feb 07.

Hemagglutinin Subtype	No. of isolates	Neuraminidase subtype	No. of isolates
H1	3	N1	11
H2	2	N2	10
H4	16	N3	15
H5	6	N4	7
H6	10	N6	16
H7	3	N7	3
H8	7	N8	5
H9	1	N9	6
H10	20		
H11	5		

Discussion

To date, 16 HA and 9 NA subtypes have been identified in wild birds [9-11] belonging to at least 90 species representing twelve different orders [12]. The aquatic birds of the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, surfbirds, sandpipers) are believed to be the main reservoirs of all of these different subtypes of influenza A viruses [13]. Our findings have revealed that a diversity of avian influenza virus exist in aquatic birds in Hong Kong. On the other hand, the non-aquatic birds did not yield virus isolates. As only LPAI H5 can be isolated from these aquatic birds in our surveillance study and only a low percentage of birds have LPAI H5 antibody, we have no proof that HPAI H5 viruses are endemic in the migratory bird populations in this region. While this surveillance program in apparently healthy wild birds yielded negative results for HPAI H5N1, surveillance of dead wild birds carried out by the Agriculture, Fisheries and Conservation department of Hong Kong regularly identifies some birds with HPAI H5N1 infection. However, these positives are not all, or even predominantly obtained in migratory bird species. Although the poultry farms within Hong Kong have remained free of HPAI H5N1 since 2004, it remains possible that these wild birds picked up infection from infected poultry in the wider region. One limitation of this study is that most swabs tested were fecal droppings. Since recent studies suggest that viral titers of HPAI H5 viruses were higher in the tracheal rather than cloacal swabs (Osterhaus A – personal communication), we may have under-estimated the presence of HPAI H5N1 viruses. Phylogenetic analysis of the HA gene of the LPAI H5 viruses in this study has revealed that they clustered together with those viruses from Hokkaido (Japan), Mongolia and Siberia. This demonstrated that there is a dynamic “mixing” of viruses longitudinally along the East Asian Pathway; as suggested previously [14]. The wide diversity of hemagglutinin

and neuraminidase subtypes identified from wild waterfowl within a rather small geographic region re-emphasizes the diversity of influenza viruses in these species. Since the previous pandemics of 1957 and 1968 did not arise from highly pathogenic avian influenza viruses, it remains important to maintain surveillance of LPAI influenza subtypes as well.

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Proceedings Topic #7

Clinical Vaccine Evaluation

**Poster
Presentations**

Antibody Responses to Influenza Vaccination in Adults and Elderly in Taiwan, 2006

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The aim of this study was to assess antibody responses against influenza viruses between adults and elderly who were vaccinated with influenza vaccine (GlaxoSmithKline) at the end of 2006. A total of 120 sera (including pre-vaccination and post-vaccination) were collected from 30 adult and 30 geriatric volunteers, respectively. For each individual, blood was collected before and after vaccination at an interval of three weeks. Antibody titers were assayed by hemagglutination inhibition (HAI) test against local circulating strains, A/Taiwan/0586/2006(H1N1), A/Taiwan/083/2006(H3N2) and B/Taiwan/0050/2006. The antibody titers of both groups were analyzed by geometry mean titers (GMTs), seroconversion rates, seroconversion factors and seroprotection rates, according to the criteria of guidelines of the European Committee for Proprietary Medicinal Products (CPMP). The results indicated that the GMTs of both groups were significantly increased after vaccination. The seroconversion factors indicated that the immune status of both groups fitted almost the criteria of guidelines of the European CPMP, except that against A/Taiwan/0586/2006(H1N1) in adults. Similarly, the seroconversion rates of elderly were greater than 30% while the rates of adults were greater than 40%, except that against the B/Taiwan/0050/2006. The seroprotection rates of both groups ranged from 70 to 100% after vaccination. In conclusion, our results indicated that the influenza vaccination program could provide a protective immunity in adults and elderly in our country.

Introduction

Influenza is one of the most important respiratory diseases that infect human globally [13]. In the last century, three pandemics occurred and caused several million people deaths [10,15]. Influenza epidemics also occurred in many part of the world annually. In the last decade, an average of 36,000 deaths occurred in the United States each year and up to 10-20% of the general population may have been infected by influenza virus each year. Populations are high risk for influenza-related complications include persons over the age of 65 and those under 3 years old [1,2,6,7]. However, in the case of 1918 Spanish flu, young adults were are high-risk of death after infection with the influenza virus [10]. By following

the trends of former pandemics, some scientists forecasted that new pandemics might happen at an interval of ten years, although this prediction has not been borne out. After the first outbreak of avian influenza that directly infected human beings in 1997, scientists warned that a pandemic might occur. From 2005 onwards, outbreaks of avian influenza have been reported throughout Asia and Europe. Thus, studies on evolution, pathogenesis, immunity, development of antiviral drugs and vaccines against influenza virus, and the efficacy of the influenza vaccination, have all become important issues as the world prepares for the next influenza pandemic [4,5,8,9,16-20]. Influenza vaccination campaigns are one of the most important strategies of public health in Taiwan government. People who are older than 65 years old, or younger than 3 years old, and those who are working in hospital are recommended to receive annual influenza vaccination. The aim of this study was to assess antibody responses against influenza viruses between adults and elderly who were vaccinated with influenza vaccine at the end of 2006.

Materials and Methods

Influenza vaccine, which was manufactured by GlaxoSmithKline Company, was provided by Taiwan Centers for Disease Control. Total 120 sera (including pre-vaccination and post-vaccination) were collected from 30 adult and 30 geriatric volunteers, respectively. For each individual, 10 mL of whole blood was collected before and after influenza vaccination at an interval of three weeks. After serum isolation, antibody titers were assayed by hemagglutination inhibition (HAI) test against local circulating strains, A/Taiwan/0586/2006(H1N1) (A/New Caledonia/20/99-like), A/Taiwan/083/2006(H3N2) (A/Wisconsin/67/2005-like) and B/Taiwan/0050/2006 (B/Malaysia/2506/2004-like), by following the standard protocol of World Health Organization [19]. The hemagglutination titers of each virus antigen was ranging from 1:64 to 1:512. The antibody titers of both groups were analyzed by statistical means and represented as geometric mean titers (GMTs), seroconversion rates, seroconversion factors and seroprotection rates, according to the criteria of guidelines of the European Committee for Proprietary Medicinal Products (CPMP) [3].

Results

Thirty adults (including 19 women and 11 men) and 30 elders (including 11 women and 19 men) participated in this project. The ages of adult group ranged from 26 to 47 years old while the ages of elderly group ranged from 60 to 77 years old (Table 1) The antibody titers of both groups were analyzed by geometric mean titers (GMTs), and the GMTs of both groups were significantly increased after vaccination (Table 2). The seroconversion factors indicated that the immune status of both groups fitted almost the criteria of guidelines of the European CPMP, except that against A/Taiwan/0586/2006(H1N1) in adults (Seroconversion factor = 2.4). In addition, both groups exerted a high value of the factor against A/Taiwan/083/2006(H3N2)

(Table 3). Similarly, the seroconversion rates of elderly against the three subtypes of the local strains of influenza viruses were greater than 30% while the rates of adults were greater than 40%, except that against the B/Taiwan/0050/2006. The seroprotection rates of both groups ranged from 70 to 100% after vaccination.

Table 1. Demographic characteristics of the subjects.

Group	No. of subjects	Age (yr)	
		Mean±SD	Range
Adult			
All subjects	30	35.2±6.6	26-47
Women	19	36.4±7.0	27-47
Men	11	33.3±6.1	26-45
Elder			
All subjects	30	67.7±4.2	60-77
Women	11	67±3.5	60-72
Men	19	68.1±4.6	60-77

Table 2. Geometric mean HAI antibody titers before and after vaccination of adult and elders.

	Adult group (N=30)	Elders group (N=30)
Geometric mean titer	value (95% CI)	
A/Taiwan/0586/2006(H1N1), (A/New Caledonia/20/99-like)		
Before vaccination	69.6 (44.4-109.5)	23 (16.3-32.5)
After vaccination	163.7 (107-239)	59.2 (44-80)
A/Taiwan/083/2006(H3N2), (A/Wisconsin/67/2005-like)		
Before vaccination	20 (15.4-26)	44.9 (33.9-60)
After vaccination	83.8 (68.7-107)	226.3 (174-294.5)
B/Taiwan/0050/2006 (B/Malaysia/2506/2004-like)		
Before vaccination	31 (22.1-41.7)	16.2 (12.9-20.6)
After vaccination	81.9 (58.2-110)	34 (24.6-47.25)

Table 3. Seroconversion rates before and after vaccination of adult and elders.

	Adult group (N=30)	Elders group (N=30)
Seroconversion factor	value (95% CI)	
A/Taiwan/0586/2006(H1N1), (A/New Caledonia/20/99-like)	2.4 (1.6-3.5)	2.6 (1.9-3.6)
A/Taiwan/083/2006(H3N2), (A/Wisconsin/67/2005-like)	4.2 (3.5-5.0)	5.0 (3.8-6.6)
B/Taiwan/0050/2006 (B/Malaysia/2506/2004-like)	2.6 (1.8-3.8)	2.1 (1.6-2.8)

For adults, the seroconversion rate must exceed 40%. For elders, the seroconversion rate must exceed 30%.

Discussion

Since the reappearance of A/H1N1 in 1977, both H1N1 and H3N2 subtypes of influenza A viruses and influenza B virus co-circulate in our world [13]. To prevent outbreaks of influenza, the trivalent influenza vaccines were developed in several countries and administer to people worldwide [12]. Influenza immunization campaigns have been carried out by Taiwan government for several years. In 1998, the level of influenza vaccine use was only 14 doses of influenza vaccine distributed per 1,000 total population in Taiwan. After promotion of a free influenza vaccination strategy for children aged less than 3, in elders whose age over 65, and first-line hospital personnel, the level of influenza vaccine use increased to 145 doses of influenza vaccine distributed per 1,000 total population in 2003 [15]. However, the immunogenicity of influenza vaccine has seldom been evaluated in our population. This study was the first to examine the immune response of the people who received the influenza vaccine in 2006 in Taiwan. In this study, we measured immune responses to three local circulating strains, A/Taiwan/0586/2006(H1N1), A/Taiwan/083/2006(H3N2) and B/Taiwan/0050/2006, which were antigenically similar to A/New Caledonia/20/99(H1N1), A/Wisconsin/67/2005(H3N2), and B/Malaysia/2506/2004, respectively, contained in trivalent inactivated influenza vaccine during the season studied. According our GMT results, both adults and elderly population had an immune response greater than 1:40, except among the elderly against B/Taiwan/0050/2006, where a lower immune response was found. Our results indicate that both adult and elderly populations exhibited a satisfactory immune response against the current circulating influenza viruses in 2006. Influenza B virus was the major circulating virus at the end of 2006 and the beginning of 2007 in Taiwan. Sequence analysis of the hemagglutinin gene indicated that the antigenicity of these strains should be similar to B/Malaysia/2506/2004. Further studies could examine whether there was antigenic drift of the 2007 isolates of influenza B virus, and also determine the HAI antibody titers against this virus in both populations. In conclusion, our results indicate that the Taiwan influenza vaccination program can provide protective antibody titers in adults and the elderly in our country. We plan to continue

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to survey the antibody responses of the subjects who were immunized with influenza vaccine over time to gather additional important data.

Acknowledgements

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Prophylaxis Against Influenza in Children With Inflammatory Bowel Diseases

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Introduction

Patients with inflammatory bowel diseases (IBD), especially if they are treated with immunosuppressive therapy, are at high risk for influenza-related complications. One of the target groups for seasonal influenza vaccination consists of patients requiring regular medical follow-up or hospitalization because of immunosuppression, including immunosuppression caused by drugs (1, 2). Patients with IBD belong to this group as they are treated with long-term immunosuppressive therapy. The incidence of IBD has been increasing with time. The aim of this study was to assess antibody responses to neuraminidase component of influenza vaccine in children with IBD. The results of the response to hemagglutinin have been already presented (3).

Material and Methods

The aim of the study was to assess response to neuraminidase component of influenza vaccine in 29 pediatric patients with IBD (mean age: 12.9 years, median: 14 years) in comparison with 36 healthy persons (mean age: 10.4 years, median: 9 years). In the epidemic season 2005/06 both groups received inactivated split influenza vaccine ('Fluarix', GSK) containing in one 0.5 ml dose 15 µg of hemagglutinin of each of the following influenza strains recommended by WHO as the components of the vaccine: A/New Caledonia/20/99-like virus (H1N1), A/California/7/2004-like virus or A/New York/55/2004 (H3N2) and B/Shanghai/361/2002-like virus or B/Jiangsu/10/2003 or B/Jilin/20/2003. Levels of anti-neuraminidase (anti-NA) antibodies were measured before vaccination, 1 month after vaccination, and 3 months after vaccination by the neuraminidase inhibition test performed at the National Influenza Center, Warsaw according to Aymard-Henry's method modified by Douglas (4, 5). Statistical analysis was performed using non-parametric U Mann-Whitney test and Wilcoxon test in Statistica computer program (StatSoft, Inc. 2001, version 6.0, USA). The results were expressed as the following serological parameters: geometric mean titer (GMT) of anti-NA antibodies before and after vaccination; mean fold increase (MFI) of anti-NA antibody levels after vaccination.

Results

In patients with IBD pre-vaccination geometric mean antibody titers (GMTs) ranged from 8.7 to 9.5 and were similar ($p>0.05$) to those in healthy persons where GMTs ranged from 9.8 to

12.1 (Table 1). One month after vaccination, antibody titers were significantly higher ($p<0.05$) than before vaccination and MFIs ranged from 2.5 to 4.9 in children with IBD and from 4.2 to 5.0 in healthy persons. Three months after vaccination, anti-NA antibody titers were significantly higher ($p<0.05$) than before vaccination and MFIs ranged from 2.7 to 4.2 in IBD group and from 3.1 to 4.8 in healthy children. Anti-N1 antibody titers measured three months after vaccination were significantly lower ($p<0.05$) than antibody titers measured one month after vaccination both in patients with IBD and in healthy children. Neuraminidase N2 antibody levels in the IBD group and in the control group of healthy children three months after vaccination were similar ($p>0.05$) to those observed one month after vaccination. In patients with IBD anti-NB antibody titers three months after vaccination were significantly higher ($p<0.05$) than those measured one month after vaccination, while in healthy children anti-NB antibody titers measured one month after vaccination and three months after vaccination were similar ($p>0.05$). Statistical analysis showed that humoral response for two of three influenza antigens included into the vaccine was better in healthy children than in those with IBD. Anti-N2 antibody levels one month after vaccination and three months after vaccination as well as the anti-NB antibody levels one month after vaccination were significantly higher ($p<0.05$) in the control group of healthy children than in the patients with IBD.

Table 1. Antibody response to influenza vaccine in patients with Inflammatory Bowel Diseases (IBD).

group	antigen	GMT ¹ of anti-NA ² antibodies			MFI ³ of anti-NA ² antibody titers	
		before	1 month after	3 months after	1 month after	3 months after
		vaccination			vaccination	
IBD	A/H1N1	9.5	46.2	25.4	4.9	2.7
healthy		12.1	50.4	37.8	4.2	3.1
IBD	A/H3N2	8.7	21.5	26.0	2.5	3.0
healthy		10.8	46.7	41.6	4.3	3.9
IBD	B	8.7	22.0	36.4	2.5	4.2
healthy		9.8	49.4	46.7	5.0	4.8

¹geometric mean titer

²antineuraminidase

³mean fold increase

Discussion and Conclusions

Immunity against influenza is associated with antihemagglutinin (anti-HA) antibodies which in titers ≥ 40 are considered to be protective, i.e. to prevent influenza infection and post-influenza complications (6-8). Antineuraminidase (anti-NA) antibodies do not prevent influenza infection, but inhibit release of the progeny virions from the infected cells limiting influenza virus spreading and may lead to a milder course of the disease (9-11). Measurement of anti-NA antibodies in the studies on the humoral response to influenza vaccination is not popular also due to a complicated laboratory technique as well as the fact that the content of neuraminidase antigen in the influenza vaccine is not standardized (12). In addition, it is not known what level of anti-NA antibodies is sufficient for

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effective reduction in viral spread (2, 9, 12). Nevertheless, it should be taken into consideration that inactivated influenza vaccines induce immunological response to both surface influenza glycoproteins: hemagglutinin and neuraminidase (13). In the present study, humoral response for two of three influenza antigens (N2 and NB) included into the vaccine was better in healthy children than in those with IBD. Nevertheless, significant increases of antineuraminidase antibody titers after vaccination as well as MFI values similar to those observed in healthy persons showed that influenza vaccine is effective in children with IBD in the aspect of the production of antibodies against one of two influenza glycoproteins which contribute to a milder course of the disease by the inhibition of the release of the progeny virions from the infected cells. Moreover it should be emphasized that optimal protection against influenza illness may be achieved through the presence of antihemagglutinin antibodies with antineuraminidase antibodies, and that the control of contents of neuraminidase in influenza vaccine may positively affect efficacy of the vaccine (8, 12).

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Assessment of Immunological Response to Influenza Vaccination in Patients With Wegeners Granulomatosis

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Introduction

Wegeners granulomatosis (WG) is a systemic disease characterized by necrotizing granulomatous inflammation of the upper and lower respiratory tract in combination with vasculitis and focal necrotizing crescentic glomerulonephritis. Treatment with cyclophosphamide in combination with corticosteroids is highly successful, although side effects may be severe and sometimes lethal. Patients receiving immunosuppressive and cytotoxic drugs risk occurrence of particularly serious complications, including post-influenza complications, and significant numbers of death cases are registered. These immunosuppressed patients are one of the target groups for which seasonal influenza vaccination has been recommended (1).

Material and Methods

The aim of this study was to assess humoral responses to influenza vaccine in 35 patients aged 20-63 years (mean: 40.5, median: 46) with WG (group A) who were in clinical and serological remission after immunosuppressive treatment, i.e. no disease activity in the clinical, biochemical and serological terms was observed. In autumn 2006 they were vaccinated with inactivated subunit influenza vaccine ('Influvac', Solvay Pharmaceuticals) containing in one 0.5 ml dose 15 µg of hemagglutinin of each of the following influenza strains recommended by WHO as the components of the vaccine for the epidemic season 2006/2007: A/New Caledonia/20/99 (H1N1)-like virus, A/Wisconsin/67/2005 (H3N2)-like virus or A/Hiroshima/52/2005 (H3N2) and B/Malaysia/2506/2004-like virus or B/Ohio/1/2005. Serum antihemagglutinin (anti-HA) and antineuraminidase (anti-NA) antibody levels were measured before vaccination and after 1 month by hemagglutination inhibition test (HAI) and neuraminidase inhibition test, respectively (2-5). Antibody levels were also determined in 28 patients aged 17-42 years (mean: 44.7, median: 47) with WG with a disease activity confirmed clinically, biochemically and serologically, who were currently treated immunosuppressively and were not vaccinated (group B) and in 35 healthy people aged 23-79 (mean: 35.1, median: 28) who received the same inactivated subunit vaccine (group C). Serological tests were performed at the National Influenza Center, Warsaw. All sera were frozen at -20°C until use and were then incubated in a 56°C water bath for 30 minutes and treated with Receptor Destroying Enzyme (RDE) from *Vibrio cholerae* to remove non-specific inhibitors of hemagglutination that may be present in

sera and cause false positive results in the HAI test. HAI test was carried out according to the technique recommended by WHO with 0.5% turkey red blood cells (2, 3). Neuraminidase inhibition test was carried out according to Aymard-Henry's method modified by Douglas (4, 5). The results were expressed as the following serological parameters: geometric mean titer (GMT) of anti-HA and anti-NA antibodies before and after vaccination; mean fold increase (MFI) of anti-HA and anti-NA antibody levels after vaccination; protection rate, i.e. the proportion of subjects with anti-HA antibody titers ≥ 1:40 before and after vaccination; response rate, i.e. the proportion of subjects with at least a fourfold increase of anti-HA antibody titers after vaccination. Statistical analysis was performed by using non-parametric Wilcoxon paired test and Mann-Whitney unpaired test in Statistica computer program (StatSoft, Inc. 2001, version 6.0, USA). This study was approved by the Bioethical Committee of the Medical University of Warsaw.

Results

Before vaccination anti-HA antibody levels were low and similar in all three study groups (p>0.05): A (GMTs from 5.9 to 6.7), B (GMTs from 5.1 to 7.6) and C (GMTs from 5.9 to 8.2) (Table 1).

Table 1. Antibody response to influenza vaccine in patients with Wegeners granulomatosis.

group	antigen	MFI ¹ of anti-HA ² antibody titers after vaccination	protection rate ³ (%)		response rates ⁴ (%) after vaccination	MFI ⁵ of anti-NA ⁵ antibody titers after vaccination
			before	after administration of the vaccine		
A	A/H1N1	12.8	5.7	74.3	74.3	8.4
B		1.4	7.1	21.4	21.4	0.9
C		24.1	2.9	88.6	88.6	5.6
A	A/H3N2	5.4	5.7	51.4	60.0	5.5
B		0.9	3.6	3.6	10.7	1.1
C		6.7	14.3	65.7	71.4	5.4
A	B	6.8	0.0	54.3	71.4	3.0
B		1.2	0.0	3.6	7.1	0.9
C		13.4	11.4	94.3	85.7	10.2

group A – vaccinated patients with Wegeners granulomatosis

group B – not vaccinated patients with Wegeners granulomatosis

group C – healthy vaccinated volunteers

¹ mean fold increase

² antihemagglutinin

³ percentage of subjects with the protective anti-HA antibody titers ≥ 1:40

⁴ percentage of subjects with at least a fourfold increase of anti-HA antibody titers

⁵ antineuraminidase

After vaccination, anti-HA antibody titers significantly increased (p<0.05) in vaccinated patients and healthy volunteers, i.e. in group A (GMTs from 36.2 to 75.4) and group C (GMTs from 51.7 to 142.1) when compared with pre-vaccination values. Mean fold increases (MFIs) of anti-HA levels ranged from 5.4 to 12.8 in group A and 6.7 to 24.1 in group C. In unvaccinated group B, GMTs values in the second serum sample obtained one month after the first sample collection ranged from 5.9 to 10.5 and MFIs ranged from 0.9 to 1.4. Before vaccination, protection rates did not exceed 15% and ranged from 0% to 5.7% in group A and 2.9% to 14.3% in group C. After vaccination, protection

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rates significantly increased and ranged from 51.4% to 74.3% and 65.7% to 94.3%, respectively. In group B, protection rates during the whole study were between 0% and 21.4%. In patients with WG who received influenza vaccine, 65.7% had protective anti-HA antibody titers against at least two of three influenza vaccine strains and 14.3% had protective antibody titers against only one vaccine strain. No protective anti-HA antibody titers were found in 7 of 35 patients (20%). In group C, all healthy volunteers had protective anti-HA antibody titers: 11.4% of them had protective antibodies against one influenza strain and 88.6% had protective antibody titers against at least two of three influenza vaccine strains. Response rates ranged after vaccination from 60% to 74.3% in group A and 71.4% to 88.6% in group C. In group B, response rates were between 7.1% and 21.4%. Similarly to anti-HA antibodies, pre-vaccination levels of anti-NA antibodies were low and did not differ significantly ($p>0.05$) between study groups: A (GMTs from 6.3 to 7.1), B (GMTs from 6.4 to 6.6) and C (GMTs from 5.2 to 7.1). One exception was found for anti-NB antibodies as their significantly higher titers ($p<0.05$) were observed in vaccinated patients of group A than in healthy volunteers. After vaccination anti-NA antibody titers significantly increased ($p<0.05$) in groups A (GMTs from 20.4 to 52.8) and C (GMTs from 38.4 to 52.8) when compared with pre-vaccination values and MFIs ranged from 3.0 to 8.4 and from 5.4 to 10.2, respectively. In group B, GMTs of anti-NA antibodies ranged from 5.8 to 7.1 and MFIs ranged from 0.9 to 1.1. The only significant difference in anti-HA antibody titers between group A and group C was found after vaccination for hemagglutinin HB, where higher values ($p<0.05$) were observed in group C. In response to neuraminidase, significantly higher ($p<0.05$) post-vaccination anti-N1 titers and pre-vaccination anti-NB titers were found in group A than in group C. Significantly higher ($p<0.05$) post-vaccination anti-NB titers were found in group C than in group A. In group B, anti-HA and anti-NA antibody levels were significantly lower ($p<0.05$) than in group A and group C. Administration of the influenza vaccine in patients with WG did not cause any serious adverse reactions or deterioration.

Discussion and Conclusions

Use of inactivated influenza vaccines, despite their safety and immunogenicity, is still significantly lower than it should be considering the broad range of recommendations for their use by many countries. Healthy people are not aware of the danger of influenza infection, being convinced that their immunological system is able to eliminate virus effectively, while patients with chronic medical conditions who are at greater risk of post-influenza complications may consider their health status as a contraindication for influenza vaccination. The National Influenza Center in Poland undertakes many activities to increase the low influenza vaccination coverage in the country; during the last epidemic seasons, approximately 86 doses of influenza vaccine per 1,000 inhabitants were distributed. There are various methods to achieve this aim, including lectures, educational brochures and leaflets, popular-

science articles, etc. Other methods include studies performed together with clinicians on influenza vaccination in patients from different high-risk groups, including immunosuppressed patients, such as those with Wegeners granulomatosis (6, 7). Immunosuppression caused by drugs affects both humoral and cellular immunity, resulting on the one hand in the increased susceptibility to any infections and on the other hand in the decreased immunological response to natural infection as well as to immunization. Nevertheless, the results of the present study showed that there were no significant differences in anti-HA antibody titers between patients with WG who were vaccinated against influenza and healthy persons, with the exception of anti-HB antibodies. Inactivated subunit influenza vaccine was immunogenic in this group of patients. One month after vaccination, 66% of these patients had anti-HA antibodies in titers considered to be protective against at least two of three influenza vaccine strains. The fact that 20% of the vaccinated patients with WG did not demonstrate protective anti-HA antibody titers against one of three strains is not a reason to reject influenza vaccination for this group. Antineuraminidase antibody levels were also assessed in this study. We showed that influenza vaccine induced their production, but HAI test remains still a gold standard used to measure response to influenza vaccination. One of the reasons for that is a strong correlation between anti-HA antibody levels and protection against influenza, while anti-NA antibodies only contribute to a milder course of the disease. MFI values of anti-HA antibody titers and response rates for all three influenza antigens were higher than 2.5 and 40%, respectively. This means that the requirements of the Committee for Proprietary Medicinal Products (CPMP) established for antibody response to influenza vaccination were fulfilled (8). The requirements of CPMP regarding protection rates, which should amount to at least 70% in healthy adults, were also fulfilled, but only in the case of antigen A/H1N1 (8). Considering the results of this study, seasonal influenza vaccination may be and should be offered to patients with Wegeners granulomatosis. This conclusion is also supported by the fact that patients with WG frequently have respiratory tract infections either at onset of the disease or before a relapse. Moreover, it is difficult, even for the experienced physician, to differentiate between a disease relapse and a new viral infection, including influenza. Such potential misdiagnosis of WG patients increases risk of complications during therapy and may lead even to death.

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Vaccination Against Influenza and Antibody Response in Patients With Coronary Artery Disease – A Single Center, Prospective, Randomized, Double-Blind Placebo Controlled Study

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Introduction

Patients with cardiovascular diseases, including those with coronary artery disease (CAD) are one of the groups for which seasonal vaccination against influenza is recommended (1). This study is a part of a project regarding the effect of influenza vaccination on the prevention of ischemic coronary events in patients with CAD that was financed by the Ministry of Science and Higher Education (2 P05B 016 27). The clinical results obtained in this project were presented during the Conference of the American Heart Association, 12-15 November 2006, Chicago (2). This study presents the results of the humoral response to hemagglutinin component of influenza vaccine in patients with CAD.

Materials and Methods

A single center, randomized, prospective, double-blind, placebo controlled study was performed in a group of 658 patients with CAD (mean age 59.9±10.3 years). The randomization schedule was 1:1 (placebo : influenza vaccine). In autumn 2004, 325 patients received inactivated subunit influenza vaccine ('Influvac', Solvay Pharmaceuticals), and 333 patients received placebo. Blood samples were collected before administration of the vaccine or placebo and 8 to 10 weeks after this intervention. Serological tests were carried out at the National Influenza Center, Warsaw. Antihemagglutinin (anti-HA) antibody levels were measured in sera by hemagglutination inhibition test performed with turkey 0.5% red blood cells and the following influenza strains recommended by WHO as the vaccine components for the epidemic season 2004/05: A/New Caledonia/20/99 (H1N1), A/Christchurch/28/03 (H3N2) and B/Jiangsu/10/03 (3, 4). Until testing serum samples were frozen at minus 20°C and were incubated in a 56°C water bath for 30 minutes and treated with receptor destroying enzyme from *Vibrio cholerae* to remove non-specific inhibitors of hemagglutination that may be present in sera and cause false positive results in hemagglutination inhibition test. Serological tests were done only for those patients from whom paired sera were obtained, i.e. for 78 patients who received influenza vaccine (24%) and for 97 patients who received placebo (29.1%). Anti-HA antibody titers were read as the reciprocal of the highest serum dilution causing complete inhibition of agglutination

of 0.5% red blood cells. To present the results the following serological parameters were calculated: geometric mean titer (GMT) of anti-HA antibodies before and after vaccination; mean fold increase (MFI) of anti-HA antibody levels after vaccination; protection rate, i.e. the proportion of subjects with anti-HA antibody titers ≥ 1:40 before and after vaccination; response rate, i.e. the proportion of subjects with at least a fourfold increase of anti-HA antibody titers after vaccination. Statistical analysis was performed by using non-parametric Wilcoxon paired test and Mann-Whitney unpaired test in Statistica computer program (StatSoft, Inc. 2001, version 6.0, USA). In the case of patients who showed influenza or influenza-like symptoms during the study, clinical material was collected to perform diagnostic tests (direct immunofluorescence test to detect antigens of influenza A and B, RSV, adenovirus, parainfluenza type 1, type 2 and type 3; RT-PCR to detect RNA of influenza virus) and confirm whether the respiratory infection was caused by influenza or other respiratory virus. Results: Before administration of the vaccine or placebo, GMTs were similar in both study groups ($p>0.05$) and ranged from 5.9 to 8.7 in the vaccinated patients and from 9.0 to 11.9 in those who received placebo (Table 1).

Table 1. Antibody response to influenza vaccine in patients with coronary artery disease.

group	antigen	GMT ¹ of anti-HA ² antibodies		MFI ³ of anti-HA ² antibody titers after administration of the vaccine or placebo	protection rate ⁴ (%)		response rates ⁵ (%) after administration of the vaccine or placebo
		before administration of the vaccine or placebo	after administration of the vaccine or placebo		before administration of the vaccine or placebo	after administration of the vaccine or placebo	
vaccine	A/H1N1	5.9	33.8	5.7	2.6	60.3	68.0
placebo		9.0	12.0	1.2	5.2	6.2	5.2
vaccine	A/H3N2	8.7	58.0	4.9	2.6	56.4	66.7
placebo		9.8	16.4	1.2	1.0	7.2	6.2
vaccine	B	7.2	64.6	5.1	0.0	60.3	62.8
placebo		11.9	19.1	1.2	2.1	8.2	7.2

¹geometric mean titer

²antihemagglutinin

³mean fold increase

⁴percentage of subjects with the protective anti-HA antibody titers ≥ 1:40

⁵percentage of subjects with at least a fourfold increase of anti-HA antibody titers

After administration of the vaccine, GMTs were significantly higher ($p<0.05$) than before vaccination and ranged from 33.8 to 64.6, while MFIs of anti-HA antibody levels were between 4.9 and 5.7. After administration of the placebo, GMTs increased when compared with values registered before this intervention and ranged from 12.0 to 19.1, while MFIs amounted to 1.2 for all antigens. The percentage of patients with protective anti-HA antibody titers, i.e. ≥40 (protection rate), ranged from 0% to 2.6% before administration of the vaccine, and from 1% to 5.2% before administration of placebo. After influenza vaccination, protection rates ranged from 56.4% to 60.3%, while in the placebo group the values of this parameter were from 6.2% to 8.2%. The percentage of patients with at least a fourfold increase of anti-HA antibody titers (response rate) ranged from 62.8% to 68% in patients who received the vaccine and from 5.2% to 7.2% in the placebo group. After administration of the vaccine/placebo anti-HA antibody titers were significantly higher ($p<0.05$) in the vaccinated patients than in the placebo

group. In patients who received the vaccine, pre-vaccination anti-HA antibody levels to antigen A/H3N2 were significantly higher ($p < 0.05$) than antibody levels to antigen A/H1N1. Similar observation was made in the placebo patients, who had anti-H3 antibody titers significantly higher than anti-H1 antibody titers before administration of placebo as well as after this intervention. Vaccine and placebo were well tolerated and no serious side effects were observed after their administration. No patients were infected with influenza virus or other respiratory pathogen, including RSV, adenovirus, parainfluenza virus type 1, type 2 and type 3.

Discussion and Conclusions

Most of the national vaccination programs, as well as different scientific societies include recommendations for seasonal vaccination against influenza for people with cardiovascular problems. In Poland, currently updated information on the influenza vaccination coverage in different target groups, including patients who especially risk occurrence of post-influenza complications as patients with heart or cardiovascular system diseases, are not available. Information collected by survey performed in 2003 in the population of 2,000 Poles showed that only 9% of persons among those with cardiovascular diseases were vaccinated against influenza (5). The results of many surveys indicate that the most important reason low influenza vaccination rates is insufficient knowledge about influenza vaccines as well as influenza infection and its serious consequences, including economic loss (5-7). Among health care workers influenza vaccination rates are also low, and this group plays a significant role as it is known that in many cases, patients decide to be vaccinated against influenza if there is a clear recommendation from physician (7-10). Considering the above facts, it is very important to provide physicians with convincing scientific data which confirm efficacy and safety of influenza vaccination in different groups of patients, including those with CAD. Clinical efficacy of vaccination is difficult to assess due to many various factors that may affect this efficacy as activity of influenza viruses in a given epidemic season, different time of exposition of the study groups to influenza virus or effect of herd immunity on not vaccinated patients (11). For the into the vaccine, values higher than those required by CPMP. This means that MFI and response rates were higher than 2.5 and 40%, respectively. The results confirm that patients with coronary artery disease should be vaccinated against influenza with any doubts regarding their antibody response to the prophylaxis of this kind. Vaccination against influenza provided this group of patients with some degree of protection and should be widely used in this group to minimize a risk of infection and serious post-influenza complications. above reasons immunological efficacy of influenza vaccination is assessed more often than clinical efficacy (11). In this study, in patients who received influenza vaccine, post-vaccination protection rates were lower than 70% for all three influenza antigens included into the vaccine. These results mean that in the case of this serological parameter, the requirements of the

Committee for Proprietary Medicinal Products (CPMP) used for the assessment of antibody response to influenza vaccination in healthy adults aged 18-60 were not fulfilled (12). Nevertheless, MFI and response rates achieved, for all three influenza antigens included.

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Immunogenicity of Influenza Vaccination in Reduced-Intensity Conditioning Allogeneic Hematopoietic Stem Cell Transplant Recipients Compared to Healthy Individuals: A Pilot Study

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Immunogenicity of the 2002-2003 inactivated influenza vaccine was compared in 5 reduced-intensity conditioning allogeneic stem cell transplant recipients and 14 healthy adults. The fold increase in hemagglutination inhibition vaccine-specific antibody titers was similar in both groups. Stem cell transplant recipients had lower rates of seroprotection prevaccination and seroconversion postvaccination.

Introduction

Respiratory virus infections are common after allogeneic hematopoietic stem cell transplantation (allo HSCT), occurring in up to 63% of respiratory tract illness episodes [1]. In a recent study [2], the incidence of influenza in HSCT recipients was 1.3%, with 29% developing pneumonia, of which 28% died within 30 days. A prospective study performed at 37 European centers showed that the overall mortality in influenza A virus infection was 23% and the direct influenza-associated mortality was 15.3% [3]. Influenza A and B accounted for 14% of episodes of respiratory virus infections in HSCT recipients after reduced-intensity conditioning (RIC) [4]. Immunological recovery after myeloablative allo HSCT takes several months and often years [5]. The Centers for Disease Control and Prevention [6] recommends annual influenza vaccination for HSCT recipients. Influenza vaccine protective efficacy among 43 patients who had received bone marrow transplant (BMT) more than 6 months prior was 80% [7]. In one study [8], 48 HSCT recipients who received 2 doses of influenza vaccination responded in 0 to 35% of cases, depending on the interval period between transplantation and immunization, and the influenza virus strain. No response was detected in patients immunized within 6 months of transplantation. Another study detected an antibody response to influenza vaccination administered within 2 years after transplantation in 13 to 21% of autologous peripheral blood SCT recipients, and 10-20% of autologous BMT recipients, depending on the influenza virus strain [9]. All allo BMT recipients in that study had no antibody response. No studies have been conducted to date to assess immunogenicity of influenza vaccination in RIC allo HSCT recipients. It was hypothesized that this nonmyeloablative protocol may allow

for a better immunological response to influenza vaccination than that seen in myeloablative allo HSCT recipients.

Materials and Methods

At our center, RIC consists of fludarabine 30 mg/m² intravenously on days -4, -3 and -2, followed by 200 cGY of total body irradiation on day 0. A prospective, open-label, case-control, pilot study was conducted in the fall and winter of 2002-2003. The hospital's institutional review board approved the study, and written informed consent was obtained from all participants. In the fall of 2002 the inactivated influenza vaccine was administered by intramuscular injection in the deltoid region. Subjects received the standard dose of the recommended Northern hemisphere 2002-03 split-virus influenza vaccine (Fluzone, Aventis Pasteur Inc. Swiftwater PA), which contained 15 micrograms of hemagglutinin (HA) for each of the vaccine strains, A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Hong Kong/1434/2002 per 0.5 ml dose. Patients were included if they had received RIC allo HSCT 3 months or more prior to enrollment, and were between the age of 18 and 65 years. They were excluded if they failed to engraft, or had relapse of the underlying disease requiring chemotherapy or immunotherapy. The comparison group consisted of health care providers at our institution, between the ages of 18 and 65 years, who were in good health, and who are routinely offered influenza vaccination. Patients and controls were also excluded if they had history of egg allergy, acute febrile illness at the time of vaccination, or influenza-like illness (ILI) within 4 weeks prior to vaccination. Prevaccination and 4 weeks postvaccination blood samples from patients and controls were tested for antibody titers specific to the 3 viral antigens included in the 2002-2003 influenza vaccine using a standardized hemagglutination inhibition (HAI) assay [10]. Since the manufacture of intravenous immunoglobulin (IVIG) is a multi-step process that takes approximately 7 months from initial plasma collection to completion of the finished product [11], it is not expected to affect the result of the antibody assay specific to the vaccine administered. In the single patient who received IVIG on the same day of vaccination, the prevaccination blood sample was collected before infusing IVIG. Sample size calculation showed that including 12 RIC allo HSCT recipients and 24 healthy controls would have 80% power to detect an effect size of ≥ 0.9 , at the 0.05 significance level, where effect size is defined as the true difference in means divided by the pooled standard deviation of the groups (UnifyPow, a macro for the SAS system [Carey, NC: SAS Institute]). Previous research conducted at our institution observed changes from baseline in the B/Yamanashi antibody level of (mean [standard deviation {SD}]) of 9 (20) for lung transplant recipients and 27 (47) for healthy controls. We expected less change in RIC allo HSCT recipients, and thus larger differences from healthy controls. That sample size would detect differences of ≥ 25 , given a SD of 15 and 35 in the 2 groups, respectively. The primary outcome was to compare healthy controls and RIC allo HSCT recipients on the fold increase in antibody titer, defined

as the postvaccination titer divided by the prevaccination titer. Seroconversion was defined as the presence of an antibody titer of 1:40 or higher. Seroconversion was defined as a 4-fold or greater increase in antibody titer from prevaccination to postvaccination. The rates of seroprotection, seroconversion, and the geometric means of reciprocals of titers (GMT) prevaccination and postvaccination were also compared.

Results

Thirty-two patients had received RIC allo HSCT at the time the study was conducted since the inception of the RIC allo HSCT program at our center in year 2000. Seventeen patients died, 2 failed to engraft, 2 had relapse of the underlying disease, 1 did not agree to participate in the study, 1 transferred care to the referring physician, 3 received influenza vaccination elsewhere, and 1 did not have a postvaccination antibody titer collected. Ten controls did not have a postvaccination antibody titer collected. Thus, only 5 patients and 14 controls were evaluable. Since this resulted in an under-powered study, statistical analysis was not presented. However, results are shown to get pilot data for future investigations. Patients were older than controls [50.2 (16.1) versus 36 (10) years {mean (SD)}]. Four patients and 9 controls were female. Three patients and 11 controls had had influenza vaccination in 2001-2002. Median interval period between the date of transplantation and influenza vaccination was 28 months (range 5-31 months). Two patients had chronic myelogenous leukemia, 2 had non-Hodgkin's lymphoma, and 1 had chronic myelogenous leukemia. At the time of vaccination, 2 patients were receiving cyclosporine A, 3 corticosteroids, 1 tacrolimus, 1 was not receiving any immunosuppressive agents, the mean IgG level was 1115 mg/dL (range 815-1380), and 1 patient had evidence of cutaneous graft-versus-host disease. Fold increase in antibody titers with vaccination was similar in both groups (Table 1.).

Table 1. Fold increase, prevaccination seroprotection and postvaccination seroconversion rates, and prevaccination and postvaccination GMT[†] of the 3 HAI[‡] antibodies.

Influenza vaccine serotype	A/H3N2		A/F
Fold increase in titer (median)	2.5	1.6	3.0
Prevaccination seroprotection rate (%)	20	64	20
Prevaccination GMT	15.1	45.2	10
Postvaccination seroconversion rate (%)	60	93	60

[†]GMT = geometric means of reciprocals of titers. [‡]HAI hemagglutination inhibition.

Patients had lower rates of seroprotection prevaccination, seroconversion postvaccination, and GMT prevaccination and postvaccination compared to controls. Influenza serotype B was less immunogenic in both groups than serotypes A/H3N2 and A/H1N1. The best antibody responses in patients occurred in the 3 patients who were 2 years or more after transplantation. None of the patients or the controls developed an ILI confirmed to be due to influenza virus during the 2002-03 influenza.

Discussion

Our pilot data suggest that influenza vaccination may be less immunogenic in RIC allo HSCT recipients compared to healthy controls. The vaccine was more immunogenic when compared to responses observed in previous studies in myeloablative allo HSCT recipients [8, 9]. Influenza B was the least immunogenic, similar to findings in a previous study [8]. Other limitations include that patients and controls were not matched by age, and that patients' underlying malignancies were diverse. Even though the 2001-2002 influenza vaccine had different H3N2 and B strains, and the 2000-2001 vaccine had a different B strain, respectively, than 2002-2003 vaccine used in this study, residual HA antibodies to these vaccines could have cross-reacted with the antibody assay specific to the 2002-2003 vaccine. Our preliminary data are consistent with a previous study (8) in BMT recipients, which showed that a longer interval period between transplantation and influenza vaccination correlated positively with seroconversion. Since the primary T cell-dependent antibody responses take 1 year or more after BMT to develop [5], the T cell-dependent humoral antibody response to influenza vaccine may not be expected to develop before this time frame. Granulocyte-macrophage colony-stimulating factor as an influenza vaccine adjuvant has not been successful in HSCT recipients [12]. Administering more than one dose of the influenza vaccine to BMT recipients did not improve its immunogenicity [8]. Donor immunization with other vaccines using polysaccharide-protein conjugate antigens or protein recall antigens prior to stem cell harvesting partially improves recipient antibody levels [13]. Intradermal influenza vaccination has been shown to enhance antibody production in healthy persons 18 to 60 years of age [14]. Influenza vaccine dose escalation improves immunogenicity in elderly persons [15]. Results of our study should be regarded as preliminary. Future studies should include a larger number of subjects, and assess the cell-mediated immune response and influenza neutralizing antibody titers.

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Immunogenicity of Trivalent Inactivated Influenza Vaccine Among Children Less Than 4 Years of Age

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This study investigated the immunogenicity of trivalent inactivated influenza vaccine. Subjects comprised 259 children under 4 years of age who visited 6 pediatric clinics to receive influenza vaccine. Age distributions were: 64 in <1.0 year, 65 in 1.0-1.9 year, 64 in 2.0-2.9 year and 66 in 3.0-3.9 year age group. Two doses of vaccine were given subcutaneously at 4 weeks apart. Dosage was 0.1 ml for children <1-year-old, while dosage was 0.2 ml for children ≥1-year-old, in accordance with standard Japanese recommendations. To measure hemagglutination inhibition (HAI) antibody titer, triplet sera were obtained before vaccination (S0), 4 weeks after first vaccination (S1) and 4 weeks after second vaccination (S2). The geometric mean of HAI antibody titer and seroprotection proportion (postvaccination titer ≥1:40) were calculated by age group. Analysis of variance was also employed to estimate the independent effects of age and prevaccination titer on the fold-rise in antibody. Geometric means of HAI titer were lower among the two younger age groups than among the two older age groups, regardless of vaccine strain or when blood samples were collected. Seroprotection proportion after 2 doses of vaccine in <1.0 year, 1.0-1.9 years, 2.0-2.9 years and 3.0-3.9 years were: 38%, 58%, 89% and 85% against A(H1); 52%, 54%, 81% and 73% against A(H3); and 23%, 49%, 67% and 71% against B, respectively. Regarding analysis of variance, prevaccination titer consistently indicated strong effects on antibody fold-rise, regardless of vaccine strain or combinations of paired sera. After 2 doses of vaccine (S2/S0), significant effects of age on antibody induction were shown against A(H1) and B (P=0.000 and P=0.002, respectively). Thus, the immunogenicity of trivalent inactivated influenza vaccine was strongly affected by prevaccination titer and age. Even after 2 doses of vaccination, a protective level of antibody could not be achieved in about 50-80% of subjects among infants aged <1-year-old, and 40-50% among children at 1.0- to 1.9-years-old.

Introduction

Recently, several studies have documented that the risk of hospitalization from influenza are higher among infants¹⁻³.

In addition, about half of influenza-related pediatric deaths from the United States during the 2003/2004 season, had no underlying medical condition previously associated with an increased risk for influenza-related complications⁴. These results lead to recognition that preventing influenza is important also in healthy children without underlying medical condition. As a result, the US Advisory Committee on Immunization Practices (US-ACIP) began recommending that infants aged 6-23-months should be vaccinated from the 2004/2005 season⁵. Furthermore, US-ACIP decided that recommendation for vaccination of children was extended from infants aged 6-23-months to all children aged 6-59-months in the 2006/2007 season⁶. However, the immunogenicity and efficacy of influenza vaccines in infants have not necessarily been established⁷⁻⁹. The reason is that infants differ from adults in terms of maturity of the immune system, history of influenza virus infections and history of vaccinations. Particularly in Japan, vaccine dosage by standard recommendation is lower than that in other countries⁶. It has been controversial whether current vaccine dosage in Japan elicits sufficient immunological responses for preventing influenza infection. However, due to the logistic difficulties, few investigations have examined immunogenicity in infants and children.

Materials and Methods

Subjects. Subjects comprised children <4-years-old who visited one of 6 pediatric clinics for vaccination from October to November 2005. Children with an acute febrile illness or signs of severe acute illness at the time of vaccination, past history of anaphylaxis due to vaccine components, or other inappropriate condition to receive vaccination were excluded. After explaining about the study, written informed consent was obtained from the legal guardian (mainly parents) of each subject. Eventually, a total of 259 children were enrolled. Age distributions were: 64 in <1.0 year, 65 in 1.0-1.9 year, 64 in 2.0-2.9 year and 66 in 3.0-3.9 year age group. All subjects completed the study protocol including collection of blood samples at three times.

Vaccination. The vaccines administered were the commercially available inactivated trivalent influenza vaccine for the 2005/2006 season (Biken HE01A). The vaccine contained A/New Caledonia/20/99 (H1N1), A/New York/55/2004 (H3N2) and Shanghai/361/2002 (B), and the antigen level for each strain was 30 µg/ml. Vaccine dosage were given according to the standard Japanese recommendation (i.e., 0.1 ml for <1-year-old and 0.2 ml for 1.0- to 5.9-years-old). Two doses of vaccine were administered subcutaneously 4 weeks apart. The second dose was completed before the end of November.

Serum collection and antibody titer measurement. Blood samples were collected for the following 3 points: before vaccination (S0), 4 weeks after first vaccination (S1), and 4 weeks after second vaccination (S2). Serum samples were stored at between -70 and -80°C, and hemagglutination inhibition (HAI) antibody titers at above triplet point were measured according to the conventional method.

Analysis. The geometric mean of HAI titer and seroprotection

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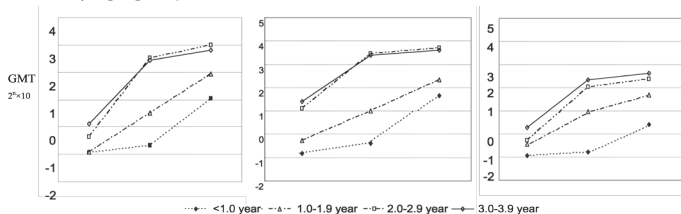
proportion (proportion of subjects with $\geq 1:40$ HAI titer)^{10,11} were calculated by age group. In these calculation, HAI titers of $<1:10$ were regarded as 1:5. The Mantel-extension method was used to assess the association between age and seroprotection proportion. Furthermore, to ascertain the effects of prevaccination HAI titer and age on immunological responses, analysis of variance was conducted using the fold-rise in HAI titer as a dependent variable, and age (4 levels) and prevaccination HAI titer (3 levels) as independent variables. In this analysis, HAI titers were subjected to logarithmic conversion. All tests were two-sided, and level of significance was set at 5%. All statistical analyses were performed using SAS version 9.1.3 (SAS Institute Inc.).

Ethical considerations. The study protocols were approved by the Clinical Study Review Board of Medical Co. LTA Kyushu Clinical Pharmacology Research Clinic.

Results

Geometric mean of HAI titer (Figure 1). Geometric means of HAI titer were lower among the two younger age groups than among the two older age groups, regardless of vaccine strain or when blood samples were collected. For children aged 2 and 3-years, geometric mean markedly increased after the first dose, confirming a favorable HAI titer rise, but degree of HAI titer rise following the second dose was not marked. Conversely, for children aged 0 and 1-year, degree of HAI titer rise following the first dose was small, while degree of HAI titer rise following the second dose was large. After 2 doses of vaccine, antibody titer for children aged 0 and 1-year was lower than that for children aged 2 and 3-years.

Figure 1. Geometric mean of HAI antibody titer against inactivated influenza vaccine by age group.



Blood samples were collected before first vaccination (S0), 4 weeks after first vaccination (S1) and 4 weeks after second vaccination (S2).

Seroprotection proportion (Table 1). The range of seroprotection proportion following 2 doses was 23-52% for infants aged <1.0 year, 49-58% for children aged 1-year, 67-89% for those aged 2-years and 71-85% for those aged 3-years. Thus, seroprotection proportion (S2) for the two younger age groups was lower than that for the two older age groups. **Analysis of variance.** Irrespective of vaccine strain or combinations of paired sera, effects of prevaccination HAI titer on fold-rise in antibody were always significant ($P=0.000-0.013$). Age and prevaccination HAI titer represented independent significant factors for titer rise after the first dose (S1/S0). After

2 doses of vaccine (S2/S0), significant effects of age on antibody induction were shown against A(H1) and B ($P=0.000$ and $P=0.002$, respectively), but not against A(H3) ($P=0.766$). Thus, the immunogenicity of trivalent inactivated influenza vaccine was strongly affected by prevaccination titer and age.

Table 1. Seroprotection proportion as frequency of subjects with postvaccination HAI titers 1:40.

Age (years)	N	A/New Caledonia/20/99/H1N1			A/New York/55/2004/H3N2			B/Shanghai/361/2002		
		S0	S1	S2	S0	S1	S2	S0	S1	S2
<1.0	64	1 (2)	2 (3)	24 (38)	2 (3)	2 (3)	33 (52)	0 (0)	3 (5)	15 (23)
1.0-1.9	65	0 (0)	18 (28)	38 (58)	9 (14)	18 (28)	35 (54)	8 (12)	22 (34)	32 (49)
2.0-2.9	64	3 (5)	50 (78)	57 (89)	23 (36)	47 (73)	52 (81)	8 (13)	39 (61)	43 (67)
3.0-3.9	66	11 (17)	51 (77)	56 (85)	29 (44)	45 (68)	48 (73)	15 (23)	43 (65)	47 (71)
Total	259	15 (6)	121 (47)	175 (68)	63 (24)	112 (43)	168 (65)	31 (12)	107 (41)	137 (53)
Trend P*		0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000

Discussion

This study shows that younger age was associated with more difficult acquisition of protective-level HAI titers. This results is consistent with that in past studies^{12,13}. Even after second vaccination, about 50-80% of 0-year-olds and 40-50% of 1-year-olds had not achieved protective-level of HAI titers. In terms of the geometric mean of HAI titer, degree of HAI titer rise following the first dose was more favorable for the two older age groups than for the two younger age groups. The reason for this was that older children already had antibodies before vaccination. In addition, degree of HAI titer rise following the second dose was lower for older children than for younger children. This represents the phenomenon called the law of initial value or negative feedback¹⁰. In other words, small immunological response following the second dose might be results from highly achieved HAI titers after the first dose among older children. 24% of total subjects possessed protective-level HAI titers against A(H3) before vaccination. The proportion of subjects with protective-level HAI titers before vaccination was higher for A(H3) than for A(H1) or B. The reason is that A(H3) represented the main epidemic strain in 2 of the past 3 seasons. If subgroup analysis is performed by limiting subjects without antibody, the effects of age alone could have been ascertained. To do such analysis, however, more sufficient numbers of children would be needed to enroll. Therefore, in this study, effects of age and prevaccination HAI titer were simultaneously considered to investigate the independent effects of these factors by using the analysis of variance. The analyses showed that the effects of prevaccination HAI titer on fold-rise in titer were always significant, regardless of vaccine strain or combinations of paired sera, and marked effects were independent of age. The effects of age on titer rise following the two doses (S2/S0) were significant for A(H1) and B, but not for A(H3). However, the effects of age for A(H3) were significant following the first dose (S1/S0) and second dose (S2/S1). This could be explained as follows: the HAI titer rise following first dose (S1/S0) was slight for younger children and favorable for older children, but degree of HAI titer rise following second dose (S2/S1) was favorable for younger children and slight for

older children. As a result, the effects of age were significant when separately analyzing first and second doses, but were not significant when combining both doses. This tendency was marked for A(H3), as prevaccination HAI titers for A(H3) were high, particularly among children aged 2 and 3-years. These findings suggest that both existing antibody and age are closely related to immunological response to vaccines.

Acknowledgements

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Intradermal Administration of a Virosomal Influenza Vaccine: A Promising Antigen Sparing Strategy

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Commonly used intramuscular injection of influenza vaccines bypasses the numerous antigen-presenting cells resident in the skin. These antigen-presenting cells, such as dendritic cells and macrophages, render the skin an attractive target for antigen administration, and may require lower antigen amounts in order to elicit an immune response comparable to that following intramuscular vaccination. In the present study, safety and immunogenicity of intradermal administration of a registered virosomal influenza vaccine (Inflexal[®] V) were compared with intramuscular administration in healthy adults. Subjects aged ≥ 18 to ≤ 60 years received single doses of Inflexal[®] V either intradermally (0.1 ml; $n = 23$) or intramuscularly (0.5 ml; $n = 57$). Evaluation of vaccine efficacy revealed comparable immunogenicity profiles for both routes of vaccine administration. Safety was assessed for three weeks after vaccination. After intradermal vaccination, the number of subjects reporting solicited systemic reactions and injection site pain was markedly reduced compared to the subjects assigned intramuscular delivery. Ecchymosis (≥ 5 mm) was reported as a result of intramuscular vaccination only. The tolerability assessment was very good, and 100 % of the subjects assigned intradermal delivery agreed to be re-vaccinated with Inflexal[®] V in the future. The present results suggest a promising antigen sparing strategy for both seasonal and pandemic influenza vaccines.

Introduction

Inactivated influenza vaccines currently in use are administered intramuscularly (i.m.). This route of administration relies on antigen circulation to the lymph nodes, or antigen recognition by transient antigen-presenting cells (APCs). Professional APCs, such as dendritic cells and macrophages, are expressed at higher levels in the skin compared to the muscle. This may render the skin an attractive site for antigen administration, and may require lower antigen amounts in order to elicit an immune response comparable to that following i.m. vaccination. According to this, intradermal (i.d.) administration was proposed as a dose-sparing strategy in case of an influenza pandemic [1]. Inflexal[®] V (Berna Biotech AG, Berne, Switzerland) is an influenza vaccine using virosomes as an adjuvant/carrier system. Virosomes interact efficiently with immunoglobulin receptors on B lymphocytes, and are avidly taken up by APCs [2], thus serving as a carrier/adjuvant system to improve the immunogenicity and efficacy of vaccines [3-6]. Inflexal[®] V has an excellent tolerability and immunogenicity profile, and is

licensed in 40 countries for immunization against influenza in all age groups. The present study assessed the immunogenicity and safety of i.d. administration of a reduced dose of Inflexal[®] V compared with a full i.m. dose in healthy adults.

Materials and Methods

Study design. The study was performed as part of the annual re-licensing study of Inflexal[®] V required by the European Medicines Agency for the Evaluation of Medicinal Products (EMA) [7] as an open-label, non-randomized, uncontrolled, single-center study in Switzerland. A total of 80 healthy adults aged ≥ 18 to ≤ 60 years were enrolled. The subjects were allocated to two groups and received on Day 1 a single dose of 0.1 ml Inflexal[®] V i.d. (Group A; $n = 23$) or 0.5 ml i.m. (Group B; $n = 57$). The study was conducted in full compliance with the principles of the Declaration of Helsinki (as amended in Tokyo, Venice, and Hong Kong) and Good Clinical Practice. The clinical trial was approved by the independent Ethical Committee, and written informed consent to participate in the study was obtained from all subjects prior to any study related activities. **Vaccine.** The virosomal influenza vaccine (Inflexal[®] V) contained purified surface antigens of A/New Caledonia/20/99 (H1N1)-like, A/Wisconsin/67/2005 (H3N2)-like and B/Malaysia/2506/2004-like viruses as recommended by the WHO for the influenza season 2006/2007 [8]. The influenza surface antigens neuraminidase and hemagglutinin (HA) were integrated into phosphatidylcholine bilayer liposomes, yielding unilamellar virosomes with an average diameter of 150 nm [9]. A single i.m. dose of 0.5 ml vaccine contained 15 μ g HA per viral strain, and a single i.d. dose of 0.1 ml contained 3 μ g HA per strain.

Immunogenicity assessment. The immunogenicity was assessed by hemagglutination-inhibition (HI) assays according to the EMA criteria for influenza vaccines, based on seroconversion rates, seroprotection rates and increase in geometric mean titres (GMT) [7]. To meet the requirements set by the EMA, at least one criterion for each viral strain had to be fulfilled. The seroconversion rate is the percentage of vaccine recipients who have an increase in serum HI titers by at least a factor of 4 and a titer of $\geq 1:40$ after vaccination. The seroprotection rate is the percentage of vaccine recipients with a serum HI titer of $\geq 1:40$ after vaccination. The final requirement to be fulfilled describes the fold-increase in GMT of HI-antibodies. Serum HI titers were determined at baseline levels (Day 1) and after three weeks (22 ± 2 days). The analyses of the serum samples were performed at the Quality Control Virology Department, Berna Biotech AG.

Safety assessment. Safety was assessed by recording of adverse events (AEs) for three weeks after vaccination. This included a subject diary in which subjects documented a set of local (pain, induration ≥ 5 mm, erythema ≥ 5 mm, ecchymosis ≥ 5 mm) and systemic (fever $\geq 38^\circ\text{C}/\geq 24$ h, shivering, malaise) solicited AEs according to the EMA specifications [7]. Subjects were also asked whether they would agree to be re-vaccinated with Inflexal[®] V in the future.

Results

Subjects. Group A comprised 14 male and 9 female subjects (mean age 38.3 years). Group B comprised 29 male and 28 female subjects (mean age 44.6 years). All 80 subjects enrolled in the study were evaluable for safety. In Group B, the intent-to-treat population for immunogenicity included 57 subjects; the per protocol population for immunogenicity included 56 subjects.

Immunogenicity assesment. Intradermal administration of one fifth of the conventional i.m. administrated dose of Inflexal® V fulfilled the EMEA criteria for the annual re-licensing of influenza vaccines in adults (aged 18 to 60 years) (Table 1). Three weeks after i.d. vaccination, GMT fold-increases ranging from 6.9 to 19.1 for all influenza strains were observed. These results far exceeded the EMEA requirements, and were comparable to the titres reached after i.m. vaccination (GMT fold-increase 11.3 to 22.7). The seroprotection rate of the B/Malaysia strain after i.d. administration (56.5%) was not in compliance with the EMEA requirements (Table 1). The seroconversion rate and GMT fold-increase for the same strain, however, met the requirements.

Table 1. Efficacy of i.d. and i.m. vaccination in healthy adults aged 18 to 60 years. To meet the EMEA requirements, at least one criterion (seroconversion rate, seroprotection rate or GMT fold-increase) for each viral strain has to be fulfilled.

Serological criteria for meeting EMEA requirements*	Vaccine administration	
	Group A i.d. (3 µg**/0.1 ml)	Group B i.m. (15 µg**/0.5 ml)
	A / New Caledonia	
Seroconversion (%) >40	69.6	75.0
Seroprotection (%) >70	100	98.2
GMT fold-increase >2.5	19.13	12.11
	A / Hiroshima***	
Seroconversion (%) >40	69.6	76.8
Seroprotection (%) >70	91.3	94.6
GMT fold-increase >2.5	13.81	22.67
	B / Malaysia	
Seroconversion (%) >40	47.8	71.4
Seroprotection (%) >70	56.5	75.0
GMT fold-increase >2.5	6.92	11.25

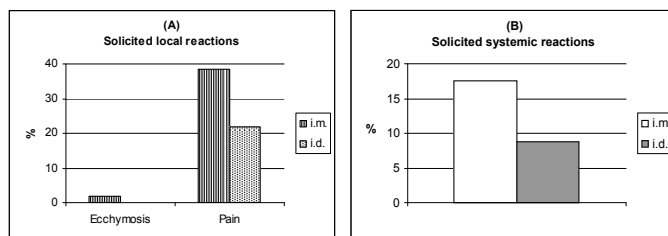
* = Applying to subjects aged 18 to 60 years, ** = Amount of HA per viral strain, *** = A/Wisconsin/67/2005 (H3N2)-like virus, GMT = Geometric Mean Titres, i.d. = intradermal, i.m. = intramuscular. Results that did not fulfill the EMEA requirements are highlighted in bold.

Safety Assessment

Most reported AEs were mild to moderate and resolved within a few days without treatment. There were no serious AEs. Subjects who received the i.d. administered vaccine frequently reported transient erythema ≥ 5 mm (87.0% in contrast to 10.5% after i.m. injection) and induration ≥ 5 mm (82.6% in contrast to 7.0% after i.m. injection). Ecchymosis ≥ 5 mm was reported for 1.8% of the subjects following i.m. vaccination only (Fig. 1A). Pain was reported less frequently after i.d. vaccination (21.7%) compared to i.m. administration (38.6%) (Fig. 1A). Solicited systemic AEs were reported for 17.5% and 8.7% of the subjects after i.m.

and i.d. injections, respectively (Fig. 1B). The tolerability of i.d. vaccination was rated as good or very good by all subjects. Re-vaccination with Inflexal® V would be accepted by 100% of the subjects receiving i.d. vaccination, compared with 98.2% of the subjects assigned i.m. vaccine delivery.

Figure 1. Percentage (%) of subjects experiencing (A) solicited local (ecchymosis ≥ 5 mm, pain) and (B) systemic (fever $\geq 38^\circ\text{C}/\geq 24$ h, malaise and shivering) reactions after i.m. (15 µg HA per strain/0.5 ml) and id. (3 µg HA per strain/0.1 ml) administration of Inflexal® V.



Discussion

The present study showed that Inflexal® V was highly immunogenic and well tolerated after i.d. administration of one-fifth of the usual 0.5 ml i.m. dose. Presumably, the high number of resident APCs in the skin, together with the efficient virosomal delivery system [10], may have contributed to the high immunogenicity observed after i.d. injection of the reduced dose. Similar results were reported in two other studies where i.d. administration of a reduced dose of influenza vaccines induced strong antibody responses in young adults (aged 18 to 40 and 18 to 60 years) [11;12]. The number of subjects reporting erythema and induration was higher after i.d., as compared to i.m. vaccination, which was expected as a result of this route of antigen administration. Intradermal vaccination was well tolerated by all subjects, and the percentages experiencing solicited systemic reactions or injection site pain and ecchymosis were lower after i.d. than after i.m. administration. As only 23 subjects were included in the group assigned i.d. delivery, further studies, also in other age groups, will be needed to confirm the results. In combination with the virosomal delivery system, i.d. administration of a reduced dose of influenza vaccine provides a promising antigen-sparing strategy for universal vaccination, and would help to meet the global need for efficient use of antigen in the case of a pandemic.

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Proceedings Topic #8

Genetic and Antigenic Evolution

**Poster
Presentations**

Genetic Analysis of influenza B (Victoria lineage) Viruses in Taiwan from 2003 to 2006: Multiple Lineages Cocirculating and Reassortment of the Internal Genes

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Introduction

The genome of the influenza B virus consists of eight RNA segments, which encode three polymerase proteins (PB2, PB1, and PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), Matrix (M1) and ion channel (M2), and nonstructural proteins (NS1 and NS2) [1]. The influenza B viruses, like other members of the family *Orthomyxoviridae* has the potential to undergo reassortment characterized by exchange of genome segments between two different strains. Thus, the severity of an influenza epidemic season may be influenced not only by variability in the surface glycoproteins but also by difference in the internal proteins of circulating influenza viruses [2]. The two lineages as defined by the phylogenetic relationship of the HA gene are represented by the reference strains B/Victoria/2/87 (B/Vic) and B/Yamagata/16/88 (B/Yam). B/Vic-like outbreaks were observed in the 2004-05 and 2006-07 influenza season in Taiwan and were associated with several severe and fatal cases in adolescents and adults. Sequencing of all eight gene segments and amino acid comparisons of 42 influenza B epidemic strains isolated during 2003 to 2006 in Taiwan were done to provide a complete profile of protein variability, as well as the evolutionary patterns of these viruses.

Materials and Methods

Throat swabs from sentinel physician networks and other sources, including hospitals, were collected. Specimens were tested for influenza by virus culture and polymerase chain reaction amplification. Sequence analyses were performed using Phylip software version 3.57c and were aligned with ClustalW software. Phylogenetic trees were generated by the neighbor-joining method using the MEGA3 program (Life Sciences, Tempe, AZ), and were carried out for the entire genome of influenza B isolates obtained from January 2003 to December 2006 in Taiwan.

Results

Taiwan B/Vic-like viruses isolated in 2003 and 2004 were phylogenetically grouped mainly around the B/Brisbane/32/2002 or B/Ohio/1/2005 clade. However, viruses isolated in 2005 and 2006 were grouped around the B/Malaysia/2506/2004 clade. Phylogenetic data shows that the B/Vic lineage has little drift from viruses similar to B/

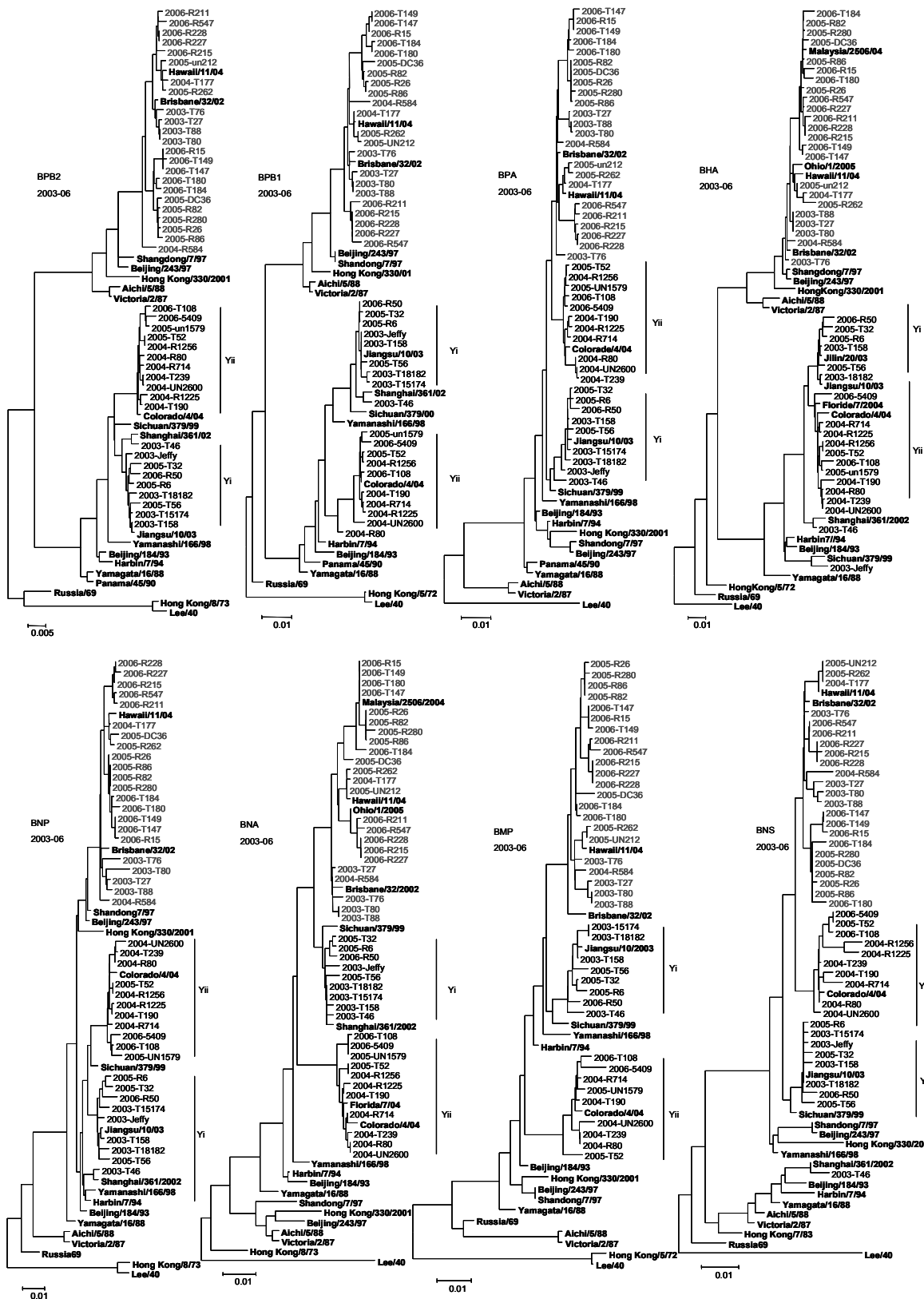
Shangdong/7/97, but more extensive drift has been seen recently. All of the B/Vic viruses analyzed at the molecular level were reassortants that inherited the B/Vic-like HA with a B/Yam-like NA during 2003-2006. Moreover, all isolates of B/Vic-like had B/Yam-like MP, NP, NS, and PA genes. In addition, the phylogenetic trees of these genes are clearly divided into two branch clusters and revealed similar patterns of genetic divergence of the HA, but belong to two different clades consisting of isolates from B/Yam HA lineage (clade Yi and Yii), respectively. For instance, the MP and PA genes revealed their divergence close to the clade Yi, as represented by B/Jiangsu/10/03, while the NS genes were closer to clade Yii, as represented by B/Colorado/4/04. However, the PB1 and PB2 genes were evolving in a manner similar to that of HA genes that remain more similar to the B/Vic lineage. The genetic analysis results indicated multiple cocirculating lineages and genetic reassortment of the internal genes between the two lineages of influenza B viruses that were observed in Taiwan during 2003 to 2006.

Discussion

A significant amount of the impact of influenza was due to the influenza B viruses during 2003 to 2006 in Taiwan. While influenza B infections are usually associated with a lower mortality than influenza A infections, occasional deaths can occur [3]. Comparison of the evolutionary profiles of all 8 gene segments of influenza B viruses revealed that the genes of recent isolates from 2003 to 2006 consistently divided into two major lineages which evolve independently. In contrast to that of influenza A viruses, the observed reassortment among variable cocirculating internal genes of influenza B viruses resulted in more pronounced protein variability. The results of this study provide evidence that genetic exchange among cocirculating B/Yam-lineage and B/Vic-lineage viruses involving gene segments coding for the internal proteins occurs naturally in the human population and that the mechanism of genetic reassortment may be important in virus evolution and pathogenicity. Changes in the internal proteins, as well as antigenic variability in the surface proteins, should be considered when analyzing and predicting newly emerging influenza viruses. In addition, our results indicate that antigenically and genetically distinct influenza B viruses co-circulated in Taiwan during 2003 to 2006 and caused seasonal outbreaks. The genetic reassortment occurring among the internal genes of these cocirculating viruses contributed to the variability of these viruses and gave rise to new variants with distinct genome constellations. Further investigation of the impact of the observed genetic diversity on influenza B virus would be important.

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Figure 1. Phylogenetic relationship of Taiwanese influenza B isolates for all eight genomic segments. Isolates of Victoria and Yamagata lineage were labeled in red and black color, respectively. Two phylogenetic groups of Yamagata lineage were labeled Yi and Yii represented by B/Jiangsu/10/2003 and B/Colorado/4/2004, respectively.



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Heterogenic Selective Pressure Acting on Influenza B Victoria- and Yamagata-Like Hemagglutinins

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As a consequence of immune pressure, some amino acids on the influenza virus hemagglutinin are under positive selection. Using a maximum likelihood method, this work intended to demonstrate the presence of positive selective pressure (PSP) and evaluate its effect on Victoria-like and Yamagata-like influenza B viruses. For this purpose, two groups of HA1 sequences (Yamagata-like and Victoria-like) were analysed with the *codeml* program from PAML 3.15 (Yang, 1997). The presence of positive selection acting on the hemagglutinin of Yamagata-like and Victoria-like influenza B viruses was demonstrated by rejecting the hypothesis of the non existence of sites under PSP when models M3 and M8 were compared to M2 and M7 using likelihood ratio tests (LRT's). The percentage of HA1 codons under PSP was almost identical for both lineages (1.0-1.1%), but with a higher acceptance rate of non-synonymous substitutions for Victoria-like viruses. Different sites were estimated to be under PSP on Yamagata and Victoria hemagglutinins, particularly sites 197 and 199 on both lineages, 75 on Yamagata and 129 on Victoria lineage with posterior probabilities higher than 0.95 (Bayes Empirical Bayes). These amino acids are located at, or near to, antigenic sites of the H3 of influenza A viruses.

Introduction

Several works [1,2] report the existence of influenza A hemagglutinin codons under PSP. However, the existence of these codons in the HA of influenza B viruses has not been a major issue of study. Nevertheless, some authors tried to determine the location of possible antigenic sites on influenza B hemagglutinin [3,4,5,6,7]. Using a codon-substitution model, this work intended to demonstrate the presence of PSP and evaluate its effect on Victoria-like and Yamagata-like influenza B viruses, estimating the acceptance rate of non-synonymous substitutions (parameter $\omega = d_N/d_S$) to describe the strength of selective pressure and identify codons that may be under positive selection.

Material and Methods

Two hundred nucleotide sequences of the HA1 gene segment of influenza viruses (140 from the Yamagata and 60 from the Victoria lineage) were selected for this study, from viruses isolated in different countries since 1987 to 2005. Of these, 67 sequences were obtained from viruses isolated at the Portuguese National Influenza Centre, and 133 were obtained from the *Influenza Sequence Database* website: www.flu.lanl.gov [8].

Nucleotide sequences from each influenza B lineage were analysed separately as two distinct groups. The base phylogenetic tree was obtained by PHYLIP [9] using F84 evolution distances and the Neighbour-Joining method. Aligned sequences with the correspondent base tree were then submitted to *codeml* program from PAML 3.15 [10]. The maximum likelihood estimates were obtained for the codon-substitution models M1a, M2a, M3, M7 and M8 [11,12]. M1a and M7 do not support sites with $\omega > 1$, considering that all sites have ω with values between 0 and 1. The other models consider sites with acceptance rate of non-synonymous substitutions between 0 and 1, but also assume the possibility that a proportion of sites could be under positive selection (ω higher than 1). For those models, which support the existence of PSP (M2a, M3 and M8), the estimated probability of each codon being under positive selection (i.e. class of sites with $\omega > 1$) was also obtained. In order to determine the existence of PSP acting on influenza B hemagglutinin, the likelihood ratio tests (LRT's) were used. Nested models, which differ only by one parameter (the class of sites with $\omega > 1$; i.e., M2a vs. M1a and M8 vs. M7) were compared. Results: M3 and M8 (both models which allow positive selection) were found to be the best-fit codon-substitution models for Victoria and Yamagata lineages, respectively, considering their log-likelihood values. Also, the M8 likelihood value for the Victoria lineage was very close to that estimated with the M3 model (Table 1).

Table 1. Log-likelihood values, acceptance rate of non-synonymous substitutions (ω) and sites under PSP in both main lineages of Influenza B viruses, and likelihood ratio tests to compare nested codon-substitution models.

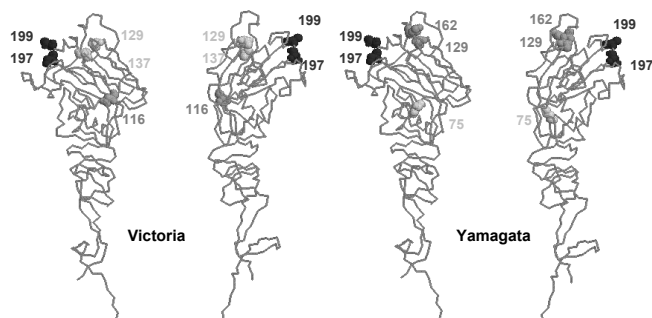
Results <i>codeml</i>	Victoria lineage (n=60)					Yamagata lineage (n=140)				
	likelihood estimates	% sites	ω	Sites under PSP (Victoria numbering)		likelihood estimates	% sites	ω	Sites under PSP (Victoria numbering)	
				NEB	DEB				NEB	DEB
M1a Nearly Neutral	-2927.214820	86.35%	0.06227			-5386.175445	85.46%	0.06659		
M2a Positive Selection	-2910.537648	86.22%	0.06967	197**	129†	-5355.248908	84.69%	0.06887	197D**	75N
		12.93%	1.00000	199T**	197D**		14.58%	1.00000	199T**	199T**
		0.85%	8.63952		199T**		0.73%	6.60086		
M3 Discrete	-2910.610940	87.73%	0.07554	68G*		-5354.888765	82.28%	0.05968	75N	
		11.48%	1.11158	73M*			16.82%	0.85152	197D**	
		0.79%	8.99703	116N**			0.91%	5.88770	199T**	
				129†						
				134V						
				197D*						
				199T**						
M7 Beta	-2929.711330	100.00%	<1			-5389.648206	100.00%	<1		
M8 Beta & $\omega > 1$	-2910.873396	98.86%	<1	129†	68G	-5354.806009	98.97%	<1	75N	75N
		1.14%	>1	197D*	73M		1.03%	>1	116N	116N
				199T**	110N				122S	122S
					129†				129†	129†
					137V				162K	162K
					197D*				197D**	197D**
					199T**				199T**	199T**
					286S				286S	286S
LRT's	$2\Delta\ell$	df	p			$2\Delta\ell$	df	p		
M2a:M1a	33.354344	2	< 0.001			61.853274	2	< 0.001		
M8:M7	37.675868	2	< 0.001			69.684394	2	< 0.001		

The models supporting the existence of sites under PSP (M2a and M8) were compared to those which consider only negative and/or neutral selection (M1a and M7) using LRT's. The hypothesis of nonexistence of sites under PSP was rejected ($p < 0.001$), conferring statistical significance to our results, thus demonstrating the presence of positive selection acting on the hemagglutinin both of Yamagata-like and Victoria-like influenza B viruses (Table 1). The results of the M8 model show 98.97% of

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codons under negative selective pressure and 1.03% of codons under positive selection for the Yamagata-like HA1 subunit. For the Victoria lineage, the percentage of codons under PSP was slightly higher compared with those of the Yamagata-like viruses (1.14%). Different sites were identified to be under PSP on Yamagata and Victoria hemagglutinins by the M8 model (Bayes Empirical Bayes). Amino acid sites 129, 197 and 199 (Figure 1) were found under PSP in both lineages. Codons 197 and 199, in particular, revealed posterior probabilities higher than 0.99 and codon 129 had posterior probabilities of $p=0.96$ and $p=0.87$ in the Victoria and Yamagata lineage, respectively. Codons 75 ($p=0.98$) and 162 ($p=0.84$) were also found to be positively selected on the HA1 subunit of B/Yamagata-like influenza viruses (Fig.1 and Table 1). Sites 137 (with posterior probability $p=0.94$) and 116 ($p=0.80$) (Figure 1 and Table 1) were detected under PSP only in the Victoria lineage. Several other codons, such as 68, 73, 122 and 286, were estimated to be under PSP (Table 1). However, according to the M8 model, they presented nonsignificant probabilities of being under positive selection.

Figure 1. Sites under PSP in Victoria and Yamagata lineages (Posterior Probabilities >0.80 in middle grey, >0.95 in light grey and >0.99 dark grey).



Discussion

Codons were detected under PSP but with a non significant posterior probability (sites 68, 73, 122, 286). Codon 75, estimated by the M8 model as being under PSP in the Yamagata lineage (with a high posterior probability $p=0.98$), seems to be under neutral selection in the Victoria lineage. Codon 116 was found under PSP in both lineages but only with a significant posterior probability ($p=0.80$) among Victoria-like strains. Amino acids 75, 116 and 122 probably form a recently discovered neutralizing epitope of influenza B hemagglutinin which does not belong to neither antigenic sites A or B [7]. In influenza A(H3) hemagglutinin they are positioned in a very close proximity to antigenic site E [13]. Codons 129 ($\omega=4.4$) and 137 ($\omega=4.3$) present high probabilities of being under a strong PSP in the Victoria lineage: $p=0.96$ and $p=0.94$, respectively. In the Yamagata lineage, site 162 with $\omega=3.1$ ($p=0.84$) was found under PSP. Codon 129 also appears to be under positive selection in this lineage but presents a lower posterior probability ($p=0.87$). Both residues 129 and 137 belong to antigenic site A of the H3

hemagglutinin. There are references in the literature that amino acid 137 may constitute an epitope of influenza B/Yamagata-like viruses [6]. Interestingly, in our Yamagata group of viruses, the M8 model results show codon 137 as a conservative (under negative selection) codon. Site 162 is located at H3 antigenic site B. However, in the influenza B structural model [14], site 162 is located very close to sites 129 and 137 (Figure 1). Sites 197 and 199 were estimated to be under a strong positive selection in both Yamagata and Victoria lineages, with the highest posterior probabilities ($p>0.9999$). They presented high values of acceptance rate of non-synonymous substitutions ($\omega=4.6$ for Victoria and $\omega=3.6$ for Yamagata viruses). In a previous work [15] we have found codons 197 and 199 under PSP along with codon 75 (the same methodology was used but with a smaller number of HA sequences not separated in 2 lineages). These codons form a potential N-glycosylation site, located in the region corresponding to antigenic site B of the H3 molecule [3,16] and near the receptor binding site. Nakagawa and colleagues [5] suggest that 197-x-199 site may constitute or influence an epitope. More than once, the glycosylation site 197-x-199 has been described by its influence in the antigenicity of hemagglutinin of influenza type B viruses [17]. It is also well known that the presence (or absence) of this glycosylation site is dependent on the host cell in which the virus is grown [18]. It is also interesting that sites detected under PSP correspond to H3 amino acid residues located near sites described as being under positive selection in influenza A viruses. Despite the identification of different amino acid sites in the two main influenza B lineages, they are apparently positioned at three common locations: 197/199 (influenza A antigenic site B), 129/137/162 (antigenic site A and probably B) and 75/116 (antigenic site E), which may possible constitute hemagglutinin antigenic sites of influenza B viruses.

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Dating the Emergence of Influenza A (H5N1) Virus

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Since the first detection of highly pathogenic avian influenza (H5N1) virus in geese in Guangdong, China, H5N1 viruses have transmitted to poultry throughout southern China. In late 2003 the first transmission wave spread the virus to multiple Southeast Asian countries. In May 2005, the second transmission wave of H5N1 virus westwards to Europe and Africa was initiated following a major outbreak in migratory birds at Qinghai Lake, China, while a third transmission wave has been initiated since mid-2005. Those viruses are now endemic in poultry populations in some affected regions and cause repeated outbreaks in poultry and increasing human infection cases, creating persistent pandemic concerns. Genetic data from systematic surveillance of H5N1 for the past seven years in marketing poultry, along with sequence data from outbreaks throughout the region, provide us with a unique opportunity to estimate the most recent common ancestor (MRCA) and postulate the dates of introduction of H5N1 variants into different affected countries. In this study, we estimated the time of emergence of those three transmission waves, based on their hemagglutinin genes, and compared these MRCA estimates with the date of detection of either human or poultry disease. These analyses indicated that the time interval between the first detection of H5N1 disease and the MRCA for the wave 1 outbreaks, in both Vietnam and Indonesia, was 5 months. For the Qinghai outbreak the MRCA was estimated at three months prior to their first detection among migratory birds. Remarkably, the mean time of the MRCA for the third transmission wave was estimated as exactly the same day as the first virus (Dk/FJ/1734/05) from this lineage was isolated. The early detection of H5N1 viruses in the second and third transmission waves, as compared to the first wave outbreaks in Vietnam and Indonesia, highlights the importance of systematic influenza surveillance in apparently healthy market poultry.

Introduction

The first outbreak of highly pathogenic avian influenza (H5N1) occurred in goose populations in southern China in 1996. Subsequently H5N1 viruses had transmitted to poultry throughout southern China, and have since become endemic in this region. In late 2003 the first transmission (Wave 1) was detected that had originated in southern China and spread to multiple Southeast Asian countries [1,2]. In May 2005, a second transmission (Wave 2) westwards to Europe and Africa was initiated following an H5N1 disease outbreak in migratory

birds at Qinghai Lake, China [3], and a third transmission (Wave 3) was initiated in mid-2005 [4]. Those H5N1 viruses are now endemic in poultry populations in affected regions and cause repeated outbreaks in poultry and subsequent human infection cases with high mortality, increasing pandemic concern. Genetic data from systematic surveillance of H5N1 for the past eight years in apparently healthy market poultry, along with sequence data from outbreaks throughout the region, provide us with a unique opportunity to estimate the most recent common ancestor (MRCA) and therefore postulate the dates of introduction of H5N1.

Materials and Methods

To estimate the time of emergence of the three major transmission waves of H5N1 viruses, we analyzed the hemagglutinin (HA) gene of representative viruses belonging to the major sub-lineages of the A/goose/Guangdong (Gs/GD)-like viruses that also included the recently sequenced viruses isolated from southern China from 2000-2003 [2]. We used the uncorrelated relaxed clock method in BEAST v1.4 using sampling dates and skyline population coalescent priors [5,6]. MCMC chains were run thrice for 20 million generations sampling every 1,000 generations under the codon based SRD06 model [7]. The times of divergence were estimated with a discarded burn-in of approximately 10% using the program Tracer v1.3 [8], and utilizing optimized operator tuning values to increase the efficiency of sampling for subsequent runs.

Results

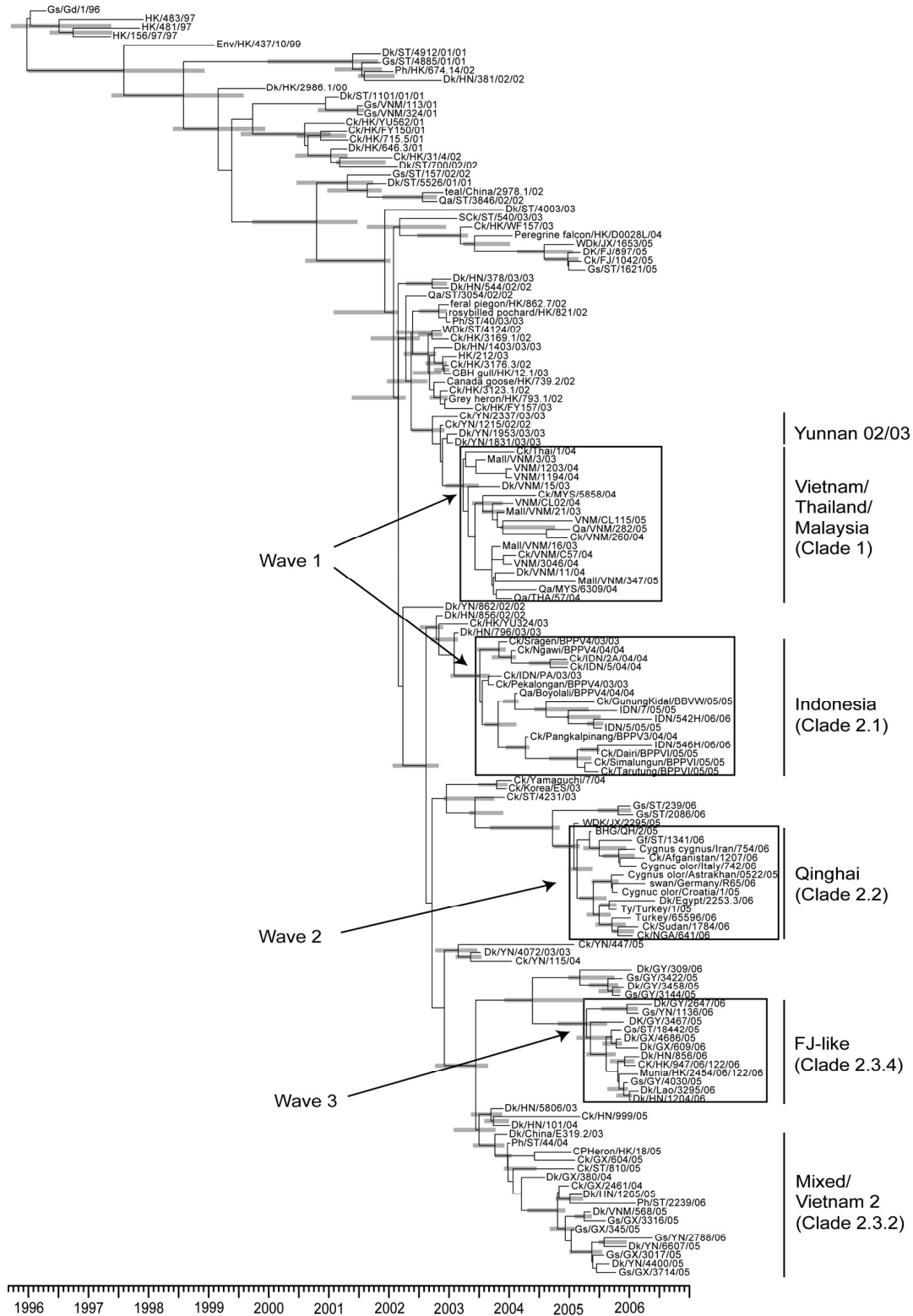
The most recent common ancestors (MRCA) for the three transmission waves were calculated for the HA gene using the uncorrelated relaxed clock method (Figure 1). The MRCA for the Vietnam, Thailand and Malaysia (VTM) lineage (Clade 1) was estimated at Mar/2003 (Highest Posterior Density {HPDs}, Dec/2002, Oct/2003), while the MRCA for the Indonesia lineage (Clade 2.1) was estimated at Apr/2003. (HPDs, Jan/2003, Aug/2003). These results indicate that Wave 1 transmission of H5N1 viruses from southern China to Vietnam and Indonesia was initiated at the same time in early 2003. The emergence of Wave 2 (Qinghai-like, Clade 2.2) and Wave 3 (Fujian-like, Clade 2.3.4) were estimated at March/2005 (HPDs, Jan/2005, May/2005) and Mar/2005 (HPDs, Oct/2004, Aug/2005), respectively, suggesting that MRCAs of the virus that caused an outbreak among migratory waterfowl in Qinghai Lake, China and the emergence of a new H5N1 variant (Fujian-like) in southern China occurred during the same time period in early 2005.

Discussion

In this study, we estimated the time of emergence of the three transmission waves, based on the hemagglutinin (HA) gene. We then compared these MRCA estimates with the date of detection of either human or poultry disease and postulated an establishment time for the virus in the absence of control measures. These analyses indicated that the time intervals

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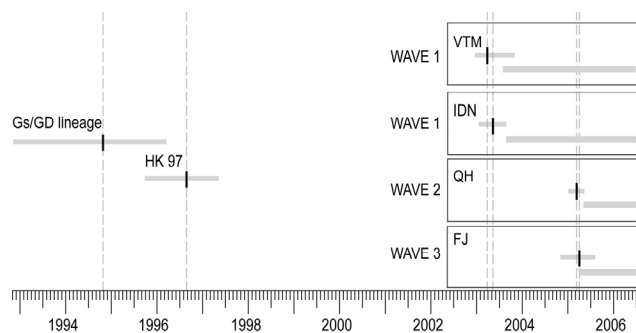
Figure 1. Phylogenetic tree of the hemagglutinin (HA) gene of representative viruses belonging to the Gs/GD lineage. The analysis was based on 963 nucleotides of the HA1 gene. The divergence times correspond to the mean posterior estimates. Bars indicate 95% confidence intervals for the divergence estimates. Tip dates correspond to the date of isolation.



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between the first detection of H5N1 disease and the MRCA for the Wave 1 outbreaks in both Vietnam and Indonesia, that were initiated from Yunnan and Hunan provinces of southern China, was 5 months (Figure 2). For the Qinghai outbreak (Wave 2) the MRCA was estimated at 3 months prior to their first detection among migratory birds. Remarkably, the mean time of the MRCA for Wave 3 was the same day that the first virus (Dk/FJ/1734/05) from this lineage was isolated (Figure 2). The early detection of H5N1 viruses in the second and third transmission waves, as compared to the first wave outbreaks in Vietnam and Indonesia, highlights the importance of systematic influenza surveillance in apparently healthy market poultry in the early detection, and potential for control, of the virus.

Figure 2. Time line of emergence of major H5N1 lineages and disease outbreaks in southeast Asia. Mean divergence times are shown in black; bars indicate 95% highest posterior density (HPD). The bars adjacent to the divergence estimates indicate outbreak/occurrence events.



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The Thousand and One Antigenic and Virologic Faces of Influenza B Virus: Ten Years of Surveillance In Italy

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Introduction

Influenza B virus' evolutionary pattern extremely differs from that of influenza A viruses, accounting for the fundamental distinctions in their epidemiology, host specificity and evolution [Lamb and Krug, 1996; Murphy and Webster, 1996]. With few exceptions, humans are the sole host of epidemiological relevance for influenza B viruses: the main consequence of this fact is that no antigenic shift has been observed in these viruses. Moreover, influenza B viruses are not classified into antigenically distinct subtypes based on the membrane glycoproteins, that consist of single HA and NA type. Even though influenza B viruses undergo antigenic drift, the evolutionary rates of HA gene are slower than those of influenza A strains [Krystal et al, 1983]. Nevertheless, despite the low rate of antigenic changes, two antigenically and genetically different lineages of influenza B viruses have co-circulated in humans since 1987, B/Victoria/2/87 and B/Yamagata/16/88 [Nerome et al, 1998]. Antigenic and genetic analysis showed gradual drift in both Victoria and Yamagata lineages, allowing the identification of two clusters into each one; i. e., B/HongKong/330/01 and B/Shangdong/7/97 for the former lineage, B/Harbin/7/94 and B/Sichuan/379/99 for the second one [Hay et al, 2002]. The proportion of influenza type A and influenza type B viruses circulating during annual epidemics vary each year and in each country. Several studies on the global spread of influenza B viruses have observed that Yamagata-lineage viruses were the predominant B strains circulating worldwide throughout the last decade. During the 2001/2002 influenza season, Victoria-lineage viruses that were mainly confined to East-Asia, reappeared in North America and Europe [Paget et al, 2002; Shaw et al, 2002]. The peculiar epidemiology of these viruses allows strains to presumably circulate silently and subsequently to reappear and co-circulate at the same time, favoring frequent reassortment events [Lindstrom et al, 1999; Hiromoto et al, 2000]. Virological surveillance is a fundamental tool to decide the annual influenza vaccine formulation, in order to obtain a better match between circulating and vaccine strains and a higher rate of protection in vaccinated people. To define the influenza B epidemiological picture in Italy, we described the antigenic and molecular characteristic of B viruses isolated during ten years of virological surveillance, by haemagglutination inhibition (HI), microneutralization (NT) assays and sequence analysis.

Materials and Methods

Influenza surveillance. Italian epidemiological and virological surveillance of influenza is routinely conducted by a national network coordinated by the Inter-University Centre for Research on Influenza (CIRI-IV) and the National Influenza Centre, Istituto Superiore di Sanità (ISS), with the collaboration of regional Health Authorities. The clinical and epidemiological sentinel network is made up of paediatricians and general practitioners, who communicate every noticed case of influenza like illness (ILI) to the surveillance centres and weekly update web publication of the frequency of patients with ILI. Paediatricians and general practitioners belonging to the sentinel network and hospital physicians collect nasopharyngeal swabs from each sick patient, in order to integrate the epidemiological and clinical surveillance with the virological surveillance. One-hundred and ninety-nine B viruses were isolated from about 3000 nasopharyngeal swabs collected from patients with an influenza-like illness since 1996. Twenty-five of them were chosen and antigenically and genetically analysed to characterise the circulating strains.

Viruses. The viruses in this study were obtained by isolation in Madin-Darby Kidney Cells (MDCK). They were chosen as representatives of the four sub-lineages circulating in the ten year surveillance period and were listed as follows: B/GE/184/03, B/GE/185/03, B/GE/191/03, B/LE/01/04, B/GE/02/04, B/GE/06/05, B/GE/07/05, B/GE/03/06 and B/GE/04/06 (B/Victoria/2/87 lineage, B/Shangdong/7/97 sub-lineage); B/GE/55/02, B/GE/57/02, B/GE/61/02, B/TS/12/02 and B/TS/19/02 (B/Victoria/2/87 lineage, B/HongKong/335/01 sub-lineage); B/GE/01/97, B/GE/01/99, B/GE/02/99, B/GE/12/99, B/GE/05/02, B/GE/12/02, B/GE/48/02 and B/GE/53/02 (B/Yamagata/66/88 lineage, B/Sichuan/379/99 sub-lineage); B/GE/56/02, B/GE/12/05 and B/GE/01/06 (B/Yamagata/66/88 lineage, B/Switzerland/6615/01 sub-lineage, an isolate belonging to the B/Harbin/7/94 sub-lineage).

Haemagglutination Inhibition Assay. The HI test was performed using whole viruses from supernatant cultures and hyperimmune sheep serum or post-infection ferret sera to influenza B HA kindly supplied by Alan Hay, W.H.O. Influenza Centre, London, UK, as described elsewhere [Kendal et al, 1982]. HI titres against vaccine and isolated strains were expressed as a reciprocal.

Microneutralisation Assay. Reference antisera used in this test were obtained from chickens challenged with B/Shangdong/7/97-like, B/Hong Kong/330/01-like, B/Sichuan/379/99-like and B/Switzerland/6615/01-like strains, as reported previously [Doowdle et al, 1979]. The test was performed as described elsewhere [Ansaldi et al, 2004].

Phylogenetic Analysis. Molecular characterisation of the globular head region of HA was carried out by sequence analysis of the HA1 subunit (amino acid 1-334), as described previously [Ansaldi et al, 2003]. Construction of a phylogenetic tree was carried out by the Neighbour-Joining method using MEGA package, version 3.1, of the Pennsylvania State University (PA).

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Results

HA and NA phylogenetic analysis performed on isolates selected for this study showed three different influenza B virus circulation patterns, as shown in Table 1.

Table 1. Lineages and sub-lineages of circulating viruses during the last decade in Italy.

Season	Type B isolates (% of total isolates)	Molecular characterization			
		HA		NA	
		Lineage	Sub-lineage	Lineage	Sub-lineage
1996/97	12 (33)	Yamagata	Sichuan/379/99	Yamagata	Sichuan/379/99
1998/99	4 (11.1)	Yamagata	Sichuan/379/99	Yamagata	Sichuan/379/99
2001/02	134 (69.1)	Yamagata	Sichuan/379/99	Yamagata	Sichuan/379/99
		Yamagata	Harbin/7/94	Yamagata	Harbin/7/94
2003/04	2 (1.9)	Victoria	Hong Kong/330/01	Victoria	Hong Kong/330/01
		Victoria	Shangdong/7/97	Yamagata	Sichuan/379/99
2004/05	26 (19.6)	Victoria	Shangdong/7/97	Yamagata	Sichuan/379/99
		Yamagata	Harbin/7/94	Yamagata	Harbin/7/94
2005/06	6 (75)	Victoria	Shangdong/7/97	Yamagata	Sichuan/379/99
		Yamagata	Harbin/7/94	Yamagata	Harbin/7/94

Until 2000/2001 season, all circulating strains belonged to Yamagata-lineage; during the following season Victoria-like viruses reappeared on Italian scene, causing the epidemic peak (69% of total isolates) and co-circulated with Yamagata strains. During the 2002/2003 and 2003/2004 influenza seasons, a low incidence of B isolates has been observed, with the emergence of HA-Victoria/NA-Yamagata reassortants. Phylogenetic analysis showed that reassortant HA and NA belong to the Shangdong and Sichuan-sub-lineage, respectively. The observed HA in circulating strains during the 2002/2003 and 2003/2004 influenza seasons does not seem linked with HA of previous circulating Victoria strains. In the 2004/2005 and 2005/2006 seasons, B viruses represented a significant proportion of circulating strains (19% and 75% of total isolates, respectively). Virological surveillance highlighted a persistent circulation of HA-Victoria/NA-Yamagata reassortants, together with HA and NA-Yamagata viruses. The 2004/05 and 2005/06 reassortant isolates belonged to Victoria lineage, Shangdong/7/97 sub-lineage, in regard to HA, and they phylogenetically grouped mainly to the vaccine strain B/Malaysia/2506/2004. HA and NA-Yamagata strains from these last seasons belonged to Harbin/7/94 sub-lineage (data not shown). Both lineages showed continue evolution with time, even though the evolution pattern was different in the two lineages. The HA belonging to Victoria lineage, Shangdong sub-lineage, showed an annual average evolution rate of 0.5% (1.5 % ± 0.4% in three years), while the two sub-lineages of the Yamagata lineage showed an evolution rate of 0.3% (1.2% ± 0.3% in the Harbin sub-lineage and 1.7% ± 0.4% in the Sichuan sub-lineage in three years). Antigenic relationships between influenza B viruses investigated by the HI and NT assays are shown in Table 2. The HI data emphasized that the two lineages had clearly different antigenic patterns, but the assay was not able to distinguish viruses belonging to different sub-lineages despite the changes over the antigenic sites (data not shown). Viruses belonging to different lineages and clusters are clearly distinguished by NT tests.

Table 2. Antigenic analysis of most representative influenza B isolates (HI and NT Test).

Virus (HA characterisation)	Haemagglutination Inhibition and Neutralization Titres									
	B/Shan/7/97		B/HK/335/01		B/Sich/379/99		B/Guan/120/00		B/Switz/6615/01	
	HI ^a	NT ^b	HI ^c	NT ^b	HI ^c	NT ^b	HI ^c	NT ^b	HI ^c	NT ^b
B/Shangdong/7/97	2560	5233	160	747	<	4	<	<	<	105
B/HongKong/335/01	1280	1023	320	3099	<	4	<	<	<	88
B/Sichuan/379/99	<	255	<	388	320	5455	320	3801	3801	3801
B/Guangdong/120/00	80	-	<	-	320	-	320	-	-	-
B/Switz/6615/01	-	5	-	499	-	1390	-	-	-	>10240
B/Victoria/2/87 lineage										
B/Shan/7/97 sub-lineage										
B/GE/184/03	160	4011	160	612	<	8	<	<	<	87
B/GE/185/03	160	4899	160	756	<	4	<	<	<	102
B/LE/01/04	320	3499	10	2479	<	963	<	<	<	1399
B/GE/02/04	40	2442	10	1270	<	7943	<	<	<	1857
B/GE/06/05	160	-	<	1288	<	2560	160	-	-	-
B/GE/03/06	20	431	80	4096	320	4096	-	-	-	2658
B/Victoria/2/87 lineage										
B/HK/335/01 sub-lineage										
B/GE/55/02	1280	240	160	3011	<	6	<	<	<	106
B/GE/57/02	1280	181	320	2888	<	4	<	<	<	88
B/GE/61/02	2560	140	320	2999	<	<2.5	<	<	<	333
B/Yamagata/66/88 lineage										
B/Sich/379/99 sub-lineage										
B/GE/01/97	<	43	<	22	160	2816	320	4729	4729	4729
B/GE/01/99	<	121	<	206	640	5120	640	2840	2840	2840
B/GE/02/99	<	154	<	129	640	4721	640	2736	2736	2736
B/GE/12/99	40	250	<	107	320	3715	320	3900	3900	3900
B/GE/05/02	80	208	<	322	320	5177	640	2344	2344	2344
B/GE/12/02	80	243	<	502	320	2748	640	3621	3621	3621
B/GE/48/02	80	177	<	198	320	5099	320	851	851	851
B/GE/53/02	80	150	<	446	320	5211	640	3235	3235	3235
B/Yamagata/66/88 lineage										
B/Harb/7/94 sub-lineage										
B/GE/56/02	80	5	<	288	160	1340	160	>10240	>10240	>10240
B/GE/12/05	<	2559	<	2500	320	1663	1280	>10240	>10240	>10240
B/GE/01/06	40	5360	160	6223	640	6309	-	6931	6931	6931

^a: post infection sheep serum.

^b: post infection chicken sera.

^c: post infection ferret sera.

In several examples, the antigenic difference between strains from different sub-lineages emerged from the antigenic relatedness of several influenza B viruses, using the Archetti and Horsfall mathematical evaluation (data not shown). Importantly, NT assays were able to distinguish Yamagata and Victoria strains from reassortants: this capacity has not been consistently shown by the HI assay. Moreover, of the viruses belonging to the B/Sichuan/379/99 sub-lineage, isolates B/Genoa/48/02 had an asparagine (N) residue at position 148, which created an additional potential glycosylation site in antigenic site A and B/Genoa/53/02 presented an aspartic acid (D) at position 197 which caused the loss of a glycosylation site in antigenic site B.

Discussion

The aim of this study was to better define the influenza B viruses evolutionary pattern in Italy during the last decade. In order to reach our goals we conducted an antigenic and molecular analysis on 25 B isolates, selected as representatives of circulating strains from 1996 to 2006 influenza season. Based on sequence analysis data, we found a heterogeneous circulation of different influenza B strains during the surveillance period. Until 2001/2002 influenza season, the unique B strains circulating in Italy were Yamagata-lineage, Sichuan-sub-lineage-like viruses, on line with the circulating strains in Europe and in North America [Paget et al, 2002]. During 2001/2002 season, Victoria-lineage, Hong Kong-like viruses appeared on Italian and on the global scene, with a heavy burden on the Italian population, in which a high rate of susceptible individuals was

present, due to the lack of circulation of this lineage during the 1990s [Ansaldi et al, 2003]. Yamagata-lineage viruses were not replaced by Victoria-like viruses and there was a co-circulation of both lineages, which had been observed during previous seasons [Kanegae et al, 1990; Rota et al, 1990; Rota et al, 1992]. Since the 2002/2003 influenza season, reassortant B viruses, possessing a Victoria-lineage HA and Yamagata-lineage NA, came to be isolated more and more frequently, but they appeared not to be the result of reassortment between the co-circulating strains during 2001/2002 season in Italy, as described previously by Puzelli et al [2004]. These mixed strains were the result of a previous reassortant event between co-circulating B viruses occurring outside Italy [Lindstrom et al, 1999; Shaw et al, 2002]. Phylogenetic analysis of the last three years of isolates confirmed the reassortants continued circulation, together with HA and NA Yamagata-lineage viruses, showing a slow, but continuous genetic drift. In regard to serological analysis, HA and NA-Yamagata strains had low or no cross-reactivity when tested with naïve ferret or chicken sera obtained by infections with a single HA and NA-Victoria virus. Likewise, the same was found when HA and NA-Victoria viruses were tested with HA and NA-Yamagata strains antisera. On the contrary, HA-Victoria and NA-Yamagata reassortants were partially cross-reactive when evaluated with antisera obtained by strains belonging to both Victoria and Yamagata lineage. Our study confirmed the limitations of the HI assay, especially in discriminating the different sub-lineages or variants of B virus, as reported previously [Medeiros et al, 2001; Hay et al, 2002; Ansaldi et al, 2003]. The NT assay was demonstrated to be a sensitive and specific tool for the antigenic characterisation of influenza B viruses, because it clearly discriminated strains belonging to the same lineage and as well as to different sub-lineages. Interestingly, NT test showed that some strains, such as the B/Genoa/48/02 and B/Genoa/53/02 isolates, had a different antigenic pattern from that expected. A possible reason for this antigenic distinction is an amino acid change which creates an additional potential glycosylation site or deletes an existing glycosylation site in the antigenic site A and B of the HA1 globular head, respectively, determining changes in the antibody affinity [Nakagawa et al, 2003; Ansaldi et al, 2004]. Furthermore, NT results highlighted the cross-reactivity of reassortant isolates versus both anti-Victoria and anti-Yamagata antisera, with a high rate of neutralizing antibodies versus all the reference strains. This observed cross-reactivity could be synonymous to cross-protection because the virus' neutralizing antibodies play a key role in preventing infection after vaccination or previous exposure. The complexity of distinguishing lineage and sub-lineage B virus circulation makes selection of the best matching influenza B strain for the annual influenza vaccine difficult. Only the Japanese manufacturers produced a quadrivalent vaccine (with an A/H1N1 and an A/H3N2 virus) containing two B viruses belonging to both Yamagata and Victoria-lineage, but WHO, manufacturers and regulators in other countries, due to impact on production capacity, costs and the lack of time to produce reagents, prefer the classic approach; producing a trivalent

vaccine with a single B component [Oh et al., 1992; WHO, 2007]. Our data support this approach: the cross-reactivity reduces the need for the presence of both lineages in the vaccine formulation, ensuring a partial cross-protection by selection of a reassortant HA-Victoria/NA-Yamagata strain as the single B component.

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A Web-Based Tool for the Clade Designation of Highly Pathogenic Avian Influenza H5N1 Viruses

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The highly pathogenic avian influenza (HPAI) H5N1 viruses are now causing worldwide concern about future pandemics due to their direct transmission from poultry to humans. The lineage information of these viruses is critical to understanding their evolutionary history and predicting their genetic changes. In the case of the HA gene of the HPAI H5N1 subtype virus, a number of clades have been defined in various publications and have been called by many different names. To facilitate resolution of this nomenclature problem and to make comparisons among virus clades easier across publications, the WHO/OIE/FAO H5N1 Evolution Working Group has developed a unified system for the HA clade designation of HPAI H5N1 subtype viruses. Here, we present two Web functions that we recently implemented and made available in FluGenome: (1) a prediction tool for the influenza A virus H5N1 HA clade designation; (2) a Web page for posting information of new sequences and their corresponding clades. The Web tool takes advantage of the clade designation recommended by the H5N1 Evolution Working Group and makes the clade prediction in a manner analogous to an NCBI BLAST search. The latest information about new H5N1 HA sequences and their predicted clades will assist in the surveillance of the geographical spread of H5N1 viruses and their continuing evolution. The above Web functions can be accessed at <http://h5n1.flugenome.org/>.

Introduction

The emergence of highly pathogenic avian influenza (HPAI) H5N1 virus as a significant veterinary and human pathogen over the past decade has been an ever increasing cause of concern because of its significant impact on the global poultry industry and its potential for initiating an influenza pandemic. Because of the rapid geographical spread of the virus and its equally expansive evolution, both in terms of its rate of mutation and level of reassortment, researchers face the challenging task of tracing the movement of the virus using molecular sequence data and, subsequently, identifying the lineage of the virus for classification [1]. As a consequence, many recent publications have used various systems and nomenclature to classify these viruses, further obscuring the relationships among divergent strains (e.g., [1-3]). As such, the classification of H5N1 strains used in phylogenetic analyses, vaccine-related studies, and other research has been complicated by the lack of a unified nomenclature system. A recent collaborative effort encouraged by the World Health Organization (WHO), World Animal Health

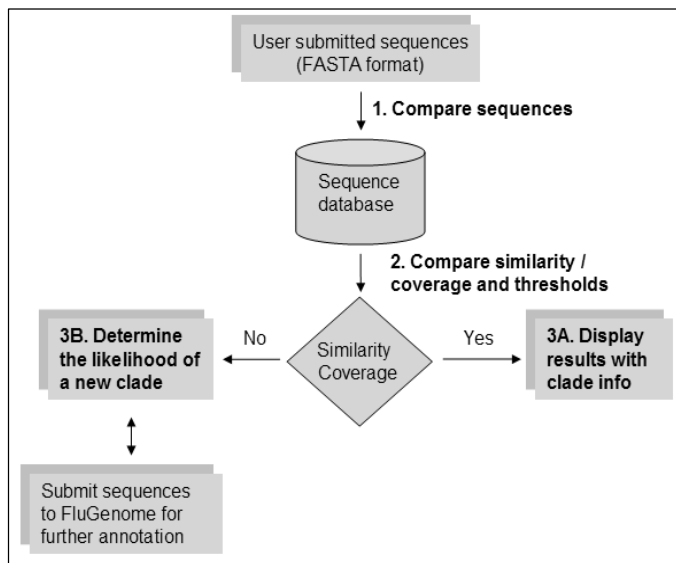
Organization (OIE) and the Food and Agriculture Organization (FAO) (herein referred to as the Evolution Working Group) has developed a nomenclature system for the HPAI H5N1 viruses that is based upon the evolution of the hemagglutinin (HA) gene for the designation of isolates by clade number (RO Donis, personal communication). Using this nomenclature system, we have added a feature to our existing FluGenome database, which allows users to predict H5N1 clade designation based on HA sequence. A clade is composed of all descendants that share a common ancestor and is a key concept in the understanding of viral diversification and evolution. Clades commonly identified through molecular phylogeny are often recognized by subtrees within a larger evolutionary tree [4]. Depending on how the subtrees are defined, different researchers may come up with dissimilar names and numbering systems to define clades even for the same phylogenetic tree. In order to circumvent this problem, the Web-based FluGenome tool has implemented a new feature in the database that will readily define the clade designation of an H5N1 isolate of interest based on the nomenclature system described by the H5N1 Evolution Working Group. For example, the system set forth by the Evolution Working Group has defined 10 unique clades with the H5N1 lineage, numbered 0-9. Within the more highly divergent clades, there may exist a number of subclades (e.g., Clade 2 is further delineated as clade 2.1-2.5). A detailed description of the nomenclature system used for the clade designation component of the FluGenome database will be published elsewhere by the H5N1 Evolution Working Group. Here, we present a new Web tool that was implemented for influenza A virus H5N1 HA clade prediction and a Web page for posting information related to the updates of new H5N1 HA sequences and their predicted clades.

Design and Implementation

The pipeline used for the development of the H5N1 HA clade prediction tool is shown in Figure 1. It involves three steps, (1) sequence comparison, (2) similarity/coverage and threshold comparison, and (3) clade assignment (Figure. 1).

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Figure 1. The pipeline for the determination of HPAI H5N1 HA Clades.



In Step 1, an improved BLAST algorithm is used for pairwise sequence comparison. The coverage and similarity resulting from Step 1 will then be compared with the cutoff values (Step 2). The default cutoff values for HA clade determination are set at 98.5% similarity and 95% coverage based upon the criteria recommended by the Evolution Working Group. These threshold values, however, may be modified as needed. In Step 3 (the clade assignment), there are two scenarios. If the similarity and coverage are larger than the cutoff values, a clade can be determined and the clade of the top BLAST hit sequence will be assigned to the query sequence. Otherwise, no possible known clade can be assigned to the query sequence. In this case, the query sequence needs further analysis for reliable clade assignment.

Sequence Database

We added a new table called H5HA to the FluGenome database. The H5HA table contains four fields, NCBI accession number, sequence name, sequence and clade. This table will be cross-linked with other tables in FluGenome using the accession numbers as foreign keys. A total of 884 publicly available sequences analyzed by the H5N1 Evolution Working Group were stored in the H5HA table. Update of New Sequences and Clades: New sequences are being generated daily. To keep pace with this trend, we created a Web page to post the updated information of new sequences and clades. A script was written to download new sequences from the NCBI influenza A virus database. These new sequences will be analyzed by an automatic lineage prediction tool, which is similar to our Web prediction tool but with some modifications. Newly downloaded H5N1 HA sequences and their predicted clade information will be stored in the local database and concurrently shown on the Web. The database also holds information on when the sequence was downloaded (Date

Processed) and whether the sequence was new and accepted (New Sequence). If a sequence was already found in the database, the sequence is marked 'Updated' and the associated data or information is compared and updated. Sequences that do not meet the criteria for any clade are marked as 'Did not meet thresholds' and processed manually for clade designations.

Discussion

We recently proposed a simple way of naming influenza A viral genotypes, where a letter is assigned to each lineage of the PB2, PB1, PA, NP, and M genes, and a number followed by a letter is assigned to each lineage of the HA, NA, and NS genes with the number representing the subtype or allele and the letter representing the specific lineage of that subtype (e.g., 5J is used to describe the HA for all HPAI H5N1 viruses in the A/goose/Guangdong/96-like lineage, whereas low pathogenic H5N1 viruses from North America are designated 5C). This nomenclature system was exploited to analyze ~2000 complete viral genomes and to date 156 unique genotypes have been identified. A preliminary experiment demonstrated that the nomenclature system for influenza A virus genotypes was particularly useful in that it facilitates the inference of genetic reassortment among viruses of different origin [5]. While the FluGenome genotyping tool allows one to characterize an influenza isolate at the genotype level, the H5N1 Evolution Working Group has developed a unified nomenclature system for the clade designation of HPAI H5N1 viruses based strictly on the HA gene. Thus, addition of the H5 HA clade prediction tool will allow researchers to further characterize the HA clade designation of an H5N1 isolate/sequence of interest. In terms of our clade prediction tool, one of our long-term goals is to improve its selectivity and sensitivity in clade prediction. Towards this end, we are improving the Complete Composition Vector method and the findings will be published elsewhere. Alternatively, the pattern or profile-based algorithms can be applied for clade prediction. For example, the HMM algorithm has been successfully applied in gene or protein family prediction. In the next version, we would like to see if we can use the HMM algorithm for H5N1 HA clade prediction. While this system will greatly facilitate the interpretation of sequence/surveillance data generated in different laboratories, the ever-changing evolution of influenza A viruses will continue to challenge researchers in this field. As new sublineages of HPAI H5N1 emerge and others become extinct, the nomenclature will require continual modification. Additionally, at least 16 HA subtypes have been identified to date and the clade designation system described here only helps to classify a subset of the HPAI H5N1 viruses (i.e., the A/goose/Guangdong/96-like lineage). Is it necessary to have a nomenclature system for each subtype or even each lineage within a given subtype? If so, is there sufficient sequence data available for this type of analysis? Also, extension of this nomenclature system to other gene segments may be possible, but perhaps unnecessary due to the different relative rates of

mutation acting on individual gene segments (i.e., the clade criteria used for the HA gene may not translate over to the M gene). Despite these and other questions concerning influenza A virus nomenclature, it is our hope and recommendation that the influenza virus community continues to work towards a unified nomenclature system for all aspects of influenza A virus classification.

Conclusion

We presented two new functions available in FluGenome, including a Web tool for the prediction of HPAI H5N1 virus HA clades and a dedicated Web page for the update of new sequences and their predicted clade designation. We also discussed the challenging issues facing influenza researchers in regards to the extension of the unified nomenclature system devised by the H5N1 Evolution Working Group to classify H5N1 viruses, as well as other influenza A viruses. It is our strong recommendation that the influenza virus community should work towards a unified nomenclature system not only to define clades within the HPAI H5N1 A/goose/Guangdong/96-like lineage but to uniformly define influenza A virus genotypes as well.

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Identification of Precursors of Indonesia and Vietnam Avian Influenza A (H5N1) Viruses From Southern China

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The transmission of highly pathogenic avian influenza (HPAI) H5N1 virus to Southeast Asian countries triggered the first outbreak wave of this virus in late 2003. Subsequently H5N1 influenza virus has become endemic in poultry in this region, which has led to the sustained transmission of those viruses and repeated outbreaks in poultry and human infection cases. This situation has raised global concern of a coming influenza pandemic sometime in the near future. Although surveillance work in market poultry had been strengthened following this initial outbreak in Southeast Asian countries, the lack of influenza surveillance prior to the outbreaks made it difficult to identify the precursors and transmission pathways of those H5N1 viruses. To determine the possible source of those H5N1 viruses responsible for this first transmission wave we recently conducted further sequencing of samples collected in live-poultry markets from Guangdong, Hunan and Yunnan Provinces in southern China from 2001 to 2003. Phylogenetic analysis of the HA gene of 50 H5N1 isolates from this period indicated that eight viruses, exclusively from Yunnan, fell as the direct progenitor to viruses isolated from Vietnam. A further two viruses isolated from Hunan were the direct precursor to those viruses from Indonesia. In general, phylogenetic analysis revealed similar relationships for the NA gene and each of the 6 internal genes, indicating that these viruses also belonged to the same H5N1 genotype Z that is predominant throughout Southeast Asia. These results clearly show a transmission of H5N1 viruses from Yunnan to Vietnam and from Hunan to Indonesia. Trade of poultry may be the major route of virus transmission between Yunnan and Vietnam, while the transmission route from Hunan to Indonesia remains unclear and could be either via migratory birds or poultry movement.

Introduction

It has been ten years since the first avian-to-human transmission of influenza virus was confirmed in Hong Kong. In the past decade, the "bird flu" caused by highly pathogenic influenza (HPAI) H5N1 virus has developed from an endemic disease in China to an epidemic disease affecting 60 countries in Eurasia and Africa. There have been 3 transmission waves of H5N1. Wave 1 involved transmission to Vietnam and Indonesia resulting in outbreaks in late 2003/early 2004 [1]. Wave 2 was initiated following the Qinghai Lake outbreak in early 2005

with subsequent spread west through Eurasia and Africa, while Wave 3 was initiated in late 2006 with spread to Laos and Malaysia [2,3]. Although surveillance in market poultry was strengthened following the Wave 1 transmission, the source of the Southeast Asian outbreak and how the virus spread from country to country remained obscure due to a lack of data prior to this period. Here we studied 50 sequences of H5N1 influenza viruses isolated from 2001 to 2003 in southern China to investigate the relationships between these viruses and those detected in Thailand, Laos, Vietnam, Cambodia, Malaysia and Indonesia during the period of the first wave of H5N1 outbreaks.

Methods

Viruses were isolated in 9- to 11-day-old embryonated chicken eggs as previously described [4]. Virus isolates were subtyped by standard hemagglutination inhibition (HI) tests using a panel of the World Health Organization reference antisera. Antigenic analysis was performed using five World Health Organization H5N1 reference antisera, monoclonal antibodies to Ck/Pennsylvania/1/83 and four monoclonal antibodies to Ck/HK/YU22/02. To visualize similarity between the antigenic reaction patterns of different viruses, numerical analysis of HI titers was conducted using PRIMER version 5.2.9 (PRIMER-E, Plymouth, United Kingdom). The data were standardized and square-root transformed, and the Bray-Curtis coefficient was used to construct a similarity matrix. Hierarchical agglomerative clustering with group-average linking was conducted and a dendrogram produced. Nonmetric multidimensional scaling was also used to produce two- and three-dimensional ordinations over 100 iterations. The two-dimensional configuration with lowest overall stress is presented. RNA extraction, cDNA synthesis, PCR and sequencing were carried out as described previously [4]. All sequences were assembled and edited with Lasergene 6.0 (DNASTAR, Madison, WI); BioEdit 7 was used for alignment and residue analysis. The program MrModeltest 2.2 was used to determine the appropriate DNA substitution model and rate heterogeneity. The generated model was used in all subsequent analyses. Neighbor-joining and maximum likelihood trees were constructed by using PAUP* 4.0. Estimates of the phylogenies were calculated by performing 1,000 neighbor-joining bootstrap replicates. All eight genes were sequenced for each virus isolate.

Results

Phylogenetic analysis. The HA gene of the 50 H5N1 isolates characterized in this study indicated that eight viruses exclusively from Yunnan (represented by Ck/YN/2336/03) fell as the direct progenitor to Clade 1 viruses isolated from Vietnam, Thailand, Malaysia and Cambodia (Fig. 1A). A further two viruses isolated from Hunan (represented by Dk/HN/795/02) were the direct precursor to those Clade 2.1 viruses from Indonesia. Phylogenetic analysis of those 10 precursor viruses revealed similar relationships for the NA gene and each of the 6 internal genes (represented by the NP gene), indicating

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Figure 1. Phylogenetic relationships of the (A) HA and (B) NP genes of influenza A viruses in Eurasia. Numbers below branches indicate neighbor-joining bootstrap values. Analysis was based on nucleotides 1-963 of the HA gene and 1-990 of the NP gene. The HA tree was rooted to Gs/GD/1/96 and the NP tree to Equine/Prague/1/56. Scale bar, 0.01 substitutions/site.



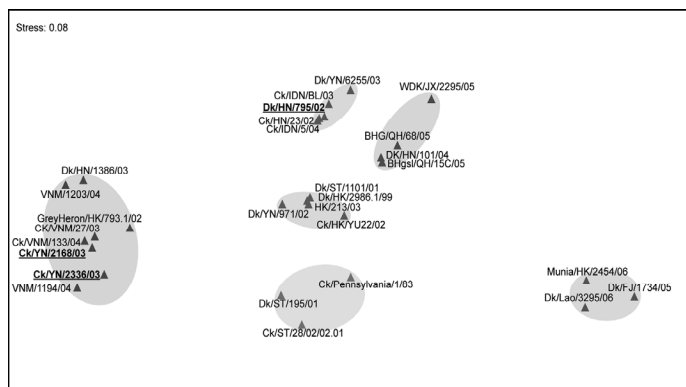
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that these viruses also belonged to the same H5N1 genotype Z that is predominant throughout Southeast Asia (Figure 1B). However, analysis of the remaining 40 viruses isolated from southern China in that period revealed the presence of multiple genotypes of H5N1 virus as previously described [1].

Molecular characterization. All of the 50 viruses characterized were highly pathogenic with variations of the multi-basic cleavage site (QRERRRKKR/G) in the HA molecule. The receptor-binding pocket of HA1 retains amino acid residues Gln 222 and Gly 224 (H5 numbering used throughout) that preferentially bind to α -2,3-NeuAcGal receptors [5]. Other amino acid residues relevant to receptor-binding sites were identical to those of HK/156/97 and Gs/GD-like viruses [5,7] in most isolates, but with some notable differences. Most of the Yunnan viruses characterized had a Ser129Leu substitution (LGVSS), which had been observed previously in viruses from Vietnam [7,8]. While most of the Hunan and Indonesia isolates had SGVSS at positions 129-133. In the NA amino acid sequences, all isolates characterized had 274Y, indicating resistance to oseltamivir. In the M2 protein, all eight Yunnan viruses that are precursors to Clade 1 had both the Leu26Ile and Ser31Asn mutations. These mutations may confer resistance to the amantadines and both are present in all Clade 1 viruses characterized to date [7]. This provides further evidence that these amantadine resistance mutations were present in the viruses that were introduced into Vietnam to form Clade 1. No amantadine resistance mutations were observed in the two Hunan viruses that are precursors to Indonesian isolates.

Antigenic analysis. The hemagglutinin inhibition reaction patterns of viruses from Yunnan and Hunan characterized in this study were similar to those of viruses from Vietnam and Indonesia, respectively (Figure 2). These reaction patterns correspond to their phylogenetic relationships and further suggest that these viruses are the precursors to those Wave 1 viruses.

Figure 2. Numerical analysis of HI titers using nonmetric multidimensional scaling.



Discussion

The outbreaks of H5N1 bird flu in Southeast Asia in late 2003 onset suddenly and spread quickly. However, the source of the virus and the route of introduction into each country were unclear. In our study, the phylogenetic trees showed a close relationship between Hunan and Yunnan with Indonesia and Vietnam, respectively. The antigenic analysis correlated strongly to their phylogenetic relationships. Molecular characterization at the amino acid level also revealed a similar pattern, especially with amantadine resistance mutations in the M2 protein of Vietnam (Clade 1) viruses. These results clearly show a transmission of H5N1 viruses from Yunnan to Vietnam and from Hunan to Indonesia. Trade of poultry may be the major route of virus transmission between Yunnan and Vietnam, while the transmission route from Hunan to Indonesia remains unclear and could be either via migratory birds or poultry movement.

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The Molecular Characteristics of the Hemagglutinin of Human Influenza A/H3N2 in China From 1995 to 2005

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We performed phylogenetic analysis of unbiased HA1 sequences from the H3N2 influenza strains isolated in China from 1995 to 2005. Some potential glycosylation sites on HA1 were very conserved but the numbers on the head of the HA have increased until 1999. Some receptor binding sites (RBS) and the sites near them have changed faster. Amino acid substitutions at all of the known antigen sites have occurred during these years. The most regular variant sites were in the proposed antigen sites or near them. Other mutant sites were also identified at HA residues 25, 50, 57, 121, 122, 124, 186, 202, and 276 HA. The HA1 sequences evolved chronologically. During a larger epidemic period the sequences of different provinces region are more consistent with each other. Between H3N2 epidemics, the HA gene of isolates appears to accumulate more polymorphism. According to these HA1 sequences, several WHO Vaccine strains were not consistent with H3N2 strains in China.

Background

Of the Influenza A viruses, only H1N1, H3N2, and occasionally H1N2 viruses are currently in circulation [1]. H3N2 influenza virus has been the dominant subtype circulating in humans since 1968. The H3N2 HA1 gene is the surface glycoprotein that contains the antibody binding sites and the receptor binding sites (RBS) [2,3] and continuous variation of the HA has been shown by HA1 sequence analysis [4-6]. HA variation is very important in influenza vaccine selection. Here we report sequence data of the HA from unbiased selected virus isolates, including 550 human H3N2 isolates from China from 1995 to 2005.

Materials and Methods

Influenza viruses: The viruses were selected depending on different provincial regions and collection period during influenza surveillance in China. Following isolation, RT-PCR and sequencing of the HA1 region was performed. All sequences were aligned by ClustalX and phylogenetic trees were constructed using Mega3.1 by neighbor-joining methods.

Results and Analyses

1. *Phylogentic tree.* The phylogentic tree based on the deduced amino acid sequence of the HA1 revealed a long trunk with short side branches suggesting that the viruses evolved

chronologically. The short branches showed that the new strains replaced the old ones quickly. Three clusters in the tree showed larger distances from the branches with strains from the previous year. These distances are marked in the figure and correspond to the three large epidemics in China (1995-1996, 1998-1999, 2002-2003).

2. *HA1 amino acid variation.* Table 1 shows that amino acid sites varied every year, but the extent of the changes at these sites was different from year to year. There are 27 antigenic sites of the 42 sites identified by this study and 12 RBS (and nearby residues). The sites of variation are mostly in the antigenic sites and RBS. The larger epidemics often occurred when many sites in antigen domains changed. Before 1995, there were 9 potential glycosylation sites on the HA1 of H3N2. The HA protein gained two potential glycosylation sites at positions 122 and 133 in 1995, while position 276 disappeared. A new glycosylation site occurred at position 144 of the H3N2 HA in 1999. Since 1999, the number of potential glycosylation sites on the HA1 has been conserved. The amino acid substitution rate of the RBS was higher from 1996 to 2005 than during previous years, especially at the 220 loop. Position 226 has changed from L>Q>I/V>I from 1968 to 2005. The RBS variation during these years may be the primary molecular basis for the decreased ability of H3N2 viruses to hemagglutinate chicken red blood cells since 1995.

3. *HA sequence uniformity.* The phylogenetic tree indicated that HA sequences were of high uniformity during 1997-1998 and 2003-2005 when there were epidemic periods in China, while the HA sequences were more divergent from 2001-2002, when China did not experience influenza epidemics.

4. *Vaccine and circulating strains.* In the past ten years in China, there have been five years when circulating viruses appeared to mutate away from the recommended vaccine. These circulating strains were different at many sites in comparison to the recommended vaccine strains.

Table 1. HA1 Amino acid substitutions between WHO vaccine strains and circulating strains from China.

Year	vaccine	China circulating strains	Different sites
1994-1995	A/Shandong/3/93	A/Harbin/3/1994	G53D,G135K,L194I,T213I,Q226I
1995-1996	A/Johannesburg/33/94	A/Nanchang/933/95	I121T, D124G, G172D, N145K, R197Q, Q226V/I, D216N, Y219S, N262S, S278N.
1996-1997	A/Nanchang/933/95	A/Hainan/10/1996	T121N, G124S, D133N, G142R, V190D, I226I/V
1997-1998	A/Nanchang/933/95	A/Syney/S/97-like	T121N, G124S, D133N, G142R, V144I, K155Q, E158K, V190D, V196A, K207K/Q, I226V, K62E, N272K
1998-1999	A/Syney/S/97	A/Guangzhou/62/1999	S142R, K160R, I196L, I226V, H233Y, I31L, R57R/Q,
1999-2000	A/Syney/S/97	A/Guangzhou/998/2000	S142R, K160R, I196L, I226V, H233Y, I31L, R57R/Q, and I72E, T192I.
2000-2001	A/Syney/S/97	A/Hebei/37/2001	all the sites of 1999-2000, and R50G, E83K, S186G, W222R, G225D
2001-2002	A/Panama/2007/99	A/Hubei/56/2002	R50G, E83K, S186G, W222R, G225D, and S22P, L25I, A131T/A, L183H.
2002-2003	A/Panama/2007/99	A/Fujian/411/2002	all the site of 2001-2002, and H155T, Q156H, S189N, S227P
2003-2004	A/Panama/2007/99	A/Jiangxi/424/2004	all the site of 2001-2002 and, Y159F
2004-2005	A/Fujian/411/2002	A/Yunan/1145/2005	K145N, Y159F, S189N, S193F, D225N, V226I, S227F.

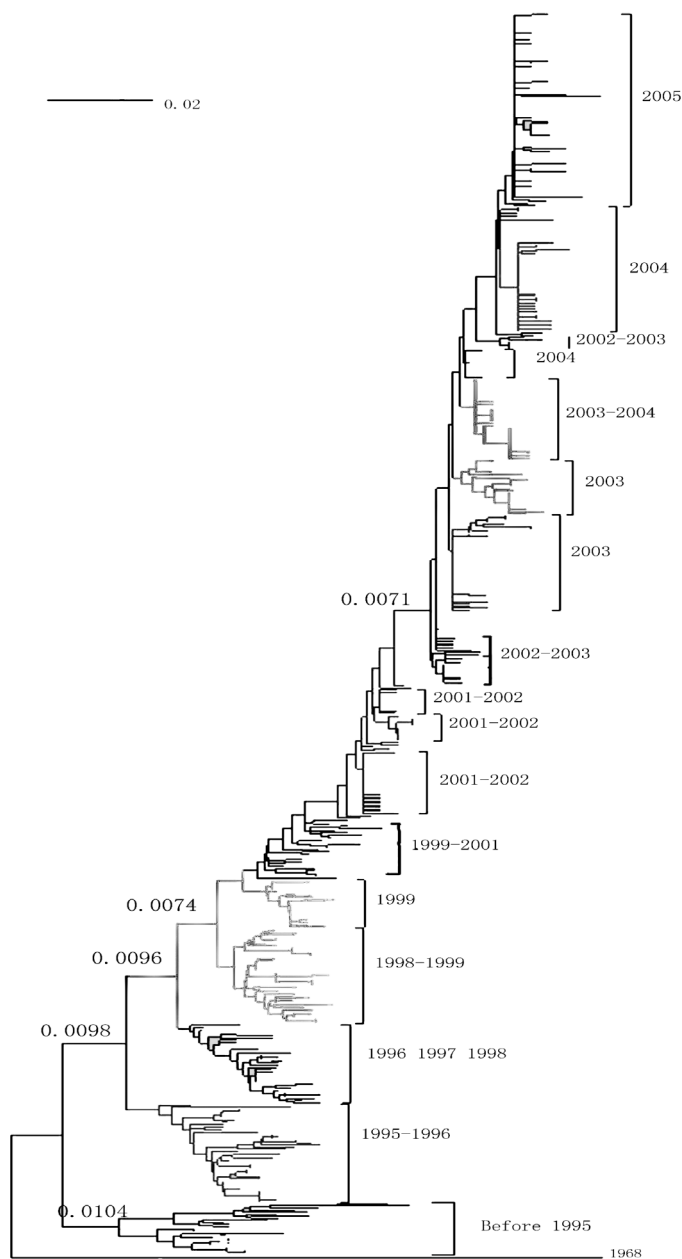
Conclusions

Based on analysis of the HA1 region of viruses from China, circulating H3N2 viruses have evolved continuously from year

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to year. Glycosylation sites on the head of the HA protein have gradually increased and residues making up the RBS and those near the site have changed more rapidly than other regions of the HA1. The 5 known antigen sites also accumulated changes during the years studied. The most regular variation was in the proposed antigen sites or near them. Between H3N2 epidemics in China, the HA sequence of circulating viruses was more polymorphic. Several of the recommended WHO vaccine strains were not very similar at the amino acid level to H3N2 viruses circulating in China according to their HA1 sequence.

Figure 1. Phylogenetic tree of the HA1 amino acid sequence of influenza A H3N2 viruses isolated from 1995 to 2005 in China.



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Genetically Destined Potentials for N-Linked Glycosylation Associated With Antigenic Changes of Influenza Virus Hemagglutinin

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The addition of carbohydrates to influenza virus hemagglutinin (HA) is believed to facilitate viral escape from neutralization by antibodies. It is well known that the number of carbohydrates in HA, especially in the globular head region, changes during circulation of the viruses in the human population. Retrospective sequence analysis confirmed a gradual increase in the number of N-linked glycosylation sequons (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro) in the amino acid sequences of human H3N2 viruses isolated from 1968 to 2006. We then analyzed the potential candidate codons that were not sequons, but able to become sequons with 1-3 nucleotide mutations (i.e., a set of three codons that required single, double, or triple nucleotide substitutions to produce sequons). All of the sites that acquired N-glycosylation sequons during the past 38 years were observed in these candidate codons in HA of the prototype strain, A/Hong Kong/1/68. These results suggest that the genetically destined potential for N-glycosylation in H3 HA could have been one of the key factors prerequisite for this virus to continuously circulate in the human population. Finally, we estimated the potentials of the acquisition of new N-linked glycosylation sites in 16 HA avian influenza virus subtypes, all of which might possibly be introduced into the human population in the future. We found that there was a difference in the capacities to acquire glycosylation sites among these subtypes. These results suggest that avian influenza viruses maintained in natural reservoirs have different pandemic potential, if introduced hypothetically into the human population.

Introduction

Hemagglutinin (HA) is a major target protein of antibodies that neutralize viral infectivity. The accumulation of a series of amino acid substitutions under the selection pressure of neutralizing antibodies results in antigenic changes of influenza virus HAs. The amino acid substitutions associated with N-glycosylation in the HA globular head region are believed to generate efficient antigenic changes. The tripeptide sequon Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro, is required for N-glycosylation of proteins. The number of N-glycosylation sequons in the HA globular head region of H3N2 virus has been increasing during circulation in the

human population for 38 years¹⁻³. Abe et al. hypothesized that the addition of new oligosaccharides to the globular head of H3 HA facilitated the virus to escape from neutralization by antibody without an unacceptable defect in biological activity². H1N1 virus has been also circulating in humans for 30 years since its reemergence in 1977. Recent H1N1 viruses possess more N-glycosylation sequons than the pandemic H1N1 strains that emerged in 1918³. In contrast, HA molecules of H2N2 viruses did not acquire a new N-glycosylation sequon during the period of H2N2 epidemics. Escape mutants of H2N2 virus selected by using the monoclonal antibodies acquired new N-glycosylation sites, suggesting that H2N2 virus also had the potentials to acquire new N-glycosylation sequons⁴⁻⁶. However, due to the additional glycosylation, these escape mutants of H2N2 virus had significantly decreased biological activities of H2 HA. These epidemiological observations and experimental results have led to the hypothesis that the addition of new carbohydrate side chains to HA were an important factor for sustained circulation of H3N2 and H1N1 influenza viruses in the human population^{2,4,7}. In the present study, we traced the history of the codon changes that resulted in the acquisition of sequons in the globular head region of H1, H2, and H3 HAs of the past human pandemic viruses. Furthermore, avian viruses of all 16 subtypes were compared to each other to assess their potentials to acquire oligosaccharide chains under immune pressure in the human population.

Methods

Sequence data of HA genes. Full-length nucleotide sequence data for HA genes of human and avian influenza viruses were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI). We excluded the sequences containing ambiguous nucleotides. We used 373, 17, and 1481 sequences of H1N1, H2N2, and H3N2 human influenza viruses, respectively, for the retrospective analyses and a total of 873 sequences of avian viruses for comparison among HA subtypes. We focused on virus strains isolated from the Anseriformes and Charadriiformes that are known as natural reservoirs of all influenza A viruses. Sequence data of human isolates that might have avian or swine origins were not included in analysis of human influenza viruses. For all HA subtypes, we defined amino acid position 52 to 277 (H3 numbering) as the globular head region. Two cysteine residues at positions 52 and 277 are linked by disulfide bridges. *Tracking of the codon changes directing to the generation of N-glycosylation sequons.* The number of N-glycosylation sequons was counted in amino acid sequences corresponding to the globular head region of HA. We then analyzed the potential candidate codons that were not sequons in HAs of the prototype viruses in previous pandemics (A/South Carolina/1/18 (H1N1), A/Japan/305/57 (H2N2), and A/Hong Kong/1/68 (H3N2)), but became sequons with 1-3 nucleotide mutations. A set of three codons that required single, double, or triple nucleotide substitutions to produce sequons are denoted as *Cand1*, *Cand2*, and *Cand3* sites, respectively. The

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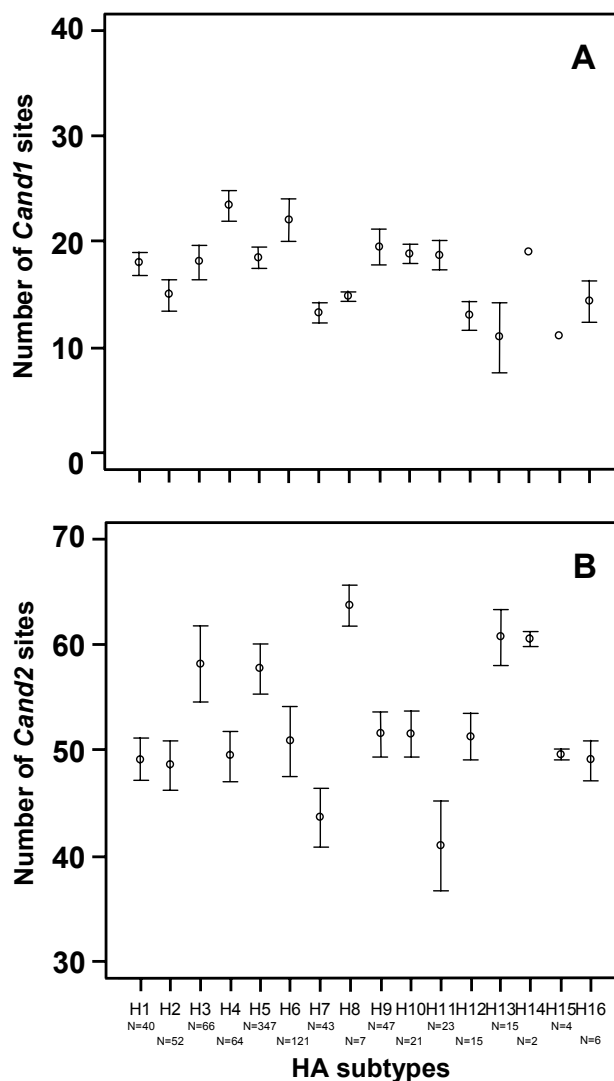
total numbers of these sites were investigated in the globular head region of each HA sequence.

Results and Discussions

N-glycosylation of human influenza virus HAs: Consistent with the previous reports^{1,2,8}, we found that the number of N-glycosylation sequon in the HA of H3N2 virus had gradually increased since its appearance in 1968. Most of H3N2 viruses that circulated in the period from 1968 to 1974 possessed only two N-glycosylation sequons at Asn 81 and Asn 165 or at Asn 63 and Asn 165 in the globular head region of HA. The former set was major and included the prototype strain, A/Hong Kong/1/68. After 1974, most of H3N2 viruses possessed three N-glycosylation sequons at Asn 63, Asn 126, and Asn 165. Then, H3N2 viruses acquired new N-glycosylation sequons at Asn 246 since 1981, at Asn 122 and Asn 133 since 1997, and at Asn 144 since 2001. In summary, the number of N-glycosylation sequons in the globular head region of H3 HA has increased from 2 to 7 in the past 38 years. Recent H1N1 viruses have more N-glycosylation sequons in the globular head region of HA than the pandemic virus that was recently sequenced from a few victims of the 1918 influenza^{2,3}. H1N1 viruses that had reemerged in 1977 possessed five N-glycosylation sequons at Asn 95, Asn 131, Asn 158, Asn 163, and Asn 271 (H3 numbering) in their globular head region of HA. After 1989, most of the H1N1 viruses had lost the N-glycosylation site at residue 158 and acquired a new site at residue 63. Recent H1N1 viruses possess four N-glycosylation sites at Asn residues 63, 95, 131, and 163 in the HA globular head region. The number of N-glycosylation sequons of the H2 HA did not change during the period of H2N2 epidemics (1957-1968)⁴⁻⁶. Most of H2N2 viruses possessed the conserved two N-glycosylation sequons that overlap each other at positions 169-172 (in H3 numbering). These viruses were reported to have only one oligosaccharide chain at residue 169 or 170 in the globular head region^{4,6}. We then investigated whether additional sequons which are found in recently circulating H3N2 and H1N1 viruses were derived either from *Cand1*, *Cand2*, and *Cand3* sites. As we expected, these additional N-glycosylation sequons were mostly *Cand1* or *Cand2* sites in the HA of the prototype virus strains, suggesting that these *Cand1* and *Cand2* sites were important motifs to rapidly acquire N-glycosylation sequons during evolution of the H3N2 and H1N1 viruses in the human population. Because N-glycosylation in the globular head region is believed to facilitate viral escape from antibody neutralization, the presence of *Cand1* and *Cand2* sites in the H3 and H1 pandemic strains could be one of the key factors prerequisite for this virus to continuously circulate in the human population. The genetic background for acquisition of N-linked glycosylation in 16 HA subtypes of avian influenza viruses: Our retrospective analysis of human influenza viruses suggested that the number of *Cand1* and *Cand2* sites may be one of the key factors for influenza viruses to rapidly acquire N-glycosylation sequons. This prompted us to investigate the number of *Cand1* and *Cand2* sites in avian influenza viruses of all 16 subtypes, which

may be potential human pandemic viruses in the future. All 16 HA subtypes were compared each other to assess their potentials to acquire N-glycosylation sequons. Figure 1 shows the average number of *Cand1* (A) and *Cand2* (B) sites for each HA subtype.

Figure 1. Comparison of the number of *Cand1* (A), and *Cand2* (B) sites, among 16 HA subtypes of avian influenza viruses. Open circles represent the mean values of the number of these sites for each HA subtype, and error bars represent the standard deviation.



The average number of *Cand1* and *Cand2* sites varied widely among the HA subtypes. These results suggest different capacities to acquire oligosaccharide chains among HA subtypes. Therefore, we hypothesized that the ability of avian viruses to rapidly acquire the N-glycosylation sequons associated with antigenic changes that allow evasion of the antibody-mediated immune pressure in the human population, may be different among the HA subtypes.

Acknowledgements

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Recent Features of Evolution of Influenza A (H1N1) Viruses in Russia

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Introduction

The circulation of influenza A (H1N1) viruses is not as intensive as it is for influenza A (H3N2) and B [1, 2, 3, 4]. In Russia subtype A (H1N1) caused two monoetiological epidemics during the observation period from 1997–1998 and from 2000–2001, and in other years these viruses co-circulated with influenza A (H3N2) and B viruses. Almost in every epidemic season, A (H1N1) viruses were isolated in Russia and their percentage of all isolated influenza strains fluctuated, but was generally minor. However, this influenza subtype did not always play a secondary role in the epidemic process. Testing of sera from older adults for influenza virus antibodies (so-called seroarchaeology) suggests that influenza in humans between 1918 (the year when the Spanish flu pandemic took place) and 1933 was caused by influenza A (H1N1) [5]. From 1933 up to 1957 the circulation of A (H1N1) was determined by virus isolation. Then after 20 years of intermission, A (H1N1) returned to the human population and remains relevant up to present time [6, 7]. Longstanding observations showed that silent pathways of evolution occur in the hemagglutinin and other genes of influenza A (H1N1) [8], such that in some years epidemic viruses have been shown to emerge not only from the preceding epidemic strain, but from earlier viruses as well. Thus, at any given time, despite the great predominance of one virus genotype, low-level transmission of a different genotype may be occurring and eventually become dominant. For example, antigenic variant A/Taiwan/1/86 (H1N1) predominated all over the world from 1986 to 1995 until two new antigenic variants appeared; strain A/Bayern/7/95 was a direct descendant of A/Taiwan/1/86 and strain A/Beijing/262/95 differed from both Bayern and Taiwan. Since 1997 A/Beijing/262/95 – like viruses have been causing epidemics and outbreaks and may reflect a similar evolutionary situation as was observed previously [9, 10].

Materials and Methods

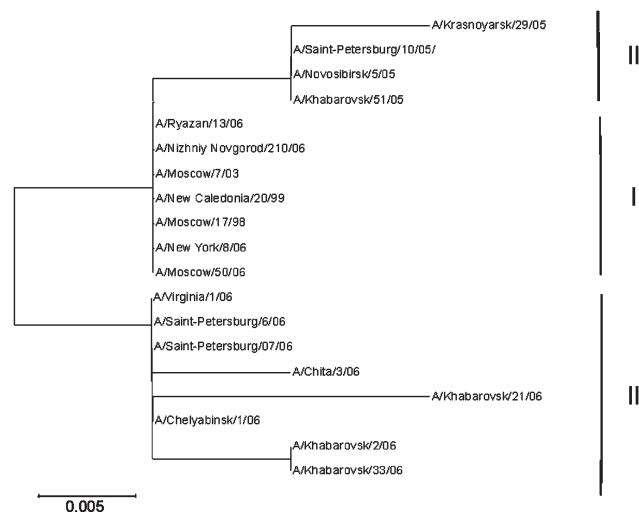
Influenza viruses A (H1N1) were isolated from patients diagnosed with influenza or ILI (influenza like illnesses) in the evolutionary variability of influenza viruses laboratory (Influenza Research Institute, Russian Medical Academy, St. Petersburg, Russia) or they were obtained from the Base Virological Laboratories (BVL) in cooperation with the Federal Center for Disease Control integrated in Influenza Research Institute. Viruses were isolated upon passage in 10-day-old embryonated eggs or in Madin-Darby canine kidney (MDCK) cells according

to the methodic recommendations [11, 12]. The antigenic structure of the viruses was analyzed in the hemagglutination-inhibition test (HI-test) using reference strains and immune sera which were obtained from the WHO Reference Centers. Immune rabbit and rat sera against epidemic and reference strains produced in the Institute for Influenza Research Institute, Russian Medical Academy were also used in the HI-test. The HI-test was performed according to WHO recommended technique: 0.5% suspension of chicken erythrocytes or 0.75% human erythrocytes of blood group 0. In our studies we used influenza strains from the collection of influenza viruses of the Influenza Research Institute. Sequencing of HA and NA was produced in NIMR, London and in the Laboratory of Molecular Virology and Genetic Engineering (Influenza Research Institute, Russian Medical Academy, St. Petersburg, Russia).

Results and Discussion

Antigenic and genetic analyses of present-day epidemic viruses showed that the influenza A (H1N1) isolates of 2004–2005 and 2005–2006 epidemic season were related to A/New Caledonia/20/99 antigenic variant (this strain is antigenically very close to A/Beijing/262/95). Nevertheless, last season the divergent changes of this subtype became evident. The antigenic analysis demonstrated that Russian strains divided into three distinct groups. According to HI (hemagglutination inhibition) tests, the viruses from the first group cross-reacted with antiserum to reference strain A/New Caledonia/20/99 with an up to 1-1/2 homological titer. Viruses of the second group were neutralized with antibodies of the same serum with 2-4 times lower HI titers, and the third ones had 4-8 times lower HI titers. The results of genetic and phylogenetic analyses corroborated the antigenic data. Three genetically distinct clusters of influenza A (H1N1) viruses were shown (Figure 1).

Figure 1. The Phylogenic tree of the observed Influenza A (H1N1).



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The first two clusters were comprised of viruses gradually evolving during the last 7 years. They accumulated 4-5 amino acid changes in the HA molecule. The substitutions took place in positions 69 *leu*→*val* (antigenic site Cb), 165 *val*→*ala* (antigenic site Sa), 251 *trp*→*arg* (significant, non-conserved amino acid substitution), 252 *tyr*→*phe* and 314 *val*→*ala*. The third cluster was distinguished from the two first groups at 9 residues: 69 *val*→*leu* (Cb site), 82 *thr*→*lys* (Cb site) and 94 *tyr*→*his* (significant, non-conserved amino acid substitution), 145 *arg*→*lys* (Ca site), 165 *val*→*ala* (Sa site), 208 *arg*→*lys*, 252 *phe*→*tyr*, 266 *thr*→*asn*, 314 *ala*→*val* as well as from reference-strain, A/New Caledonia/20/99 (in 4 residues). These were changes in positions 82 *thr*→*lys* (Cb site) and 94 *tyr*→*his*, 145 *arg*→*lys* (Ca site) and 165 *val*→*ala* (Sa site). It's important to note, that the registered amino acid substitutions were unique for this viral cluster. Additionally, it is important to mention several strain specific changes. Isolates A/Novosibirsk/5/05 (I cluster) and A/Khabarovsk/21/06 (III cluster) have lost a potential glycosylation site because of changes at residues 54 and 125, accordingly. Substitution 109 *ser*→*pro* (significant, because of appearance of proline, that makes the formation of α -helix impossible) took place in the amino acid sequence of the hemagglutinin of two strains (III cluster) isolated in Khabarovsk in 2006, numbers 2 and 33. Also, it is worth pointing out that the majority of substitutions have happened in antigenically important areas and/or the magnitude of the substitutions have been defined by biochemical features of the variable amino acids. Most of the substitutions also led to the accumulation of positively charged amino acid residues at the surface of the hemagglutinin globular head. Moreover, according to the Kendal and Cox criteria [3], which say that new antigenic variants can be distinguished if there are 4 and more substitutions accumulated in more than 2 antigenic sites, we also can speculate about the need for a new reference strain. The third group was formed only with the strains isolated in 2006. As previously reported by WHO in 2007, influenza A (H1N1) viruses were associated with outbreaks in several countries. Many isolates were antigenically similar to the current reference virus, A/New Caledonia/20/99, but an increasing proportion of recent viruses were more closely related to A/Solomon Islands/3/06, which is recommended to be used in vaccines for the next epidemic season. A similar situation was observed in Russia. During the current epidemic season, the population of influenza A (H1N1) viruses has divided into three groups according to antigenic analysis (Table 1). The strains of the first group were drift variants of A/New Caledonia/20/99 and cross-reacted with antiserum to this reference strain by up to 1/4-1/8 homological titer. The second group was similar to a new reference strain, A/Solomon Islands/3/06 (in HI-test; 1-1/2 homological titer). Finally, the third viral group comprised strains which didn't interact with any contemporary reference antisera at high titers. At present time, we can propose that the last group is composed of genetically diverse influenza A (H1N1) viruses, but that antigenic analysis does not distinguish them.

Table 1. Antigenic structure of the representative influenza viruses A (H1N1) isolated in Russia during epidemic season 2006-2007 in HI test.

№	Influenza Viruses A(H1N1)	Reference rat antisera					
		A/N.C./20/99	A/St.Pb/66/03	A/Nov/5/05	A/St.Pb/7/06	A/St.Pb/46/07	A/Sol.Is/3/06
1	A/N.Caledonia/20/99	1280	640	640	160	160	160
2	A/St.Petersburg/66/03	640	640	320	320	160	160
3	A/Novosibirsk/5/05	320	320	640	640	320	80
4	A/St.Petersburg/7/06	160	320	640	640	320	80
5	A/St.Petersburg/46/07	80	80	160	80	640	80
6	A/Solomon Islands/3/06	40	40	80	80	160	640
7	A/St.Petersburg/41/06	40	80	160	160	160	320
8	A/St.Petersburg/77/07	80	80	160	160	640	640
9	A/Tver/512/07	40	40	80	160	640	640
10	A/Tula/2/07	40	80	320	160	320	320
11	A/Moscow/1/07	320	160	320	320	160	40
12	A/Murmansk/7/07	160	320	320	160	40	40
13	A/Astrakhan/57/07	160	320	160	160	160	40
14	A/St.Petersburg/3/07	80	320	320	320	80	40
15	A/Voronezh/31/07	80	160	160	320	80	40
16	A/Irkutsk/121/07	80	80	160	80	160	40
17	A/Kaliningrad/14/07	80	80	160	160	80	80
18	A/Tula/12/07	40	80	160	160	40	40

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Genetic Analysis of Two Influenza A (H1) Swine Viruses Isolated From Humans in the Philippines and Thailand in 2004 and 2005

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Influenza viruses A/Philippines/341/2004 (H1N2) and A/Thailand/271/2005 (H1N1) were isolated from two males with mild influenza providing evidence of sporadic human infection by contemporary swine influenza. Both viruses were antigenically and genetically distinct from influenza A (H1N1 and H1N2) viruses that have circulated in the human population. Genetic analysis of the haemagglutinin genes found these viruses to have the highest degree of similarity to the classical swine H1 viruses circulating in Asia and North America. The neuraminidase gene and the internal genes were found to be more closely related to viruses circulating in European swine, which appear to have undergone multiple reassortment events. Using the FluGenome web tool, the genotypes of both viruses were determined. However, no previously reported influenza viruses shared the same genotype indicating that these may represent new swine influenza genotypes. Although transmission of swine influenza to humans appears to be a relatively rare event, swine have been proposed as the intermediate host in the generation of novel influenza viruses that may have the capacity to cause human epidemics resulting in high morbidity and mortality. Thus, when humans are infected with swine viruses these viruses should be carefully evaluated and if possible their lineages determined.

Introduction

A wide variety of species, such as humans, swine and birds, are known to be infected by influenza A viruses. Swine are thought to have an important role in the interspecies transmission of viruses as they carry receptors for both human and avian viruses, therefore giving rise to the possibility of acting as an intermediate host able to create novel reassortant viruses of interspecies origin [1]. Influenza A subtypes, H1N1, H3N2 and H1N2 have been reported circulating in swine (2,3), whereas H1N1 and H3N2 are known to infect the human population on a regular basis. To a lesser extent H1N2 has also circulated briefly in the human population [4]. Although

infection of humans with swine influenza is known to occur this still remains a relatively uncommon occurrence, although humans infected with H1N1 and H3N2 of swine origin have been reported previously in North America, Europe and Asia [5,6,7]. The classical H1N1 swine viruses which originally infected European swine herds were replaced in 1981 by 'avian' like H1N1 viruses and these have since co-circulated with H3N2 swine viruses [8,9]. Genetic reassortment between these two subtypes has produced reassortant H3N2 viruses containing six internal genes from the 'avian-like' H1N1 virus along with swine derived HA and NA genes. A H1N2 swine virus derived by reassortment between H1N1 and H3N2 viruses, has also been isolated [10]. Asia has reported all three subtypes H1N1, H1N2 and H3N2 circulating in swine with studies of the prevalent viruses circulating in pigs in southern China showing that the H3N2 swine viruses were closely related to the human H3N2 that had circulated during 1976-78 and in 1982 [11, 12]. In contrast, during 1977- 1980, the classical H1N1 swine strain accounted for most infections in pigs. During the intervening period there appeared to be variation in the prevalence of each subtype and while there was co-circulation of the H1N1 and H3N2 subtypes, the incidence of reassortants was low and there was no evidence of the acquisition of avian influenza virus segments during this period [13]. However, in 1993, there was antigenic and genetic evidence that two different groups of H1N1 were circulating in China, one group, similar to the original classical lineage and a second group more closely related to the 'avian-like' H1N1 viruses circulating in pigs in Europe since 1979 [14]. Japan, Korea and Thailand have variously all reported H1N2 and or H1N1 swine viruses circulating at different times since the 1970s which were related to swine viruses circulating in the USA [15,16,17,18]. During February 2004, influenza A/Philippines/344/2004 (H1N2), was isolated at the Research Institute for Tropical Medicine, Manila and in July 2005 and influenza A/Thailand/271/2005 (H1N1), was isolated by the WHO National Influenza Centre, Bangkok. Both samples were forwarded to the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne for testing where they were found to be antigenically and genetically distinct to the viruses circulating in the human population at the time.

Methods

Viruses. The viruses were passaged in MDCK cells and growth was monitored by CPE and the presence of haemagglutination activity was determined using turkey red blood cells (RBC's) as previously described [19]. The isolates were tested using a standard haemagglutination inhibition assay (HAI) against a panel of reference viruses and their homologous ferret antisera [19]. The BD Directigen™ EZ Flu A+B (New Jersey, USA) and Remel Xpect Flu A&B rapid test kits (Kansas,USA) were used to confirm that cell cultures with CPE contained influenza virus. **Sequencing and phylogenetic analysis.** RNA extraction, RT-PCR and sequencing were performed as previously described [19]. Sequences were assembled using the Lasergene Seqman

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package IV (DNASar 6). The gene segments of each virus were identified by the highest degree of homology based on nucleotide comparisons using the BLAST function on the Influenza Sequence Database [20]. The viruses were further genotyped using the Influenza A Virus web based Genotyping Tool [21] to determine the gene segment lineage and to identify any circulating viruses with a similar genotype. The filter options set were 95% coverage and 85% identity and the segments were searched on the program on 4 June 2007.

Results

Two viruses, one from a 25-year old male from Manila, Philippines (A/Philippines/344/2004) and one from a 4-year old male from Bangkok, Thailand (A/Thailand/271/2005) were sent to the Melbourne WHO Influenza Centre. When the viruses were re-passaged in MDCK cells the cells exhibited typical CPE for influenza and agglutinated turkey RBCs. When tested using two rapid Immunochromatographic-based influenza detection kits, both isolates gave positive results for influenza A. In HI tests using specific ferret antisera raised against contemporary human influenza A, H1 and H3 viruses the isolates were both negative. When the isolates were further tested against broadly reactive hyperimmune rabbit and sheep antisera, they were identified as Influenza A viruses of the H1N1 subtype. RT-PCR using gene specific primers provided further confirmation of influenza A (H1) and full sequences were obtained for all gene segments. These sequences are all available on Genbank (<http://www.ncbi.nlm.nih.gov/>). The HA gene sequences of A/Philippines/344/2004 and A/Thailand/271/2005 were subjected to a BLAST search (www.flu.lanl.gov) that revealed the sequence with the greatest degree of similarity was A/Wisconsin/4755/94, a virus isolated from a human but of swine origin (Table 1).

Table 1. Percentage of similarity between A/Philippines/344/2004 and A/Thailand/271/2005 and viruses with the highest degree of homology based on nucleotide comparisons and also the genotype classification as determined using the Flugenome web server program.

Gene	A/Philippines/344/2004	Geno-type	A/Thailand/271/2005	Geno-type
PB2	94% a/sw/germany/2/81 (H1N1)	F	94% a/swi/italy/2064/99 (H1N2)	F
PB1	93% a/sw/cotes d'armor/790/97 (H1N2)	G	94% a/sw/italy/1521/98 (H1N2)	G
PA	94% a/sw/cotes d'armor/2433/98 (H1N2)	I	94% a/sw/cotes d'armor/790/97 (H1N2)	?
HA	94% a/wisconsin/4755/94 (H1N1)	?	90% a/wisconsin/4755/94 (H1N1)	?
NP	95% a/turkey/germany/3/91 (H1N1)	F	96% a/turkey/germany/3/91 (H1N1)	I
NA	96% a/sw/Belgium/220/92 (H3N2)	2A	94% a/sw/England/195852/92 (H1N1)	F
MP	98% a/turkey/germany/3/91 (H1N1)	F	95% a/sw/hong kong/5200/99 (H3N2)	?
NS	96% a/sw/germany/8533/91 (H1N1)	1E	91% a/sw/tennessee/26/77 (H1N1)	?

The NA gene of A/Philippines/344/2004 was found to be of the N2 subtype and of swine origin, while the NA gene of A/Thailand/271/2005 was found to be of N1 subtype and also of swine origin (Table 1). The internal genes of A/Philippines/344/2004 were found to have the highest degree of homology with swine viruses for the PB2, PB1 and PA genes, whereas for the MP and NP genes had the highest degree of similarity with viruses of avian origin. For A/Thailand/271/2005

the internal genes had the highest degree of homology with swine derived viruses, with the exception of the NP gene which had the highest match with an avian virus NP gene (Table 1). Phylogenetic analysis of the nucleotide sequences of the HA genes of the two viruses indicated that they were related to swine viruses from the classical H1 lineage, which include swine viruses isolated in North America and Asia. When the sequences were compared to that of a contemporary human H1N1 virus, influenza A/New Caledonia/20/99, the HA gene of each virus was found to be clearly distinct with only 74% similarity to the HA gene of A/New Caledonia/20/99. The HA of the two viruses were also only distantly related to each other with 86% nucleotide similarity. When the amino acid differences of the two swine A(H1) HA genes were compared to a contemporary human A(H1) virus A/New Caledonia/20/99 there were some 106 amino acid differences (20.2%) for the A/Philippines/344/2005 and 113 amino acid differences (21.5%) for the A/Thailand/271/2005 HA genes, respectively (Figure 1).

Figure 1. Amino acid alignment of the HA genes of the two human derived swine viruses A/Philippines/344/2004 And A/Thailand/271/2005 compared to a contemporary human influenza A(H1N1) virus A/New Caledonia/20/99.

```

NewCal/20/99 MKAKLLVLLCTFTATYADTCIGYHANNSTDTVDVLEKQVTVTHSVNLLSDSHNGKLCCLLKGAPLQNCVAGWILG 80
Phil/344/04 ...T.V...Y...TAN...L...
Thail/271/05 ...I...LA...AN...L...

NewCal/20/99 NPECELLISKESWSYIVETPNPENGTCYPGYFADYELREQLSIVSSVFERFEIIPKESWPHVT-T-GVSASCSHGKSS 160
Phil/344/04 ...L...TAR...S...SD...D...IN...
Thail/271/05 ...FAVN...S...SD...D...TS...

NewCal/20/99 FYRNLLWLTGKNGLYPNLSKSYVNNKEKVLVWGVHPPNIGDQRLAYHTENAYVSVSHSRRFTPEIAKRKPKVRDQ 240
Phil/344/04 ...I...K...GNS...K...G...I...TST...QS...Q...NAD...F...GT...K...NKK...N...T...
Thail/271/05 ...I...VR...GNS...K...K...I...TNA...QS...Q...NAD...F...G...K...NKK...K...G...

NewCal/20/99 EGRINYYWTLLEPGDTIIFEANGNLIAPWAFALSRGPGSGIITSNAPMDECDACKQPPQGANSSLPFQVHPVTIGEC 320
Phil/344/04 ...A...M...I...T...T...V...R...MK...S...I...D...VHD...NTT...R...T...
Thail/271/05 ...A...M...V...T...T...V...R...MN...DP...D...IHD...N...T...K...T...I...I...

NewCal/20/99 PKYVRSAKLRMTGLRNIPSIQSRGLFGAIGAGIEGGTGMVDGQWGYHQEQSGSYAADQKSTQNAINGITKNVNSVI 400
Phil/344/04 ...K...T...A...
Thail/271/05 ...K...TR...A...

NewCal/20/99 EKMMTQPTAVGKPEFNKLERMENLNKRVDDGFLDIWYNAELLVLENERITLDFHDSNVKQNLKYEKVSQLNKAKEIGNG 480
Phil/344/04 ...H...K...I...R...
Thail/271/05 ...I...H...H...I...V...T...R...

NewCal/20/99 CFEFYHKNCNEMESVKNGTYYDPKYSESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISSPWCMSNGSL 520
Phil/344/04 ...DDT...
Thail/271/05 ...DDT...I...K...E...Q...TRL...A...G...

NewCal/20/99 QCRICI
Phil/344/04 .....
Thail/271/05 .....

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Genetic relationship analysis of the NA nucleotide sequences for the viruses showed that the N1 gene of influenza A/Thailand/271/2005 was more closely related to the 'avian-like' European lineage of H1N1 swine viruses. The N2 gene of influenza A/Philippines/344/2004 also appeared to be more closely related to H3N2 and H1N2 swine viruses circulating in Asia. The nucleotide sequences for the six internal genes of both viruses were compared with sequences available on public databases. The six internal genes of influenza A/Philippines/344/2004 were found to have the greatest degree of similarity to the internal genes of H1N1, H1N2 and H3N2 viruses circulating in European swine. With the exception of the NS gene of A/Thailand/271/2005 the remaining internal genes also appear to have originated from the European lineage. The NS gene differed in that it appears to be more closely related

to North American swine viruses. The two swine viruses were also examined using the FluGenome web based program. The A/Philippines/344/2004 virus had the genotype [F, G, I, ?, F, 2A, F, 1E] corresponding to the [PB2, PB1, PA, HA, NP, NA, MP, NS] gene segments. The A/Thailand/271/2005 virus had a [F, G, ?, ?, I, F, ?, ?] genotype, indicating there were only three shared segment lineages with the A/Philippines virus, the PB2, PB1 and NP segments. The two swine genotypes were clearly different from the classic swine A(H1N1) virus and the recent swine-human-avian triple reassortant viruses which have the respective genotypes of [B, A, C, 1A, A, 1B, A, 1A] and [C, D, E, 3A, A, 2A, A, 1A].

Discussion

Influenza viruses, A/Philippines/304/2004 (H1N2) and A/Thailand/271/2005 (H1N1) were isolated from patients with influenza-like illness and were found, following sequence analysis of the HA gene, to be swine-like viruses. These viruses most closely resembled viruses that have circulated in swine of North America in the 1990s and more recently in Asia. Little is known of the epidemiology of influenza in swine in the Philippines as outbreaks have not been reported to date, therefore it is unknown if this strain is representative of strains that are currently circulating in swine in the Philippines. The isolation of a swine-like influenza virus from man appears to be the first such case reported from the Philippines. Thailand first reported the isolation of influenza A H1N1 viruses in swine in 1988 and these viruses were found to be related to those prevalent in the USA, Japan and Hong Kong [18]. The NA and internal genes of the two viruses isolated in this study were found to have a closer genetic relationship to viruses circulating in European swine, which appear to have undergone multiple reassortment events [22]. When the two swine viruses were analysed using the FluGenome web server program, the lineages of 7/8 gene segments from the A/Philippines virus could be determined, but only 4/8 of the A/Thailand virus. The genotypes were clearly different to each other and to both the classical swine A(H1N1) influenza and the swine-human-avian triple reassortant that has circulated recently in a number of countries. This indicates that both of the swine viruses described in this report are new genotypes that have not been described in previously circulating strains of influenza A. This report highlights two recent infections of humans with swine influenza viruses, who both appeared to recover without further complications following their illness. No other cases of swine influenza were identified in humans at a similar time in these locations, suggesting that there was no human to human transmission. It is not known how the swine influenza viruses were contracted, as neither patient appeared to have direct contact with swine, however, as pigs are commonly raised in backyard farms in these regions, incidental contact with pigs cannot be excluded. While such infections with swine influenza viruses appear to be relatively rare, they highlight the potential for these viruses to cross the species barrier and possibly reassort with other influenza

viruses to enable the efficient transmission of novel viruses from person to person. Thus, it is important to rapidly identify and fully characterise novel influenza viruses such as the swine viruses reported in this study that can cross the species barrier into humans and it is also necessary to monitor the disease severity and transmission rates that these viruses cause in individuals and in the community.

Acknowledgements

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Phenotypic and Genetic Peculiarities of Influenza B/USSR/60/69 Master Donor Strain Based Reassortants

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Our investigation has revealed that the influenza virus B/USSR/60/69 (B/60) master donor strain for live, attenuated influenza reassortant vaccine (B/60-MDV) is a mixed population of cold adapted (*ca*) variants of B/60 virus displaying different levels of temperature sensitivity (*ts*) at 37°C. Part of the *ca* clones isolated in eggs from master donor stock possess *ts* phenotype at 37°C, but the other portion of the clones are *non-ts*. A unique mutation at Val - 644 - Ile in PB1 that is specific for the *ts* clones has been found. Multiple reassortants were generated between the B/60-MDV clone and wild type influenza B *non-ca* viruses. Their reproductive capacity (RCT25) in eggs was evaluated as difference between virus infection titers (log EID50/ml) at optimal (32°C) and low (25°C) temperatures. All the reassortants containing PB1 and PA genes of B/60-MDV expressed *ca* phenotype. RCT25 of this group of reassortants and of B/60-MDV was 1.5–2.0 log EID50. The reassortants containing NP gene of B/60-MDV had better growth capacity at 25°C (RCT25 3.0–3.5 log EID50) compared to wild type virus, but it was lower than that of B/60-MDV. These investigations describe the multicomponent characteristics of the B/USSR/60/69 master donor strain and the stability of live, attenuated reassortant vaccines.

Introduction

B/USSR/60/69 (B/60) influenza virus is the master donor strain (B/60-MDV) for the “B” component of Russian live, attenuated trivalent influenza reassortant vaccine [1-4]. B/60 master strain was derived from the wild type virus after 60 passages in eggs at suboptimal temperature 25°C. It possesses a cold-adapted, temperature sensitive at 38°C and attenuated phenotype. Live influenza vaccine 6/2 reassortants with 2 genes expressing HA and NA antigens from epidemiologically important viruses inherit temperature-sensitivity (*ts*), cold-adaptation (*ca*) and attenuation (*att*) for humans with the 6 internal genes of B/60-MDV. The B/60 master strain temperature sensitivity at 38°C, nevertheless, revealed various degrees of temperature-sensitivity at 37°C in different experiments when propagated in embryonated chicken eggs. To find out the reason for that, the B/60-MDV stock has been cloned and nucleotide sequencing of 2 clones with opposite (*ts* – *non-ts* at 37°C) properties has been performed. In order to define the role of individual mutant genes of B/60-MDV in inheritance of the *ca* phenotype, multiple reassortants were generated between B/60-MDV clone and wild type influenza B *non-ca* viruses.

Methods

Clones of B/60 master donor virus. Plaque assay was carried out

in MDCK cells [5]. After adsorption of virus dilutions, the MDCK monolayers were overlaid with 0.8% agarose containing 2 mg/ml TPCK-trypsin (Sigma) and incubated at 32°C. Three days after infection, clones were obtained from plaques. Plaque assay with isolated subclones was performed once more.

Genetic reassortment. A subclone of donor strain clB/60/15 was used for reassortment, as the *ca* phenotype carrier and another parent was used as the *non-ca* wild type (*wt*) influenza B viruses: B/Harbin/07/94, B/Saint Petersburg/92/95, B/Johannesburg/05/99, B/Jilin/20/03, B/Shanghai/361/02, B/Malaysia/2506/04 or B/Florida/07/04. Cloned B/60-MDV and *wt* virus were co-infected into embryonated chicken eggs. Five rounds of selective propagations were performed. The production and selection for reassortants was performed in the presence of rat anti-B/60-MDV serum. Cloning by endpoint dilution was performed in each of the last two passages. *Determining ca and ts phenotype.* Incubation at 32°C, 25°C, 37°C and 38°C temperatures were used for the characterization of *ca* and *ts* phenotypes of viruses in eggs. Viral growth was expressed as virus infection titers in log EID50/ml. The log EID50/ml calculation was based on Reed and Muench [6]. The reproductive capacity (RCT25 and RCT37,38) were evaluated as difference between virus infection titers at optimal (32°C) and low (25°C) or upper permissive (37, 38°C) temperatures [7].

Genome composition analysis. Viral RNA was extracted from virus containing allantoic fluid using Qiagen viral RNA mini kit (Qiagen Inc., USA). The genome compositions of reassortants were analyzed by RT-PCR and RFLP genotyping [8]. Sequencing reactions were performed using the ABI BigDye terminator cycle sequencing kit with products were resolved on an ABI 3100 Genetic analyzer. Sequences of primers used for amplification and sequencing are available upon request.

Results

Clones of B/60 master donor strain. B/60-MDV stock has extremely heterogeneous phenotype at 37°C (3.5 ± 2.8 log EID50/ml) (Figure 1a). Cloning of B/60 master strain by plaques in MDCK cells has revealed that the B/60-MDV stock is a mixed population of *ca* variants of B/USSR/60/69 virus displaying different levels of temperature sensitivity. All tested clones were temperature sensitive at 38°C in eggs. Though at 37°C part of the tested *ca* clones possess a *ts* phenotype (Figure 1b), but the other part are *non-ts* (Figure 1c). Comparative sequence analysis of the PB1 gene segment of *non-ts* clone B/60/15 and *ts* clone B/60/22 identified a mutation unique to *ts* clone B/60/22:

Gene	Nucleotide position	Amino acid changes in polymerase protein PB1		
		B/60 stock	clB/60/22, <i>ts</i>	clB/60/15, <i>non-ts</i>
PB1	644	Val	Ile	Val

Genome composition analysis and phenotype of reassortants between MDS-B donor strain and current wt viruses. Data obtained from the reassortants studies reveal that PA is the critical mutant gene in conferring *ca* in the B/USSR/60/69 master donor virus. Single wild type PA gene inheritance by B/60-MDV is enough

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for the loss of the *ca* phenotype (Table 1). All the reassortants containing PB1 and PA genes of B/60-MDV expressed the *ca* phenotype. RCT25 of this group of reassortants and B/60-MDV was 1.5–2.0 log EID₅₀. The reassortants containing NP gene of B/60-MDV had better growth capacity at 25°C (RCT25 3.0–3.5 log EID₅₀) compared to wild type virus (RCT25 4.0–7.5 log EID₅₀), but it was lower than that of B/60-MDV. No influence on the *ca* phenotype of wild type M and NS genes were found. (It was shown once that the reassortants containing M gene of B/60-MDV had $\pm ca$ phenotype, but we didn't confirm this result later). We didn't analyse the *ts* phenotype of the reassortants in this study because epidemic strains that were used for reassortment mostly have *ts* phenotype by themselves.

Figure 1. Reproductive capacity of B60-MDV and its subclones at different temperature.

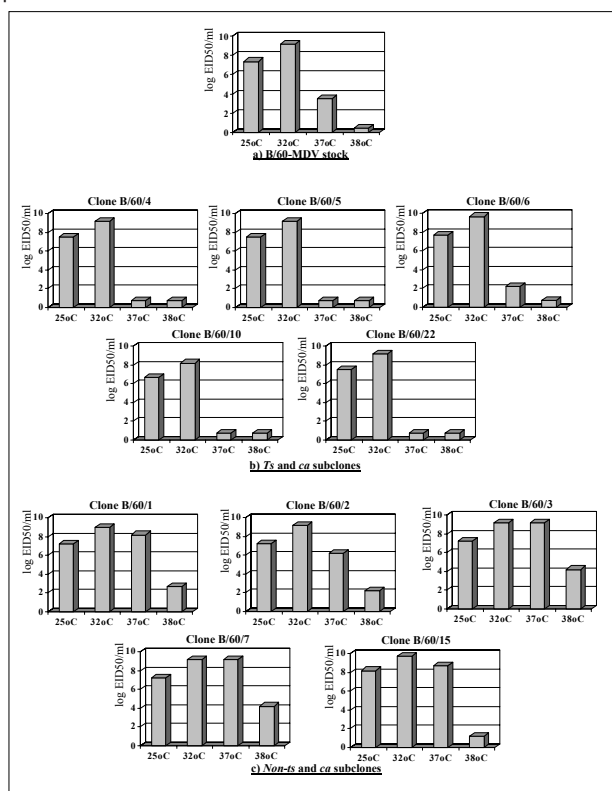


Table 1. Gene constellation and *ca* phenotype of B/60-MDV and wild type viruses reassortants.

Group of clones #	Virus clone genome constellation						<i>ca</i> phenotype
	PB2	PB1	PA	NP	M	NS	
1	60 ¹	60	60	60	60	60	<i>ca</i>
2	60	60	WT ²	60	60	60	<i>non-ca</i>
3	60	60	60	60	WT	WT	<i>ca</i>
4	WT	60	60	WT	WT	60	<i>ca</i>
5	WT	WT	WT	WT	60	60	<i>non-ca</i>
6	WT	WT	WT	60	60	WT	$\pm ca$
7	60	WT	WT	WT	WT	WT	<i>non-ca</i>
8	WT	60	WT	WT	WT	WT	<i>ca</i>

¹Gene belongs to B/60-MDV.

²Gene belongs to wt parental virus.

Conclusion

It was shown previously that mutations in PB2 and PA genes are responsible for *ts* phenotype manifestation of influenza B/60 master donor strain [9]. Comparative sequence analysis of *ts* and *non-ts* B/60-MDV clones identified a unique mutation, specific for *ts*₃₇ phenotype, in PB1 gene segment (Val-644-Ile). The PA and PB1 polymerase genes confer *ca* characteristics. NP internal gene segment from the B/USSR/60/69 donor virus apparently plays an additional role in conferring *ca*. Investigations indicate the essential role of influenza B/USSR/60/69 master donor strain polymerase genes in manifestation of the temperature sensitivity and cold-adapted phenotype of reassortant viruses.

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Phylogenetic Analysis of Hemagglutinin and Neuraminidase Genes of A/Domestic Goose/Pavlodar/1/05 (H5N1) Avian Influenza Virus Strain Isolated in Kazakhstan

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In August of 2005, A/domestic goose/Pavlodar/1/05 avian influenza H5N1 virus was isolated in the infection focus in Kazakhstan. To characterize the virus, the hemagglutinin and neuraminidase genes were amplified and sequenced. Comparative analysis of hemagglutinin and proteolytic cleavage site shows A/domestic goose/Pavlodar/1/05 to be a highly pathogenic strain. According to the results of the neuraminidase gene genetic analysis, the strain has been designated as genotype Z. Phylogenetic analysis of genes demonstrated high homology of A/domestic goose/Pavlodar/1/05 strain neuraminidase gene with that of A/turkey/Suzdalka/1/05 (H5N1) strain isolated in 2005 on the territory of Novosibirskaya oblast (Russia) with a sequence identity of over 99.6%. A high degree of sequence identity (over 99%) of the A/domestic goose/Pavlodar/1/05 hemagglutinin gene with those of viruses isolated in 2005 in Qinghai province (China) was also demonstrated.

Introduction

Avian influenza virus belongs to the family of Orthomyxoviruses, has a spherical structure sized 80-120 nm, and contains 8 segments of single-stranded negative sense RNA that encode 10 viral proteins [1, 2]. Type A influenza viruses are divided into multiple subtypes on the basis of antigenic differences between the envelope hemagglutinin and neuraminidase glycoproteins. At present 16 hemagglutinin and 9 neuraminidase subtypes are known [3, 4]. Lately, outbreaks of the highly pathogenic avian influenza (AI) H5N1 occur worldwide, in particular in China, Korea, Vietnam, Thailand, Japan, Cambodia, Indonesia, Pakistan, and in countries of the European and African continents. It is known that in the course of the avian influenza epizootics in 1997 in Hong Kong and in 2003-2004 in the South-Eastern Asia there were cases of human mortality [5-8]. In Kazakhstan the outbreak of avian influenza H5N1 was reported in 2005 in Pavlodarskaya oblast. The staff members of the Research Institute for Biological Safety Problems have isolated the A/domestic goose/Pavlodar/1/05 (H5N1) AI strain at the focus of the outbreak. This paper presents data on the genetic characterization of this new AI virus isolated in August 2005 in Pavlodarskaya oblasts, the Republic of Kazakhstan. To determine the similarity with AI strains isolated in Russia, China and Mongolia, the hemagglutinin and neuraminidase genes were sequenced and phylogenetic analysis against database of Genbank was conducted.

Materials and Methods

Viruses. The influenza virus used in the research was isolated from a dead goose during the outbreak of highly pathogenic AI in Pavlodarskaya oblast (Republic of Kazakhstan) in August of year 2005.

RNA Isolation. The viral RNA was extracted with use of the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the instructions from the manufacturer. The RNA was eluted with 40 µL of RNase free water.

Gene Amplification. A set of sequencing primers designed by Vector NTI Suite 9 and Oligo 6 programs were used to sequence the hemagglutinin and neuraminidase genes. Primers were synthesized by oligonucleotide synthesizer Expedite 8909 (Applied Biosystems, USA) according to the manual enclosed. Gene fragments were produced with use of the OneStep RT-PCR kit (Qiagen, Germany) in the reaction mixture of 50 µL in volume. Extraction of PCR fragments from agarose gel and their purification was performed by using QIAquick Gel Extraction Kit (Qiagen, Germany).

Virus Genome Sequencing. Sequencing was performed with the ABI PRIZM 310 Genetic Analyzer (Applied Biosystems, USA). Nucleotide sequence was determined by using BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 1.1 (Applied Biosystems, USA). The sequenced DNA fragments were analyzed and integrated with the Sequencher 4.5 program. *Phylogenetic Analysis.* Alignment and phylogenetic analysis of nucleotide sequences derived from sequencing was performed by the neighbor-joining method with use of Vector NTI Suite 9 and BioEdit v.7.0 programs.

Results and Discussion

The influenza virus genome contains 8 RNA segments each of them encoding different proteins involved in the processes of replication and assembly as well as outer envelope glycoproteins and nucleocapsid proteins. The hemagglutinin and neuraminidase proteins that are responsible for attachment and cell to cell spread and govern the virus pathogenicity are among the most important proteins. At the initial stage of sequencing the hemagglutinin and neuraminidase genes, a set of primers were generated that allowed us to obtain overlapping cDNA fragments. Eight pairs of primers were selected for sequencing HA and NA genes. The size of the HA gene sequenced was 1737 nucleotides, and the NA gene was 1339 nucleotides long. Comparative analysis of NA gene against sequence data of Asian and Russian AI H5N1 viruses using the BLAST program showed that the sequences identity between them exceeded 99%. The HA gene analysis gave similar results. The data obtained with the BLAST program was confirmed by the Vector NTI Suite 9 program using the same viruses for comparative analysis. Currently there is a large amount of data on gene sequences of AI virus strains isolated in different countries [9-11]. Therefore, we focused our analysis on viruses that were likely to provide information regarding the introduction of this virus into the Republic of Kazakhstan. Alignments of the NA and HA genes of A/Domestic Goose/Pavlodar/1/05 (H5N1) strain was carried out with the strains of the avian influenza virus isolated in

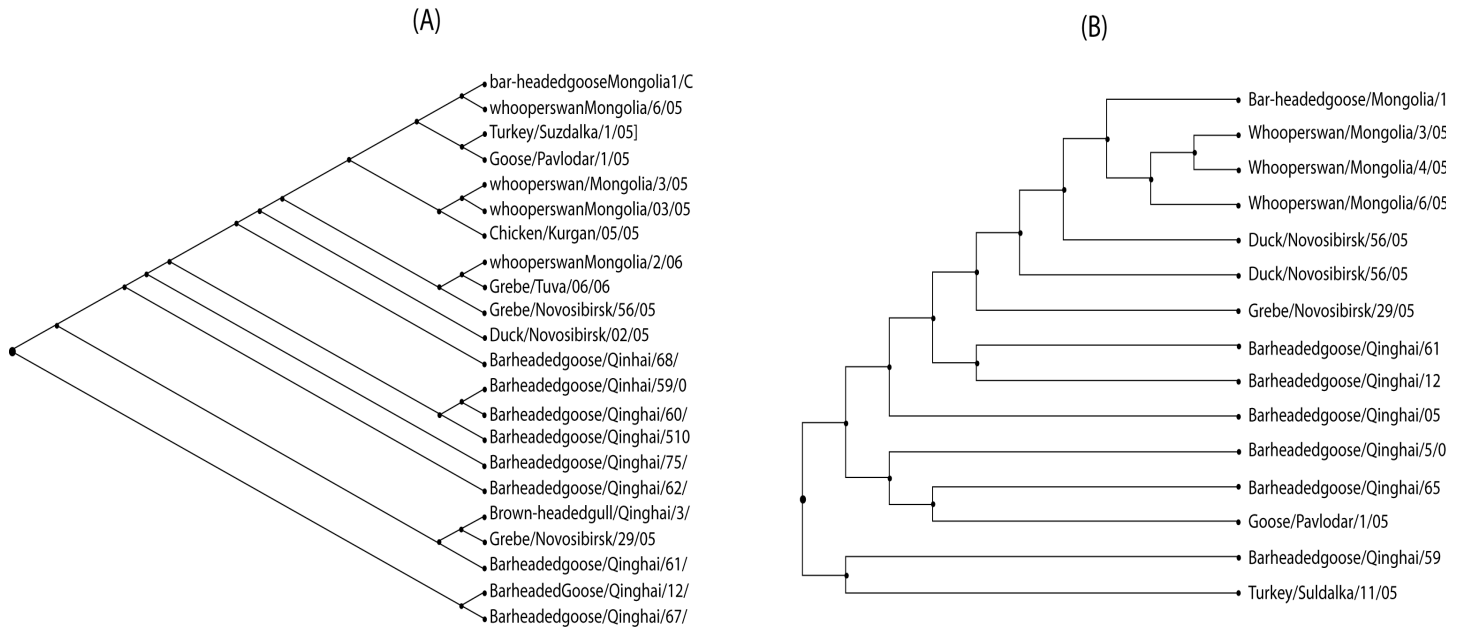
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Novosibirskaya oblast (Russia), Qinghai province (China) and in Mongolia in 2005.

Phylogenetic analysis of A/domestic goose/Pavlodar/1/05 strain (H5N1) showed that the neuraminidase gene is highly homologous to to all strains presented on Figure 1-A but the highest nucleotide identity was demonstrated to the Russian strain A/turkey/Suzdalka/Nov-01/05(H5N1) isolated in 2005 in Novosibirskaya oblast, Russia, as well as to the strains A/whooper/swan/Mongolia/6/05(H5N1) and A/bar-headed goose/Mongolia/1/05(H5N1) isolated in 2005 in Mongolia. Phylogenetic analysis for hemagglutinin revealed a somewhat different picture. Like the analysis of the neuraminidase gene, the hemagglutinin gene exhibited high identity with those of strains isolated in the territories of Russia, China and Mongolia in 2005 (Figure 1-B), but exhibited the highest degree of homology

to strains isolated in Qinghai province (China) in 2005, such as A/Bar-headed Goose/Qinghai/65/05(H5N1) and A/Bar-headed Goose/Qinghai/5/05(H5N1). These results confirmed that the hemagglutinin of the AI virus strain isolated in Pavlodarskaya oblast, Kazakhstan, in 2005 belongs to subtype H5 and N1 subtype. Pathogenicity of the virus substantially depends on the structure of the site of proteolytic cleavage of the hemagglutinin molecule [12]. As such, the amino acid sequence of the hemagglutinin cleavage site was analyzed to assess the potential of the virus pathogenicity. Figure 2 presents the differences in the site of the hemagglutinin proteolytic cleavage of A/domestic goose/Pavlodar/1/05 strain and other highly pathogenic avian influenza H5N1 virus with high hemagglutinin sequence identity.

Figure 1. Phylogenetic analysis of HA (A) and NA (B) genes of the AI virus strains isolated on the territories of Kazakhstan, China, Mongolia and Russia.



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Figure 2. Comparative analysis of amino acid sequence of the proteolytic hemagglutinin cleavage site in different H5N1 strains.

	320	330	340	350	360	370
A/goose/Pavlodar/1/05	ECPKYVKSNRLVLA	SLRNS	PKSRRRKKR	ELFGAIA	SPIEE	SWQKMQVDEWTCYI
A/Cygnus olor/Astrakhan/Ast05-2-2/2005 (H5N1)
A/duck/Kurgan/08/2005 (H5N1)
A/chicken/Kurgan/05/2005 (H5N1)
A/duck/Novosibirsk/56/2005 (H5N1)
A/Bar-headed Goose/Qinghai/5/05 (H5N1)
A/Cygnus olor/Czech Republic/5170/2006 (H5N1)
A/goose/Krasnoozerska/627/2005 (H5N1)
A/chicken/Crimea/08/2005 (H5N1)
A/mute swan/Bavaria/12/2006 (H5N1)
A/wild duck/Omsk/103-01/05 (H5N1)
A/whooper swan/Mongolia/3/05 (H5N1) R
A/chicken/Sudan/1784-7/2006 (H5N1)
A/chicken/Guangdong/174/04 (H5N1) R
A/Dk/Indonesia/M8/2004 (H5N1) R
A/chicken/Jilin/9/2004 (H5N1) R
A/duck/Guangxi/50/2001 (H5N1) R
A/chicken/Bangkok/Thailand/CU-3/04 (H5N1) R
A/duck/Shanghai/xj/2002 (H5N1) R
A/mallard/Guangxi/wt/2004 (H5N1) R
A/Hong Kong/213/03 (H5N1) R
A/chicken/Viet Nam/LD-080/2004 (H5N1) R
A/chicken/jiyuan/1/03/ (H5N1) R
A/duck/Zhejiang/52/2000 (H5N1) R
A/duck/Hong Kong/821/02 (H5N1) R
A/chicken/Thailand/Kanchanaburi/CK-160/2005 (H5N1) R
A/chicken/China/1204/04 (H5N1) R

As the figure shows A/Domestic Goose/Pavlodar/1/05 AI virus strain has the same amino acid sequence of the proteolytic hemagglutinin cleavage site P₃₃₇QGERRRKKRGLF₃₄₉ as other H5N1 strains isolated in Russia and China in 2005. The determination of the structure of the proteolytic cleavage site allowed us to confirm that the strain under investigation was indeed a highly pathogenic avian influenza virus [13]. To determine the genotype of A/domestic goose/Pavlodar/1/05 virus, neuraminidase gene sequences were compared. A/Domestic Goose/Pavlodar/1/05 strain possesses a 20- amino acid deletion at positions 49-68 and this is evidence that it belongs to the genotype Z. This 20 amino acid deletion in the neuraminidase stalk region is considered to be a key adaptation that occurs in H5N1 viruses as they spread among domestic birds. This deletion in the neuraminidase stalk region occurs in many highly pathogenic AI virus isolated after 2001 however this mutation was not found in strains isolated before the year of 1999 [14,15].

Conclusion

We have described nucleotide sequence characterization of the hemagglutinin and neuraminidase genes of A/Domestic Goose/Pavlodar/1/05 virus. The comparative analysis has shown that the isolated virus is of subtype H5N1 and possesses a specific site of proteolytic cleavage that is typical for highly pathogenic strains. Phylogenetic analysis of the strain demonstrated a high level of nucleotide sequence identity with A/turkey/Suzdalka/Nov-01/05(H5N1) virus for the neuraminidase gene and to A/Bar-headed Goose/Qinghai/65/05(H5N1) virus for the hemagglutinin gene. The presence of a 20 amino acid deletion

in the neuraminidase of A/Domestic goose/Pavlodar/1/05 is evidence that it belongs to genotype Z of H5N1 viruses. Highly pathogenic avian influenza has been reported in the territory of the Republic of Kazakhstan in August 2005 following the outbreaks of the highly pathogenic avian influenza in China and Russia. The information about the high level of homology of the neuraminidase gene A/Domestic Goose/Pavlodar/1/05 virus with that of H5N1 strains isolated in Russia strongly suggests that the virus was introduced from Novosibirskaya oblast of Russia. Furthermore, the high degree of nucleotide sequence identity of the HA gene with viruses from Qinghai, confirms that the virus originated from China.

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Phylogenetic Analysis of the HA1 Coding Region of Influenza B Strains (From 1994 to 2006)

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Nucleotide sequences of the HA1 region of influenza B isolates were aligned by the Clustal method and their phylogeny was constructed by the Neighbour-Joining algorithm. The best-fit nucleotide substitution model was found using maximum likelihood estimates (Yang, 1997). Synonymous and non-synonymous substitutions per site were estimated using the method of Yang and Nielsen (2000). In order to understand which domains of influenza B hemagglutinin are influenced by positive selection, nucleotide sequences were analysed by a maximum likelihood method using the program *codeml* (Yang, 1997). The null hypothesis that selective pressure along the sequence is either neutral or deleterious was rejected, demonstrating the existence of positive selective pressure acting on hemagglutinin of type B viruses.

Introduction

The influenza type B hemagglutinin gene segment encodes the main surface antigen responsible for inducing the immune response to influenza infection. Influenza B viruses present different epidemiological and evolutionary patterns when compared to influenza type A viruses, such as slower evolution rates, co-circulation at the same time of different lineages (Victoria87 and Yamagata88) and their evolution seems to be related with an insertion-deletion mechanism and reassortment of gene segments [1,2]. We have conducted sequence and phylogenetic analysis based on the HA1 domain-coding region aiming to describe the evolution of influenza B viruses isolated in Portugal since 1994/1995 until 2005/2006 winter season.

Material and Methods

Seventy eight sequences of the HA1 region were obtained from isolated influenza B viruses (since 1994 to 2006) after RT-nested PCR amplification [3] and then aligned by the Clustal method. Evolutionary distances (estimated with F84 nucleotide-substitution model) and the base tree file (by Neighbour-Joining algorithm) were obtained by PHYLIP [4]. Aligned sequences with the correspondent base tree were then submitted to *baseml* and *codeml* programs from PAML 3.15 [5]. The best-fit nucleotide substitution model was found using maximum likelihood estimates [5]. Hierarchical likelihood ratio tests (LRT's) were used for comparison between nested evolution models, starting from the simplest model Jukes-Cantor 69 (JC69) to the most complex model, General

Time Reversible (GTR). The phylogenetic tree was constructed with estimated nucleotide distances obtained by the best-fit nucleotide-substitution model. Synonymous and non-synonymous substitutions per site were estimated between sequences obtained in this study and their respective prototype strain B/Victoria/2/87 or B/Yamagata/16/88, using the YN00 method [6] included in PAML, and then plotted against the time of divergence since these are reference strains. The *codeml* programme was used to find the codon-substitution model that better explained our sequence data. To test the presence of positive selective pressure (PSP), nested models differing only by one parameter (class of sites with $\omega > 1$), were compared by LRT's. This program also estimated the positively selected codons.

Results

Maximum likelihood estimates were obtained for several nucleotide-substitution models, varying in complexity (GTR+ Γ , GTR(REV), TN93+ Γ , TN93, HKY85+ Γ (clock), HKY85+ Γ , HKY85, K80+ Γ , K80, F81+ Γ , F81, JC69+ Γ , JC69). Hierarchical LRT's have confirmed the Hasegawa-Kishino-Yano 85 plus Gamma (HKY85+ Γ) as the best-fit evolution model. This model assumed a transition/transversion rate ratio $k=7.6$ and rate heterogeneity among sites ($\alpha=0.27$). Based on nucleotide distances estimated by the best-fit model (HKY85+ Γ , $k=7$), a new phylogenetic tree was constructed (Figure 1) by the Neighbour-Joining algorithm. The estimated synonymous substitution rates were similar between the two influenza B lineages (Figure 2): 6.94×10^{-3} (95% CI: $6.68-7.19 \times 10^{-3}$) synonymous substitutions/synonymous site/year for Yamagata lineage and 7.56×10^{-3} (95% CI: $7.40-7.70 \times 10^{-3}$) synonymous substitutions/synonymous site/year (Victoria-like viruses). Concerning the rates of non-synonymous substitutions per non-synonymous site, Yamagata-like influenza B viruses presented a higher rate than viruses from the Victoria lineage (Figure 2): 2.17×10^{-3} (95% CI: $2.05-2.28 \times 10^{-3}$) non-synonymous substitutions/non-synonymous site/year (Yamagata-like viruses) and 1.09×10^{-3} (95% CI: $1.08-1.11 \times 10^{-3}$) non-synonymous substitutions/non-synonymous site/year (viruses from Victoria lineage). The *codeml* program revealed M3 as the codon-substitution model that better fitted our sequence data. Also, the M8 likelihood estimate (-3934.280607) was very similar to the value obtained by M3 (-3934.003794). Both these models (M3 and M8) allow for the existence of positive selection acting on specific sites of the molecule. The M3 model considers 3 classes of sites with different values of ω . One larger class (66.56%) of very conservative sites ($\omega=0.01251$), a second group which represents 31.14% of sites under moderate negative selective pressure ($\omega=0.53314$) and a third class of sites (2.29%) under PSP with an $\omega=3.21$. This model detected sites 129, 197 and 199 under PSP (by Naïve Empirical Bayes) with posterior probabilities higher than 0.99. The results of the M8 model reveal 98.16% of codons under negative selective pressure and 1.84% of codons under PSP with acceptance rate of $\omega=3.4747$. Sites estimated to be under PSP by the M8 codon-substitution model (by Bayes Empirical

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Bayes) were the same as for the M3 model: sites 129 and 199 with posterior probabilities higher than 0.95 and site 197 with $p > 0.99$. Likelihood Ratio Tests between models 1a and 2a (p -value=0.003) and models 7 and 8 (p -value<0.001) rejected the null hypothesis that selective pressure along the sequence is either neutral or deleterious, thus demonstrating the existence of positive selective pressure acting on the hemagglutinin of type B viruses.

Figure 1. Phylogenetic tree based on HA1 subunit of influenza B viruses isolated in Portugal from 1994 to 2006 (neighbour-joining algorithm using HKY85+ Γ evolution distances).

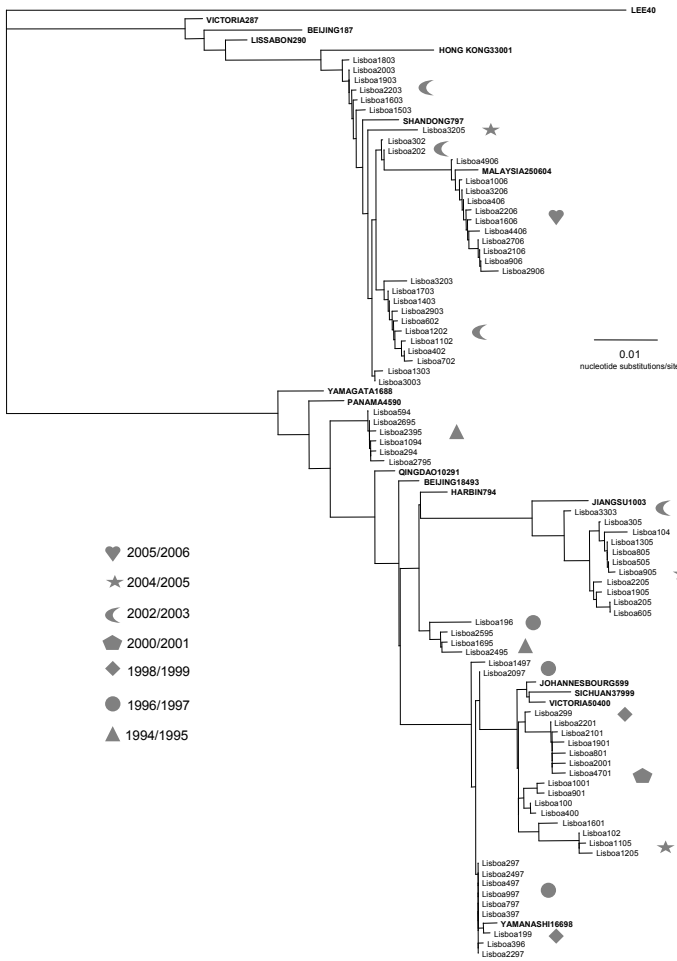
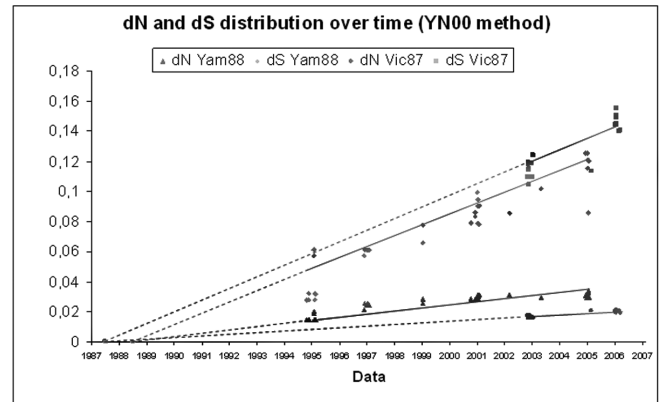


Figure 2. Distribution, over time, of the number of non-synonymous (dN) and synonymous substitutions (dS) per site (HA1 Subunit) between analysed strains and their prototype virus (B/Victoria/2/87 or B/Yamagata/16/88).



Discussion

The best-fit model obtained for our influenza B hemagglutinin sequence dataset was Hasegawa-Kishino-Yano 85 plus Gamma (HKY85+ Γ). This nucleotide-substitution model considers different nucleotide base frequencies and also a transition/transversion rate ratio. In this study, it was found to be $k=7$. This is to say that transitions occur 7 times more frequently than transversions in our dataset. This model also assumes a site-dependent rate variation (i.e., nucleotide substitution rate varies along the nucleotide sequence). The parameter α describes the degree of rate heterogeneity among sites: when $\alpha < 1$, which means that nucleotide sequences have strong rate heterogeneity, a fact that occurs with our group of sequences ($\alpha=0.27$). The phylogenetic tree obtained (Figure 1) with parameters from the best-fit model displays an important epidemiological characteristic of influenza type B viruses: the co-circulation, during the same winter season, of genetically distinct influenza B strains [7]. The synonymous and non-synonymous substitution rates were very similar to previously obtained values [3], despite the use of a different method. The rate of non-synonymous substitutions was lower than other values described in the literature [1,2,8]. Victoria-like viruses reveal a non-synonymous substitution rate two times lower when compared to strains of Yamagata lineage (Figure 2). This fact may be explained by the limited circulation of Victoria-like influenza B viruses [8] during most of the period of this study (influenza B viruses from Victoria lineage reappeared in Portugal only in 2002/2003 winter season). Results show that M3 and M8 were the best-fit codon-substitution models. The M3 model considers 3 discrete classes of sites with different values of ω , one of which is representative of positively selected sites ($\omega > 1$). The M8 model represents a β -distribution of site classes plus a class with sites also under PSP. The presence of positive selection along the hemagglutinin sequence of influenza B strains isolated in Portugal was statistically demonstrated by LRT's. M3 (Naïve Empirical Bayes) and M8 (Bayes Empirical

Bayes) codon-substitution models identified the sites 197, 199 and 129 under PSP with high posterior probabilities. The method of Bayes Empirical Bayes is not available for the M3 model, but according to the program's author it identifies less false-positive sites under PSP. Other codons (68, 73, 75, 116, 122, 137, and 163) were identified as being under PSP, although presenting low posterior probabilities. Site 129 belongs to antigenic site A of H3 hemagglutinin of influenza A viruses. However, except from our own experience (unpublished data) we have found no references to antigenicity of amino acid 129. Codons 75, 197 and 199 were found under PSP in a previous study [3]. Sites 197 and 199 form a potential N-glycosylation site located in the region corresponding to antigenic site B of H3 molecule [9,10] and near the receptor binding site. There are references that 197-x-199 site may constitute or influence an epitope [11]. This glycosylation site has been described by its influence on the antigenicity of type B hemagglutinin [12,13]. The presence (or absence) of this glycosylation site is also dependent on the host cell in which the virus is grown [14,15].

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Influenza A H3 Virus: A Ten-Year Genetic Analysis

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Genetic analysis of circulating influenza strains is essential for influenza surveillance, not only by contributing to a better understanding of molecular epidemiology and evolutionary relations of influenza viruses in circulation, but also predicting virus evolution for influenza vaccine optimisation. This study presents a genetic analysis based on HA1-coding region of influenza A(H3) viruses, isolated in Portugal over the last ten influenza winter seasons. Nucleotide sequences of HA1 region were obtained from isolated influenza A(H3) viruses after RT-nested PCR amplification. Complete HA1 nucleotide sequences were aligned by the Clustal method, and their phylogeny was constructed by the Neighbour-Joining algorithm. Using maximum likelihood (Yang, 1997), we found Hasegawa-Kishino-Yano 85 plus Gamma (HKY85+ Γ) evolution model to best fit our H3 sequence data, assuming rate variation among sites ($\alpha=0.7$) and a transition/transversion ratio $k=3.99$. Influenza A(H3) viruses, isolated between 1996/97 and 2001/02 seasons belonged to the same lineage of the correspondent vaccine strains. The first A/Fujian-like viruses were detected in Portugal at 2003/04, presenting a strong antigenic drift relative to the vaccine strain A/Panama/2007/99. Strains detected in that season had origin on two distinct phylogenetic groups, one similar to A/Kumamoto/102/2002 and another closer to A/Wyoming/3/2003. At 2004/2005, the isolated viruses were A/California/7/2004-like, that would become the vaccine strain for the next 2005/06 winter. At the latest, we have isolated only one A/Wisconsin/67/2005-like strain. Hemagglutinin of circulating strains presented several amino acid substitutions relative to vaccine strains, the majority of them located at antigenic sites.

Introduction

Genetic analysis of circulating influenza strains is essential for influenza surveillance, not only by contributing to a better understanding of molecular epidemiology and evolutionary relations of influenza viruses in circulation, but also in predicting virus evolution and contributing to influenza vaccine composition. This study presents a genetic analysis based on HA1-coding region of influenza A(H3) viruses, isolated in Portugal over the last ten influenza winter seasons.

Material and Methods

Naso-pharyngeal swabs were collected by General Practitioners (participating in the National Influenza Surveillance System) from cases of influenza-like illness between 1996 and 2006. Fifty nine nucleotide sequences of HA1 region were obtained

[1] from isolated influenza A(H3) viruses after RT-nested PCR amplification [2]. Nucleotide sequences of vaccine strains were obtained from the *Influenza Sequence Database* at www.flu.lanl.gov [3]. Complete HA1 sequences were aligned by the Clustal method. The base phylogenetic tree file (Neighbour-Joining algorithm) was obtained by PHYLIP using F84 evolution distances [4]. Aligned sequences with the correspondent base tree were then submitted to *baseml* program from PAML 3.15 aiming to find the best-fit nucleotide-substitution model using maximum likelihood estimates [5]. Hierarchical likelihood ratio tests (LRT's) were used for comparison between nested evolution models, starting from the simplest model Jukes-Cantor 69 (JC69) to the most complex model General Time Reversible (GTR). The phylogenetic tree was then constructed with estimated nucleotide distances obtained by the best-fit nucleotide-substitution model.

Results

The results obtained by *baseml* are shown in Table 1.

Table 1. Log-likelihood estimates, k ratio of transition/transversion rate, values of α (Γ -Distribution) and likelihood ratio tests to compare and determine the best-fit nucleotide-substitution model.

Nucleotide-substitution Models	k	α	Maximum Likelihood estimates
GTR+ Γ	Average Ts/Tv = 1.9713	0.745750	-2.910,046402
GTR (REV)	Average Ts/Tv = 1.9649		-2.917,850057
TN93+ Γ	3.35306 4.49099	0.73621	-2.916,828338
TN93	3.27108 4.44797		-2.924,726485
HKY85+ Γ	3.99643	0.71251	-2.918,089004
HKY85	3.990650		-2.926,298479
K80+ Γ	3.958490	0.6912	-2.945,254398
K80	3.847050		-2.953,764645
F81+ Γ		0.6811	-2.967,338392
F81			-2.975,813527
JC69+ Γ		0.70466	-2.993,970225
JC69			-3.002,327872
LRT	$2\Delta\ell$	df	p
JC69:K80	97.126454	1	<0.001
K80:HKY85	54.932332	2	<0.001
HKY85:TN93	3.143988	1	0.076
GTR:TN93	13.752856	3	0.003
HKY85+ Γ :HKY85	16.41895	1	<0.001
JC69+ Γ :K80+ Γ	97.431654	1	<0.001
K80+ Γ :HKY85+ Γ	54.330788	2	<0.001
HKY85+ Γ :TN93+ Γ	2.521332	1	0.112
GTR+ Γ :TN93+ Γ	13.563872	3	0.004

The Hasegawa-Kishino-Yano 85 plus Gamma (HKY85+ Γ) evolution model achieved the highest maximum likelihood

estimate (Table 1). This model was confirmed as the best-fit nucleotide-substitution model (Table 1) by Hierarchical LRT's. This model assumed transition/transversion rate ratio of $k=3.99$ and a value of $\alpha=0.7$ (parameter relative to the nucleotide substitution rate heterogeneity over sites). Influenza A (H3) viruses, isolated between 1996/97 and 2001/02 seasons belonged to the same lineage of the correspondent vaccine strains (Figure 1). The single viral isolate genetically analysed in 1996-1997 winter season revealed 8 amino acid substitutions comparing to the influenza H3 vaccine strain, A/Nanchang/933/95: T121→N, G124→S, D133→N, G142→R, V190→D, L194→I, R220→S, I226→V. In the winter of 1999/2000, 13 amino acid replacements were detected in HA1 subunit relative to the vaccine strain A/Sydney/5/97. The viruses circulating during 2001/2002 flu season were distinguishable from A/Panama/10/99 (strain included in influenza vaccine since 2000/2001) by 6 replacements on hemagglutinin amino acid residues. The first A/Fujian-like viruses were detected in Portugal at 2003/2004, presenting a strong antigenic drift relative to the vaccine strain A/Panama/2007/99. Except for one isolated A/Panama-like strain (A/Lisboa/26/2003), genetically, 2003/2004 isolated viruses also presented high dissimilarity from A/Panama/2007/99. Strains detected in that season had origin on two distinct phylogenetic groups (Fig.1); one branch included shared amino acid substitutions S45→I and N126→D and the second branch (closer to A/Kumamoto/102/2002 and A/Wyoming/3/2003) with amino acid changes R142→K and S189→N. At 2004/2005 flu season, all isolated viruses were A/California/7/2004-like (that would become the vaccine strain for the next 2005/06 winter) differing from the 2004/2005 vaccine strain (A/Wyoming/3/2003) in 6 amino acid substitutions. The peptide sequence of A/Lisboa/10/2006, one of two influenza A(H3) strains isolated in 2005/2006, revealed its similarity with A/Wisconsin/67/2005 hemagglutinin, showing 8 amino acid changes comparing to influenza H3 vaccine strain -A/California/7/2004: N81→K, S95→R, C97→R, S138→A, N188→D, S193→F, T196→A and D225→N.

Discussion

The Hasegawa-Kishino-Yano 85 plus Gamma was found the best-fit nucleotide-substitution model for our H3 sequences. This model assumes different base frequencies and different rates for transitions and for transversions, in our study with $k=3.99$, i.e. transitions are 3.99 times more frequent than transversions. The model HKY85+Γ also considers site-dependent rate variation (nucleotide substitution rate varies along the HA1 sequence). The parameter α describes the degree of rate heterogeneity among sites: when this parameter is <1 , means that nucleotide sequences have strong rate heterogeneity, which occurs with our sequence dataset ($\alpha=0.7$). In every studied winter seasons, part of registered amino acid replacements has occurred at antigenic sites of the H3 hemagglutinin (data not shown). Influenza A(H3) viruses isolated in 1996/1997, 2001/2002, 2004/2005 and 2005/2006 winters were closer related with correspondent

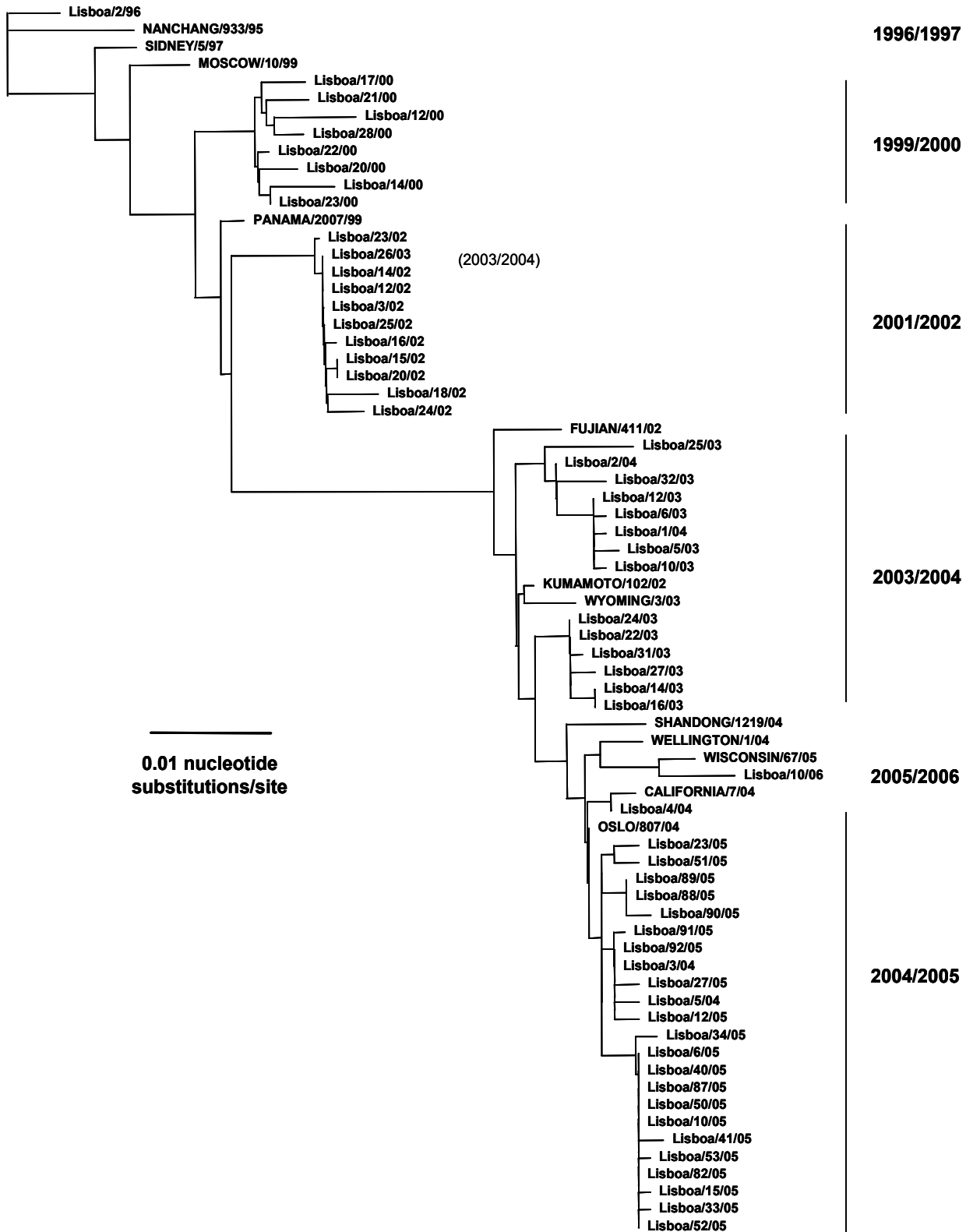
vaccine strain, differing by 6-8 amino acids substitutions. However, strains circulating in 1999/2000 and 2003/2004 presented higher number of amino acid substitutions (13 and 11 changes of amino acid residue, respectively) comparing to the correspondent vaccine strains A/Sydney/5/97 and A/Panama/2007/99, which is displayed on the phylogenetic tree configuration. A/Fujian/411/02-like viruses, emerged in Portugal at 2003/2004, presented strong antigenic and genetic differences from A/Panama/2007/99. Genetic analysis revealed 11 amino acid substitutions since this vaccine strain, 7 of them in antigenic sites. However is considered that two amino acid changes (155 and 156) were responsible for this antigenic drift [7] (Jin *et al.*, 2005).

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Figure 1. Phylogenetic tree obtained by neighbour-joining method based on Hasegawa-Kishino-Yano 85 Plus gamma evolution distances.



Influenza Virus Research Data Management and Analysis Resource: A New Collaboration of the Influenza Sequence Database and BioHealthBase

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In the past 14 months, the number of genomic influenza sequences in the public domain has increased from ~22,000 to ~47,000, driven by the need to better understand the nature of influenza virus evolution and, if possible, to anticipate a future pandemic. In conjunction with this increased attention to the evolution of the influenza virus, there has been an increased study of influenza viral infections in humans and in animal models, bringing insights into the complexities of host-pathogen interactions. Clearly, there is a great need for resources that support efficient analysis of these data in efforts to develop improved vaccine strategies, therapeutics and diagnostics. Recently, the National Institute of Allergy and Infectious Diseases (NIAID), USA, funded a consortium of the University of Texas Southwestern, Northrop Grumman, Vecna, and AMAR International (USA) to provide bioinformatics support for researchers on influenza virus and four other infectious agents with public health consequences. The resultant BioHealthBase (BHB <http://biohealthbase.org>) Bioinformatics Resource Center (BRC) was opened to the public in January 2006. Recently, the BioHealthBase BRC entered into a collaboration with the pioneering Influenza Sequence Database (ISD: <http://www.flu.lanl.gov>) at Los Alamos National Laboratory (USA). The BHB-ISD collaboration will result in integration of the ISD's high-quality curation pipeline and tools for molecular epidemiological studies of the influenza virus into the BioHealthBase BRC database and analysis portal. An additional collaboration with the recently funded Centers of Excellence for Influenza Research and Surveillance (CEIRS) serves the purpose of providing a repository of CEIRS research and surveillance data. The goal of these collaborations is to provide a comprehensive, public resource for the management and analysis of data from these related influenza research activities. As a foundation, the BioHealthBase BRC provides an integrated set of basic influenza viral gene and protein sequence, structure and function data from a variety of public database resources, including NCBI (GenBank, RefSeq), UniProt (TrEMBL, Swiss-Prot), and Pfam. Through an ongoing collaboration with the Immune Epitope Database (IEDB: www.immuneepitope.org), influenza B-cell and T-cell (CD-4, CD8) epitope information is layered into the BioHealthBase BRC. In

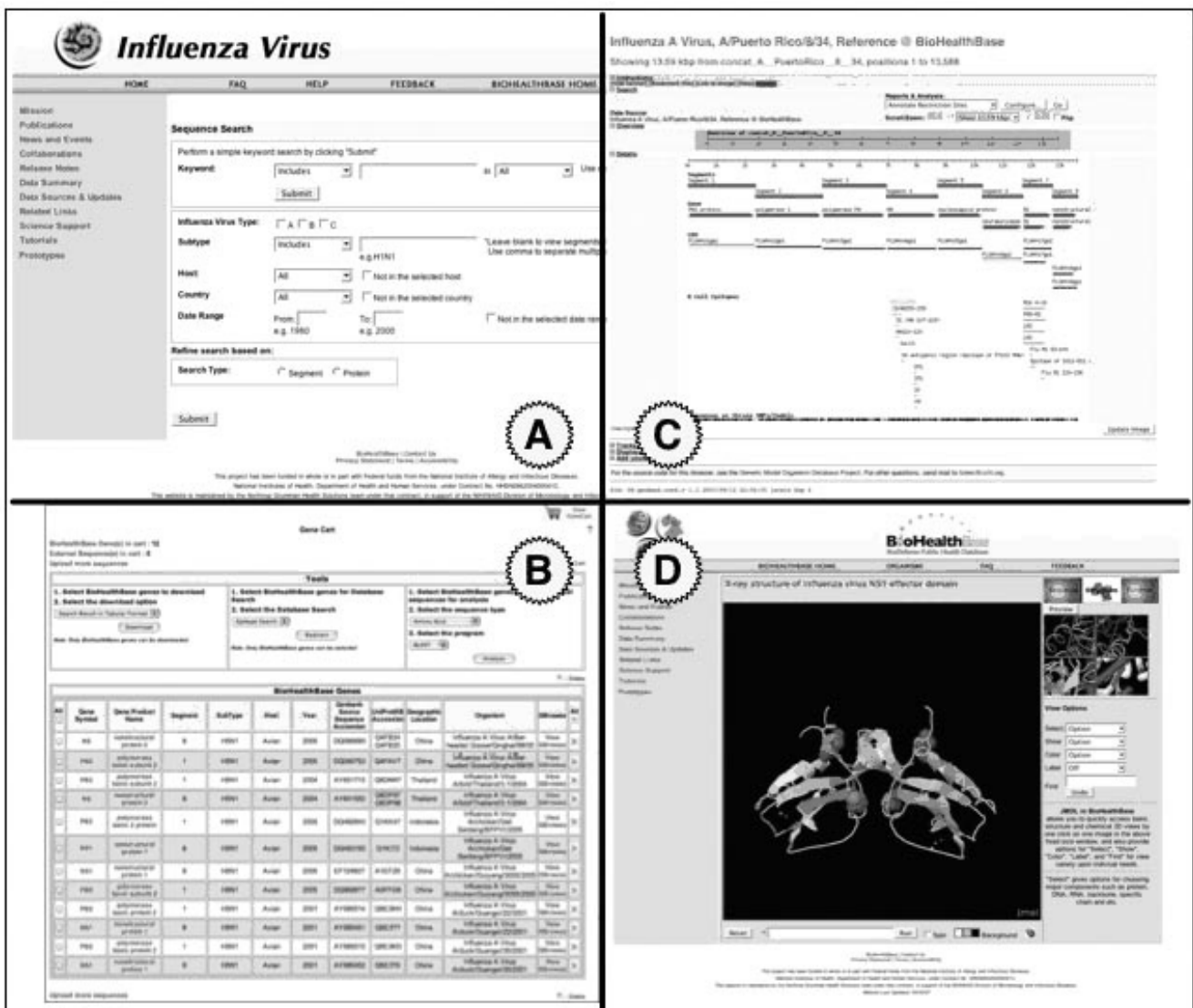
addition to data from these external sources, the BioHealthBase BRC computes and integrates additional information, including sequence similarity (BLAST) results for all influenza sequences; predicted cytotoxic T lymphocyte (CTL) epitopes utilizing the NetCTL algorithm; single nucleotide polymorphism and consensus sequence data using the MUSCLE multiple sequence alignment software and custom algorithms (e.g. concatenated reference genome sequences for the A/Puerto Rico/8/34 strain). The BioHealthBase BRC offers two options for searching the large database: quick keyword search, or a more detailed search incorporating advanced search logic; e.g. "exact matches" searches or results that exclude (Boolean "NOT") any records that include a specific search term (see Figure 1A). Advanced search options allow researchers to specify combinations of attributes such as influenza virus type, subtype, host, country or year of isolation, for determining inclusion or exclusion criteria for a record. The search interface provides mechanisms for handling obstacles such as partial sequences and partial genomes that can make searches inefficient. Search results may also be limited to particular segments, minimum segment length, or specific proteins. Once the advanced search is constructed, users may designate which attributes they wish to view in the search results as well as which attributes they wish to sort the results by. Sequence selections may be saved to a desktop in GFF3 or FASTA file format. Alternatively, the user may choose to view detailed information about sequences or BLAST or align one or more sequences. The "Gene Details" page is the central location for the integrated information pertaining to a particular sequence. The "Gene Details" page is structured as follows: strain and genomic details come first followed by genomic location details (including a glimpse of the genome viewer), protein and protein-related features (motif, domains, epitopes, pre-computed BLAST results), with references and external links at the bottom of the page. The "Strain Identification" at the top includes information about the particular strain that the current sequence belongs to as well as a link to the data source. The "Gene Identification" section includes gene symbol, gene product name, locus name as well as the Entrez ID and associated comments. The "Genomic Location" section includes start and stop positions for the gene and a link to view (and download) the sequence. The "Gene Features" section lists known CDS regions and proteins associated with the sequence. The "Genomic View" section shows a graphical representation of the gene while also serving as a link to the genome browser view of the sequence feature data. Single nucleotide polymorphism (SNP) sequence variation data completes the genomic section. The protein section of the "Gene Details" page mirrors the genomic section with "Protein Identification" information integrated from UniProt at the top of the section, followed by Pfam and other protein domains, including transmembrane and signal peptide regions. The top ten results from pre-computed sequence similarity (BLAST) searches are shown followed by both predicted and curated immune epitopes with database cross reference details completing the section. Another view of the sequence feature

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data is presented by the genome viewer, located by clicking the "Genome View" graphic in the "Gene Details" page or from the search results by clicking the "View Gbrowse" link. The genome browser allows researchers to view all of the sequence feature information in the context of the complete segment structure. In the "Details" section of the genome viewer researchers can see the location of protein coding regions (CDS), as well as SNP and epitope information. At the bottom of the screen researchers may customize the genome viewer by turning each informational "track" off or on, as well as uploading their own sequence feature data into a custom Gbrowse track to view in relation with the sequence feature provided by the BioHealthBase BRC. A special version of the Genome viewer that displays a concatenated version of the A/Puerto Rico/8/34 strain along with all associated information (see Figure 1C) is located through a link on the left hand menu. In addition to providing a

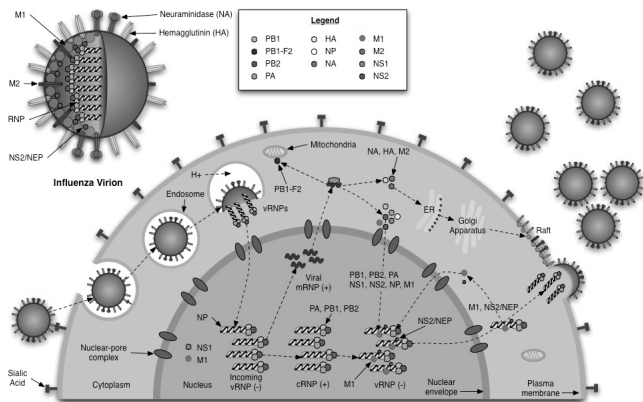
wealth of integrated influenza data, the BioHealthBase BRC offers the capability to analyze selected sequences through the use of a workspace or "Gene Cart" that disappears at the end of a session. The contents of the "Gene Cart" can be downloaded onto a desktop or fed into BLAST or multiple sequence alignment tools. Additional tools, such as tools for computing phylogenetic trees, are planned for a future release of the BioHealthBase BRC. Another important example of BioHealthBases data integration and visualization features is the 3D protein structure visualization capability (Figure 1D) that provides researchers the ability to visualize and manipulate protein structures from the Protein Database (PDB) in real-time. A protein structure can be displayed in one of many built-in views (cartoon, ball-and-stick, spacefill, line, primary structure in residue or N->C colors, rocket, composition, hydrophobicity and charge). Researchers may also select and highlight sequence

Figure 1. BioHealthbase BRC screenshots of A) The influenza virus search page, B) A search results page, C) A custom concatenated genome view of the A/Puerto Rico/8/34 strain with curated B cell epitopes for the IEDB displayed along with a sequence variation track and D) A 3D view of the NS1 effector domain displayed as a ribbon diagram with the functional amino acid residues 95 and 197 space-filled.



features of interest from the BioHealthBase through easy-to-use drop down menus or by finding the features using the “find text” field. Expert 3D modelers also have access to a console for executing Jmol commands. Finally, in collaboration with the Reactome (www.reactome.org) project, the BioHealthBase has assisted in the addition of the entire influenza life cycle pathways into the Reactome database, as well as the relevant host infection response pathways involving TLR-3 and RIG-I. The influenza life cycle pathways detail the individual reactions that make up each of the seven major events of the influenza life cycle (Figure 2). These events include the binding of the influenza virion to the host cell, entry of the influenza virion into the host cell via endocytosis, fusion and uncoating of the influenza virion, transport of the ribonucleoprotein into the host nucleus, influenza viral RNA transcription and replication, export of the viral ribonucleoprotein from the nucleus and finally virus assembly and release.

Figure 2. A schematic of the influenza life cycle showing the major events of the influenza life cycle.



In summary, the BioHealthBase BRC is a public, integrated database and analysis resource for influenza virus researchers designed to support efforts to develop improved vaccine strategies, therapeutics and diagnostics for influenza virus.

The Properties of Influenza Viruses Isolated in Russia in 2004-2006

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Introduction

The Influenza Ecology and Epidemiology Center (IEEC) located in the D.I. Ivanovsky Institute of Virology RAMS in Moscow has been observing influenza circulation in different regions of Russia. This study was devoted to the analysis of biological properties of epidemic strains isolated in the 2004-2006 period in Russia to identify the main trends in the variability of influenza viruses and the evolutionary link between past and modern influenza viruses circulating in the world.

Materials and Methods

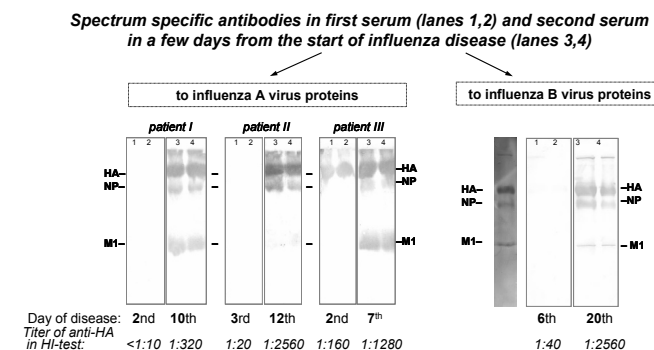
All strains were isolated from nasal/throat swabs of patients with influenza or ILI diagnosis in MDCK cells [1]. Some strains were attempted to isolate in embryonated chicken eggs (ECE). The paired sera of 200 ill patients were investigated in hemagglutinin (HA) inhibition tests (HI test) and then some of them were analyzed by Western blot [2]. To account for differing sensitivity of epidemic strains (238) to different erythrocytes in the HA test, we used 1% chickens, 1% guinea pigs and 0.75% humans red blood cells. Bromelain treatment of purified viruses was performed according to Brand and Skehel [3] using Sigma bromelain. SDS-PAGE analysis of viral proteins was carried out according to Laemmli [4]. We also isolated M1 proteins from the core of influenza viruses A and B [5], which had been treated by trypsin, which breaks the link between amino acids arginine and lysine. The hydrolysates were analyzed by RPLC [6].

Results

Influenza A(H1N1), A(H3N2) and B viruses co-circulated in Russia in various combinations in the seasons 2004-2005 and 2005-2006. The spread, number and antigenic properties of isolates depended on strain specificity. Influenza A(H1N1) viruses (16 strains) have been isolated in both epidemic seasons in Russia. In 2004-2005 there were 3 strains in sporadic cases from 2 regions; in 2005-2006 there were 13 strains. The HA antigenic properties of most isolates were similar to those of the reference strain A/New Caledonia/20/99 (H1N1). Two isolates which poorly reacted with sera to this reference strain in the HI test were antigenic variants of the modern reference strain, A/Solomon Islands/3/06, of season 2006-2007 [7]. Influenza A(H3N2) viruses (191 strains) were isolated in 16 regions of Russia. The virus population was heterogeneous in 2004-2005. There were strains related to the previous reference strain, A/Fujian/411/02 (13 strains), and to the following reference strain, A/California/7/04 (65 strains). In 2005-2006,

most of the isolates were closely related to the reference strain, A/California/7/04 (113). Some of them were antigenic variants of reference strain, A/Wisconsin/3/06 of season 2006-2007 [7]. Influenza B viruses (143 strains) were isolated in 10 regions of Russia in 2004-2005. Antigenic analysis of the HA has shown that most of these isolated strains were related to the B/Yamagata/16/88 lineage (84) and that only 3 of them were related to the B/Victoria/2/87 lineage and were B/Hong Kong/330/01-like. Antigenic analysis of the HA of the epidemic strains isolated in 2005-2006 has shown that all of them (56) were related to the B/Malaysia/2506/04 (B/Victoria/2/87 lineage). These strains circulated during the next epidemic season 2006-2007. These data indicate that evolutionary changes took place in the antigenic domain of the HA of all epidemic influenza A and B viruses, as has been noted before [8]. The increase of antibodies to the HA of A(H1N1), A(H3N2) and B viruses was detected by HI test in ill patients' paired sera that was received from 2 regions. They varied depending on the season and were identified in 2.5-13.9% of sera as A(H1N1), 14.9-27.8% as A(H3N2) and 38.0-21.5% as influenza B viruses in 2004-2005 and 2005-2006, accordingly. The presence of antibodies to virus proteins - hemagglutinin (HA) and internal proteins - nucleoprotein (NP) and matrix M1 protein have been identified in these sera by Western blot investigations, as well. In comparison, the results obtained in HI testing and by Western blot showed that the intensity of HA-lines on the membrane correlated with the amount of rising anti-HA in the serum of ill patients. In the study, secondary patient serum, as determined by Western blotting, showed that most are in the process of forming antibodies to three major peptides of influenza virus (NA, NP and M). Thus, the data indicate that the first antibodies in the blood appear to be anti-HA, followed by anti-NP. Antibodies to internal protein M1 were actively recognized in sera at the 7th day of disease onset. This research is important because it illustrates the importance of recognizing the antigenic determinants of virus internal proteins, NP and M1, which are common for human and bird influenza A viruses.

Figure 1. Detection of antibodies to influenza A and B virus proteins in paired sera of an ill patient by western blot.



Poster Presentations: Genetic and Antigenic Evolution

Analysis of the Moscow region population immunity dynamics on donor immune sera revealed that during the period from 2003 to 2006, which included 3 epidemic seasons, the levels of antibodies to circulating strains varied depending on the phase of the epidemic circle (before, during and after active isolation of viruses). All of the epidemic strains were isolated in MDCK in 2004-2006. The next line of our investigation was to examine the sensitivity of various biological systems to modern epidemic strains. We were able to isolate A(H1N1) from nasal/throat swabs (2 strains of the 14 MDCK-positive swabs) and ECE (six of the 12 strains isolated in MDCK). There were negative results for A(H3N2) virus isolates during two years: 48 throat swabs were negative for isolation in ECE and positive in MDCK. Only one A(H3N2) isolated in MDCK cells has been successfully adapted to the ECE. During two seasons only one strain of influenza B virus was isolated from 24 MDCK-positive swabs and 6 of 14 strains isolated in MDCK cells were transmitted to eggs. Interestingly, the adaptability of the influenza viruses B/Yamagata/16/88 lineage to ECE was found in strains B/Sichuan/379/99-like (20 out of 30 studied), while influenza viruses that were B/Hong Kong/330/01-like did not have this ability. In 2005-2006 the B/Malaysia/2506/04-like epidemic strains that circulated had antigenic structures different from the antigenic structure of B/Hong Kong/330/01. Seven of them have been adapted to ECE from 14 strains isolated in MDCK. These data suggest that the evolutionary process involves not only the antigenic domain, but also the receptor site of hemagglutinin influenza viruses of Victorian lineage, which had increasing tropism to ECE. Evolution of human influenza A and B viruses from the bird reservoir to the human may involve genetic changes that make them less competent for avian cells. These investigations support this conclusion based on the increase of virus isolations in the ECE at the end of the 20th century and the ability to actively isolate viruses in MDCK cells culture [9]. It should be noted that in the past, the percentage of viruses that interact with "bird" receptors SA α -2,3Gal decreased from 40% in 1968 to 10% in 1975 [10]. Our results showed that this process has continued. Our studies conducted from 1999 to 2004, as presented in this article, showed that tropism towards human cells and isolation in cell culture versus traditional cultivation systems in ECE is different for influenza A(H1N1), A(H3N2) and B viruses. The study of epidemic strains of influenza viruses isolated in 2004-2006 showed that this property is maintained by influenza A(H1N1) virus. The above mentioned data raises the question of a possible link of epidemic strains tropic to ECE and MDCK cells and their ability to interact with cells of animals, birds and humans. Therefore we conducted a study on the sensitivity of human, O(I) and A(II) human blood groups, guinea pig and chicken erythrocytes to the epidemic strains (238), isolated in MDCK from 2002 to 2006. Analysis of the results revealed that human erythrocytes had higher sensitivity than chicken erythrocytes to the epidemic strains of A(H3N2) and A(H1N1) isolated in MDCK (Table 1). In the case of Influenza B viruses, significant differences were not found. The average differences

between GMT hemagglutinin titers of epidemic strains with erythrocytes from humans and chickens were greatest for influenza viruses A(H1N1), (H3N2) and minimal for influenza B viruses (from 3.1 to 0.1 log₂ in different epidemic seasons). The least susceptible viruses to chicken erythrocytes were B/Hong Kong/330/01-like epidemic strains, which correlates with failure of virus isolation and adaptation to ECE in 2002-2003 [11]. However, the epidemic strains, B/Shanghai/361/02- and B/Sichuan/379/99-like (B/Yamagata/16/88 lineage), have been isolated and adapted to the ECE (Table 1).

Table 1. The sensitivity of different erythrocytes to epidemic strains of influenza viruses isolated in 2002-2006.

Epidemic season	Reference strains	N investigated	GMT viruses hemagglutinin activity to erythrocytes (M \pm m log ₂)				Δ GMT of human O(I) and chicken erythrocytes
			Human blood group		guinea-pig	chicken	
			O(I)	A(II)			
2002-2003	A/New Caledonia/20/99	30	7,0 \pm 0,2	6,8 \pm 0,2	2,8 \pm 0,2	<1,0 \pm 0,1	6,0
2003-2004			No virus isolation				
2004-2005	A/New Caledonia/20/99	3	7,7 \pm 0,3	6,7 \pm 0,3	4,8 \pm 0,2	<1,0 \pm 0,1	6,7
2005-2006		5	6,6 \pm 0,2	7,2 \pm 0,3	4,0 \pm 0,0	<1,0 \pm 0,2	5,6
2002-2003	A/Moscow/10/99	44	5,9 \pm 0,2	6,6 \pm 0,2	3,2 \pm 0,4	<1,0 \pm 0,5	4,9
2003-2004	A/Fujian/411/02	18	5,6 \pm 0,4	5,9 \pm 0,5	3,3 \pm 0,5	<1,0 \pm 0,4	4,6
2004-2005		34	3,6 \pm 0,4	no investigate		1,1 \pm 0,4	2,5
2005-2006	A/California/7/04	33	5,1 \pm 0,3	4,8 \pm 0,3	3,4 \pm 0,2	1,1 \pm 0,4	4,0
2002-2003	B/Hong Kong/330/01	11	6,4 \pm 0,5	7,0 \pm 0,7	4,7 \pm 0,4	3,3 \pm 0,6	3,1
2003-2004			No virus isolation				
2004-2005	B/Shanghai/361/02	51	6,3 \pm 0,1	no investigate		5,2 \pm 0,3	1,1
2005-2006	B/Malaysia/2506/04	8	6,5 \pm 0,1	6,9 \pm 0,2	5,0 \pm 0,3	6,6 \pm 0,2	0,1

Analysis of viral surface antigen properties in the bromelin digestion reaction showed that the degree of HA removal of viral particles was the same for influenza B viruses of both evolutionary lineages in spite of the significant difference of structure and antigenic properties. Influenza B viruses of both lineages were more sensitive to proteolytic treatment by the enzyme bromelin than influenza A(H1N1), A(H3N2) viruses. Our interest in the internal protein M1 is related to its conservative structure and the possibilities to use it in test systems for diagnosis of disease caused by bird flu viruses in the human population. A feasibility study the sensitivity of trypsin treatment was carried out with reference strains A (H1N1), A (H3N2) and B isolated in 1999-2002 and the reassortant A (H3N2), A/X-31. Differences between chromatographic profiles of M1 protein tryptic hydrolyzates of influenza A(H1N1), A(H3N2) and B viruses of two lineages were found. This was confirmed by sequencing of M1 genes, which demonstrates that the evolutionary processes in the internal protein gene M1 are conservative [12].

Discussion

These studies showed that epidemic strains circulating in Russia in 2004-2006 were similar to reference strains circulating in the world and that evolutionary changes took place in influenza A and B virus proteins. Antigenic drift and the increase of affinity to mammalian cells was mostly expressed by influenza A(H3N2) viruses.

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Phylogenetic Analysis of Influenza Viruses Circulating in South Africa During 2006-2007

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The purpose of this study was to monitor the genetic drift in South African influenza viruses by sequencing of the genes coding the surface glycoproteins of representative viruses circulating in 2006. Several viruses isolated early in 2007 were also analysed. In 2006, the predominant subtype in South Africa was influenza A H3N2, followed by influenza B, while H1N1 activity was sporadic. This is in contrast to 2005 where H1N1 accounted for 55.6% of the isolates made. Sequence analysis of the HA1 region of representative H3N2 isolates from the Gauteng, Kwa-Zulu Natal and Western Cape provinces revealed significant drift from the A/California/7/04 southern hemisphere vaccine strain. Some drift in antigenic sites A and B was also observed compared to the A/Wisconsin/67/05 vaccine strain for the 2006/7 season. Influenza B viruses from both the B/Victoria/2/87 and B/Yamagata/16/88 lineages were isolated with the majority being B/Malaysia/2506/04-like (Victoria lineage). Phylogenetic analysis of the HA1 subunit of representative South African 2006 influenza B viruses showed that the 2006 B/Victoria/2/87-like isolates were closely homologous to the B/Malaysia/2506/04 vaccine strain. In the B/Shanghai-like viruses, substitutions were seen at four common residues and sporadic changes were seen at several additional other residues. A single H1N2 reassortant virus was identified in the six influenza A H1 viruses isolated in 2006 by RT-PCR typing of the neuraminidase genes. This is the first time an H1N2 reassortant has been detected in South Africa since it circulated in this country in 2002 and 2003.

Introduction

Surveillance and monitoring of the antigenicity of influenza viruses in circulation each year is necessary to identify any new variant strains so that vaccines can be updated annually. The influenza laboratories at the National Institute for Communicable Diseases (NICD) serve as one of the WHO National Influenza Centres (NICs) and provide data annually to the WHO Collaborating Centres (WHO CCs). South Africa is one of the few countries in Africa that has the capability of characterizing influenza virus isolates at the genetic level. The purpose of this study was to first monitor the drift in the HA1 gene human influenza viruses circulating in South Africa in 2006 and 2007 for vaccine evaluation purposes. The second objective was to characterize an H1N2 reassortant virus which was isolated in 2006.

Methods

Patients and sources of specimens. Clinical specimens were

obtained mainly from the active viral surveillance programme at NICD which monitors influenza activity mainly in Johannesburg, but also includes sites in the KwaZulu-Natal, Western Cape and Eastern Cape provinces. Viruses were isolated from specimens sent to NICD during March - October 2006 with the peak of activity corresponding to early June. Sporadic isolates were also made early in 2007 from imported cases of influenza from travelers who had returned from the northern hemisphere. Viruses were isolated locally in the country from April onwards. *Virus isolation and molecular characterization.* Viruses were detected using the shell vial method described by Besselaar et al [1]. Cell culture supernatants of the influenza-positive specimens were typed by haemagglutination inhibition (HAI) with reference antisera supplied by the WHO CC in Melbourne. A number of the influenza A isolates did not agglutinate guinea pig, human or turkey erythrocytes and were subtyped by RT-PCR instead. HA1 genes of representative influenza A and B isolates were amplified by RT-PCR and sequenced. N2 genes of several influenza A isolates were also amplified using N2 - specific primers and partially sequenced. Recent HA1 sequences from other countries were obtained from the Influenza sequence database at Los Alamos National Laboratories [2]. BLAST searches were done and phylogenetic trees were constructed by the Neighbor-joining method using MEGA software version 3 [3].

Results

The total number of isolates made in South Africa between March and October 2006 was 554. Both influenza A subtypes as well as influenza B viruses were detected, with H3N2 being predominant (76.71%). Six H1 viruses were identified (1.08%) and of these one was found to be H1N2 as determined by neuraminidase typing by RT-PCR using N2 specific PCR primers. 11.73% of the influenza A viruses isolated could not be subtyped, while 58 isolates (10.48%) were influenza B. Of these, 48 were B/Malaysia/2506/04 - like, 7 were B/Shanghai/361/02 - like and 3 could not be typed. During 2007, two H3N2 isolates were made from patients in February that had contact with travelers from the northern hemisphere in February. In April/May both subtypes of influenza A and influenza B were detected sporadically in the local population, with the majority being H3N2. Phylogenetic analysis of the HA1 subunit of representative H1N1 isolates revealed little drift from the A/New Caledonia/20/99 vaccine strain as was observed with viruses isolated in Egypt, Israel and Sweden in 2006 (data not shown). In contrast, the H3N2s circulating in 2006 showed extensive genetic drift from the A/California/7/04 vaccine strain and shared greater homology with the A/Wisconsin/67/05-like viruses which are characterized by amino acid changes 193 (S-F) and 225 (D-N) (Figure 1). Common amino acid substitutions in the 2006 isolates relative to the Wisconsin strain were seen at residues 122 (D-N), 195 (H-Y) and 223 (I-V). Several of the 2007 isolates had additional substitutions at 140, 142 and 144 (site A) while others had changes at residues 50 (G-E) (site E) and 175 (D-N) (site D). The local South African 2007 isolates made in

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April and May were most closely related to A/Washington/1/07 as determined by BLAST searches but differed at residue 83 (K-R) in site E.

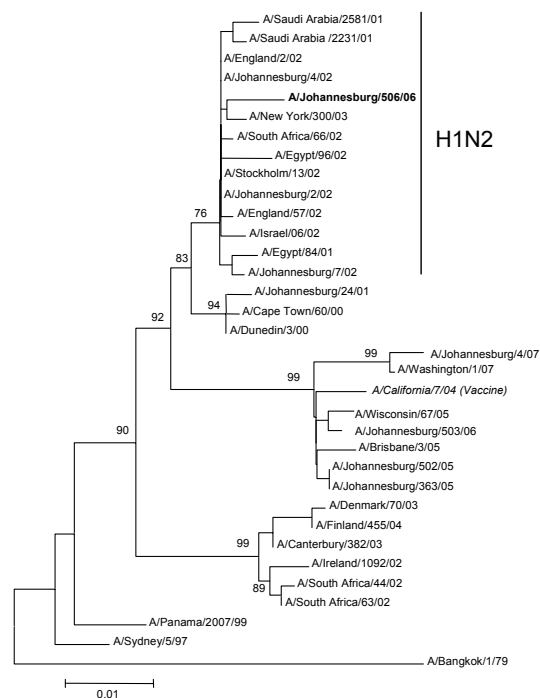
Figure 1. Phylogenetic tree of the HA1 gene sequences for H3N2 viruses (923 bp). The tree was generated using neighbor-joining analysis with MEGA software. (*Vaccine Strain*). Numbers at the branch nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value.



The HA1 genes of representative 2006 South African viruses from both the B/Victoria/2/07 and B/Yamagata/16/88 lineages were sequenced. The B/Malaysia/2506/04-like isolates grouped with the clade 2 viruses and shared close homology with the B/Malaysia/2506/04 vaccine strain, with only one common amino acid change at position 109. The 2006 South African B/Yamagata/16/88B-like viruses were distinguishable from the B/Shanghai/361/2002 strain and shared greater homology with the B/Florida/7/04 reference strain and a 2005 Malaysian isolate (B/Malaysia/53/05) (data not shown). Phylogenetic analysis of the partially sequenced N2 gene of the 2006 South African H1N2 reassortant strain showed that it clustered in the subgroup together with the earlier H1N2 strains that were isolated globally between 2001 -2003 (Figure 2) and shared

common amino acid substitutions with the earlier H1N2 reassortants. Several additional changes were observed in the 2006 Johannesburg isolate indicating that some drift had occurred in the N2 from the previously circulating H1N2s. The N2 of a representative 2006 H3N2 isolate was closely related to that of the 2006/7 vaccine A/Wisconsin/67/05 strain while an early 2007 isolate shared higher homology with the A/Washington/1/07 strain.

Figure 2. Phylogenetic tree of the partial N2 gene sequences for H3N2 and H1N2 viruses (700 Bp). The tree was generated using neighbor-joining analysis with MEGA software. (*Vaccine Strain; 2006 H1N2 reassortant virus*). Numbers at the branch nodes indicate confidence levels of bootstrap analysis with 1000 replications as a percentage value.



Discussion

Human influenza A H1N2 reassortant viruses were detected globally during the 2001 – 2003 influenza seasons but have only been detected very sporadically since then. The 2006 reassortant virus is the first one identified in South Africa since 2002 and suggests that these reassortants may still be circulating at a very low level but have not been detected. It remains to be seen whether H1N2 viruses will be maintained in circulation in low numbers in humans in the future. The HA1 subunit of the South African H1 isolates that were analyzed did not show substantial antigenic drift from the A/New Caledonia/20/99 vaccine strain. This is similar to the majority of H1N1 viruses isolated globally in 2006 where only a small proportion were genetically distinct from the vaccine strain and reacted poorly in the HAI tests with ferret antiserum raised against A/New Caledonia/20/99 [4]. The H3N2 viruses isolated from three different provinces in South Africa were very similar to each other. The extensive drift seen in the

H3N2 South African 2006 isolates from A/California/7/04 suggested the 2006 vaccine was not a good match to the circulating strains. The early 2007 isolates were closely related to the A/Wisconsin/67/05 vaccine strain for the northern hemisphere 2006/7 season but did show some genetic drift from this strain. Antigenic characterization with ferret antisera will help determine just how significant this drift is. The close relationship between the 2006 South African B viruses from the B/Victoria/2/87 lineage and other clade 2 viruses isolated from various countries during the same year showed a good match with the B/Malaysia/2506/04 vaccine strain.

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H5N1 Clade 2.2 Polymorphism Tracing Identifies Influenza Recombination and Potential Vaccine Targets

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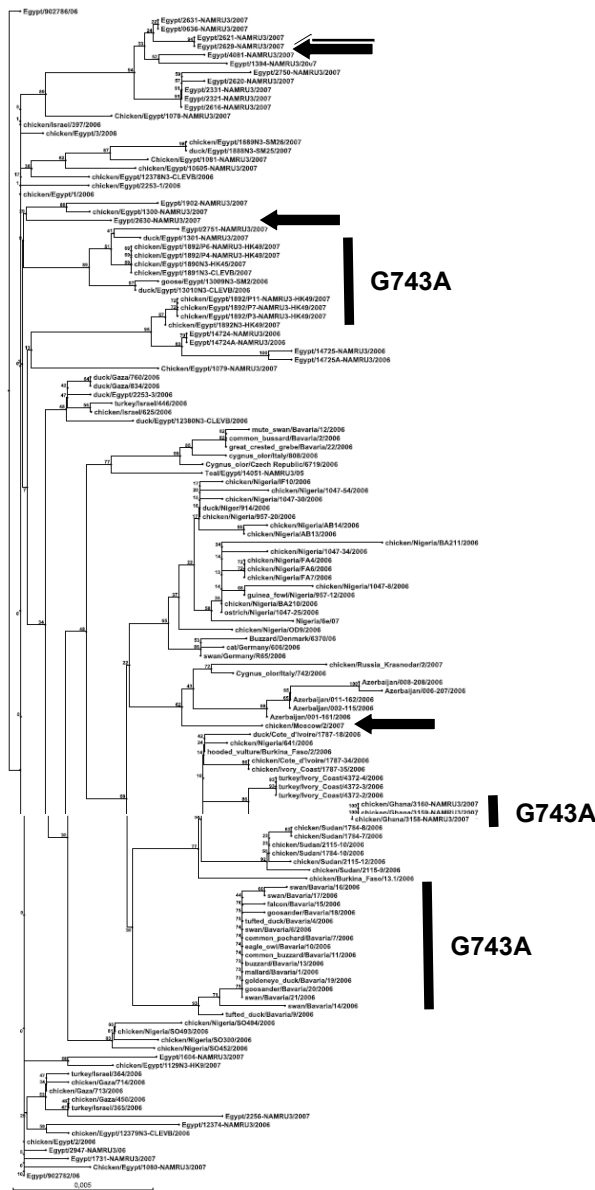
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Highly pathogenic Influenza A H5N1 was first identified in Guangdong Province in 1996, followed by human cases in Hong Kong in 1997¹. The number of confirmed human cases now exceeds 300 and the associated Case Fatality Rate exceeds 60%². The genetic diversity of the serotype continues to increase. Four distinct clades or sub-clades have been linked to human cases^{3,4}. The gradual genetic changes identified in the sub-clades have been attributed to copy errors by viral encoded polymerases that lack an editing function, thereby resulting in antigenic drift⁵. We traced polymorphism acquisition in Clade 2.2 sequences. We report here the concurrent acquisition of the same polymorphism by multiple, genetically distinct, Clade 2.2 sub-clades in Egypt, Russia and Ghana. These changes are not easily explained by the current theory of "random mutation" through copy error, and are more easily explained by recombination with a common source. This conclusion is supported by additional polymorphisms shared by Clade 2.2 isolates in Egypt, Nigeria and Germany including aggregation of regional polymorphisms from each of these areas into a single Nigerian human hemagglutinin gene. The study of influenza evolution in nature has been aided by the emergence of a new strain (Clade 2.2) first identified at Qinghai Lake in central China in the spring of 2005. Sequencing of all eight genes^{6,7} showed that isolates from migratory waterfowl were easily distinguishable from previous isolates linked to poultry and human infections in eastern and southeastern Asia. The new strain was subsequently found in outbreaks in Russia, Kazakhstan and Mongolia⁸. Prior to these Clade 2.2 outbreaks the highly pathogenic Asian version of H5N1 had never been reported west of China. The H5N1 detection in migratory waterfowl in summer 2005 in Russian and Mongolian migratory bird sanctuaries signaled the start of a major geographical expansion of H5N1. In the following 12 months, almost 50 countries west of China reported H5N1 for the first time. All infections were Clade 2.2. The expanded geographical reach included Europe, the Middle East and Africa. This expansion offered a unique opportunity to study the evolution of H5N1 as it migrated into new regions, including human cases in Turkey, Iraq, Azerbaijan, Egypt, Djibouti in 2006 and Nigeria in 2007. Sequence analysis indicated all cases were Clade 2.2. The outbreaks were due to multiple introductions and isolates had regional specific polymorphisms. We isolated H5N1 from patients and poultry in Egypt. The first poultry

isolates were collected February 2006. The first human case developed symptoms in March 2006. Analysis of the H5N1 isolates collected between February and May 2006 defined a series of HA and NA regional markers. These markers were also found in the human case from Djibouti and poultry isolates from Israel and Gaza. After a lull in reported infections over the summer, H5N1 re-emerged in Egypt in September 2006. The more recent isolates had the same regional markers seen the previous season. However, the more recent poultry and human isolates had acquired a series of new polymorphisms. Non-synonymous polymorphisms were identified in samples collected from a cluster of three family members from the Gharbiya governorate (Nile Delta). HA gene polymorphisms were identified in or near the receptor binding domain, including V223I and M230I, as well as the oseltamivir resistance polymorphism, N294S (NA gene). The patients first developed symptoms in December 2006 and all three infections were fatal (a detailed report on patients and polymorphism tracing will be described elsewhere). Additional cases in early 2007 included HA sequences with a 3 BP deletion of the nucleotides encoding Ser at position 133 (H3 Numbering), and a case with a novel HA cleavage site, REGRRRKR. The changes were found in multiple patients in central and southern Egypt. The above non-synonymous changes were associated with additional synonymous and non-synonymous changes in the HA and NA sequences. However, these isolates from the 2006/2007 season maintained the regional markers seen in early 2006 in isolates from Egypt, Djibouti, Israel and Gaza. Chicken isolates from Gharbiya samples collected on February 15, 2007 included one sequence that was closely related to the sequences from the human Gharbiya cluster. The sequence, A/chicken/1892N3-HK49/2007, had the regional markers previously seen in the 2006 and 2007 isolates, as well as HA non-synonymous changes, V223I and M230I. Additionally, an NA synonymous polymorphism, G743A, was appended onto the genetic background of the human Gharbiya cluster, as seen in an NA cladogram (data not shown). This polymorphism was found in two additional chicken isolates, A/chicken/1890N3-HK45/2007 and A/chicken/1891N3-CLEVB/2007, collected the same day in the Gharbiya governorate, but these two isolates fell onto a separate branch of the tree. The G743A was subsequently found in human isolates from patients who developed symptoms in April 2007. Included were siblings with HA sequences that had the 3 BP deletion seen in earlier patients from central Egypt. Like the chicken sequences above, the G743A polymorphism was appended onto sequences identified earlier in Egypt. Similarly, distinct sequences from another patient, A/Egypt/2630-NAMRU3/2007, that acquired G743A, also fell onto a separate branch. The distinct branches displayed in the NA cladogram were also seen in the HA cladogram (data not shown). The isolates with G743A are also located at the tips of the branches, supporting a recent polymorphism acquisition. In February 2007, an H5N1 Clade 2.2 outbreak occurred near Moscow, Russia. Isolates from infected chickens were most closely related to 2006 sequences from Azerbaijan. Figure 1

is an expanded NA cladogram with isolates from Europe, the Middle East and sub-Saharan Africa.

Figure 1. NA Phylogram of clade 2.2 isolates. NA phylogram of positions 43-1337. Isolates with G743A marked either with black arrows or black bars.



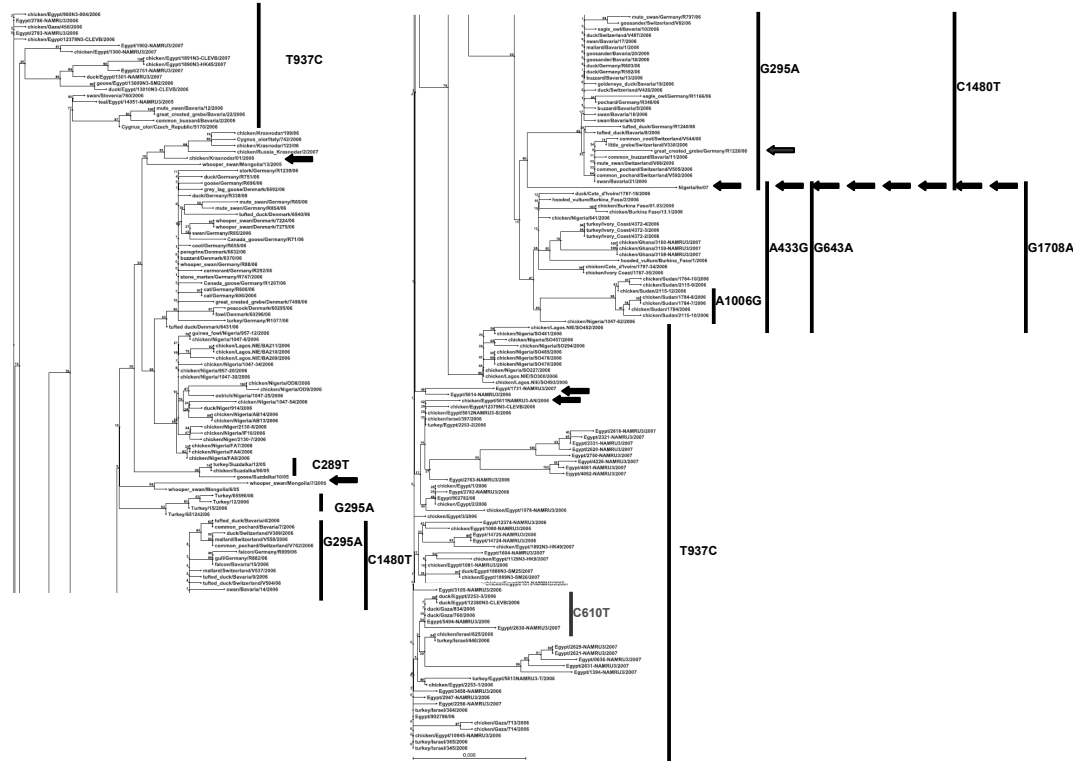
mapped to the tip of a branch containing earlier isolates that did not have the polymorphism. Additional HA polymorphisms are noted in the HA phylogram (data not shown). Isolates that had the NA polymorphism, G743A, also had a synonymous HA polymorphism, C689T. This polymorphism was also in human and bird isolates from the Nile Delta. Another polymorphism, G754A, that encodes M230I, is in one of the German isolates⁹, A/eagle owl/Germany/R166/2006, and maps to another branch with Egyptian human and poultry isolates from the Nile Delta. A third polymorphism, C1614T, that encodes T517I, is in another German isolate, A/mute swan/Germany/R797/2006, and another branch with human isolates from southern Egypt. The isolates also have the novel HA cleavage site initially found in whooper swan isolates in Mongolia (2005). The polymorphisms found in German isolates in 2006 were in Russian Clade 2.2 isolates in 2005. The NA G743A polymorphism can be traced through public H5N1 sequences¹⁰. The polymorphism was identified in the first reported sequences linked to the spread of H5N1 in Asia in 2003/2004 in South Korea and Japan. The polymorphism was subsequently identified in Clade 1 isolates in southeast Asia, as well as Clade 2.1 isolates in Indonesia and Clade 2.3 isolates in China. The first reported Clade 2.2 isolates were in wild birds in Germany collected in February 2006. The isolates in Germany formed distinct HA and NA branches due to a series of regional markers. The first human isolate from Nigeria had several markers that were regionally distributed in the 2006 Clade 2.2 isolates. These shared HA polymorphisms are labeled in the HA cladogram (Figure 2).

HA phylogram of positions 93-1688. A/Nigeria/6e/2007 shared polymorphisms annotated with colored arrows and bars. One of the Nigerian human polymorphisms, T937C is an Egyptian regional marker. A/Nigeria/6e/2007 also has T610C matching a small subset of the Egyptian isolates. G881A is in another small Egyptian subset, and C980T is in isolates from Egypt and Nigeria. Similarly, the Nigerian isolate has two regional markers from Germany, G295A and C1480T, and another marker, A778G, found in one of the German bird isolates, A/great crested grebe/Germany/R1226/2006. The pattern continues in this same Nigerian isolate with the inclusion of sub-Saharan African regional markers, A433G and G643A, as well as another marker from a subset of the Sudan isolates, A1006G. Finally, G1658A from A/whooper swan/Mongolia/7/2006 appears in A/Nigeria/6e/2007. Hence the demonstration shows that the Nigerian isolate has recently aggregated H5N1 Clade 2.2 sequence information, including twelve Clade 2.2 SNPs, from a minimum of three geographically distinct areas. The cobbling together of this series of 2006 regional markers from multiple, geographically distinct locations into a single sequence is most easily explained by recombination. Similarly, the concurrent acquisition of the same polymorphism by multiple Clade 2.2 sub-clades challenges the current theory of influenza evolution that invokes random mutations as a mechanism for the generation of antigenic drift. The isolates with the newly acquired polymorphisms map to the tips of

Like the acquisitions in Egypt, the isolates with G743A mapped onto the tip of a branch composed of earlier isolates that did not have the acquisition. Similarly, in April 2007, an H5N1 clade 2.2 outbreak occurred near Tema, Ghana. Sequences from three chickens were most closely related to turkey isolates collected in December 2006 in the Ivory Coast. Like the Egypt and Moscow isolates above, the recent isolates with G743A

Options for the Control of Influenza VI

Figure 2. HA Phylogram of H5N1 isolates showing polymorphisms shared with Nigerian isolate.



the branches of the phylogenetic trees, indicating recent acquisition. All referenced isolates on the tips of the branches were collected over a short time frame between February and April 2007 and in three geographically distinct regions. These data do not support a common progenitor sequence because the most closely related sequences to each of the respective recent isolates do not have this change. Similarly, concurrent mutation / selection by eleven isolates that map to six branches in three countries and were collected over a short time frame is also unlikely. An alternative explanation for this concurrent acquisition of G743A in multiple Clade 2.2 sub-clades and for the Nigerian isolate aggregating previously dispersed regional markers is homologous recombination between closely related sequences. Polymorphism tracing demonstrates that most of the newly acquired polymorphisms can be traced to the same serotype identified recently at locations that are linked together by migratory bird flyways, raising the possibility that the distribution and acquisition of the polymorphism is linked to recombination between H5N1 sequences transmitted and transported by migratory birds. The individual polymorphisms recombine to generate sequences that create antigenic drift. Mapping of these pathways and associations can be used to develop novel vaccine targets representing rapidly evolving genomes.

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Proceedings Topic #9

Antivirals and Resistance

**Poster
Presentations**

Amantadine-Resistant Influenza A (H3N2) Virus in Argentina, 2001-2006

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Introduction

The cornerstone of influenza prevention is annual vaccination. However, antiviral drugs are an important adjunct to vaccination for influenza prevention and control. Adamantane derivatives, such as amantadine and rimantadine, have been used successfully for the prevention and treatment of influenza A virus infection for more than 30 years worldwide. These drugs, known as M2 channel blockers, inhibit influenza A virus replication by blocking the M2 protein ion channel thereby preventing fusion of the virus and host-cell membranes and the release of viral RNA into the cytoplasm of infected cells. Human and animal studies have shown the frequent occurrence of amantadine-resistant influenza virus, and drug resistant viruses can be transmitted from one person to another without apparent loss of pathogenicity. Strains of influenza A (H3N2) virus with a specific mutation (Ser31Asn) associated with amantadine resistance have recently shown a dramatic increase in communities in Asia and North America. As in many countries, the use of these antivirals is not common in Argentina. Given the infrequent use of adamantane drugs, the globally increasing frequency of adamantane resistance is not attributable to direct drug selection pressure. Adamantane virus resistant data available from South America are poor. There is a necessity to establish the effectiveness of adamantane antivirals in Argentine population for treatment or prophylaxis in the event of influenza outbreaks. Because of that, in this study we report preliminary results of a surveillance study for resistance to adamantanes among circulating influenza viruses collected in Argentina from 2001 to 2006 and a few ones from 2007 season. Besides, we chose some of these strains to study the HA1 segment and try to match all the information available in order to know the evolutionary pattern of these viruses.

Materials and Methods

Routinely, the National Network of Influenza and Respiratory Viruses Laboratories send to the National Influenza Center nasal swabs and nasopharyngeal aspirates positive for influenza viruses. Samples are collected from pediatric and adult outpatients and inpatients presenting respiratory infection. None of these patients had received previous treatment with amantadine. Viruses were propagated in Madin-Darby Canine Kidney (MDCK) cells and the isolates frozen at -80°C (as stock). For this special work; we selected up to now 55 influenza A H3 strains isolated between 2001 and 2007 to study the segment 7 M2 region: 6 strains from 2001, 2 from 2002, 9 from 2003, 9 from 2004, 8 from 2005, 16 from 2006 and 5 from 2007. From

them, we chose 25 strains to study the HA1 portion of the HA segment. We extracted RNA from 140 µl of virus culture using the QIAmp viral RNA mini kit (Qiagen) according to the manufacturer's protocol. We undertook reverse transcription-PCR (RT-PCR) of the M2 region (286 bp product encompassing nucleotides 741-1027) and HA1 portions of the HA gene (929 bp encompassing nucleotides 55-984) with a one-step RT-PCR kit (QIAGEN). We examined PCR products (5 µl) on a 1% agarose gel to confirm amplification of an appropriately sized DNA band. cDNA products were purified with the SigmaSpin™ post-reaction clean-up columns procedure (Sigma). Sequencing reactions were done with the ABI BigDye 3.1 Terminator Cycle Sequencing kit (Applied Biosystems); products were resolved on an ABI 3100 avant (Applied Biosystems). Sequence data were edited and analyzed using Clustal X (version 1.8). The analysis of the genomic data was made by the construction of phylogenetic trees using MEGA program (version 2.1).

Results

A total of 55 influenza A H3 field isolates were screened for specific aminoacid substitutions in the M2 region known to confer drug resistance. Of these viruses, 20 (36%) were identified with the resistance-conferring substitution at amino acid 31 (serine to asparagine [S31N]). Any resistant virus was detected between 2001 and 2005. All strains isolated in 2006 were resistant and from 2007, four up to five isolates were resistant. See figure 1. Analysis of the hemagglutinin gene revealed, as it was described by other authors, the existence of a distinct lineage (N-lineage) of adamantane resistant viruses. The isolate A/Salta/94/07 (salta9407) clusters with the N-lineage, but does not carry the S31N mutation. There are two changes only present in the resistant viruses that are not in the sensible ones. Those changes are at position 193 serine to phenylalanine (S193F) and at position 225 aspartic acid to asparagine (D225N), which falls into the receptor-binding region. See figure 2.

Conclusions

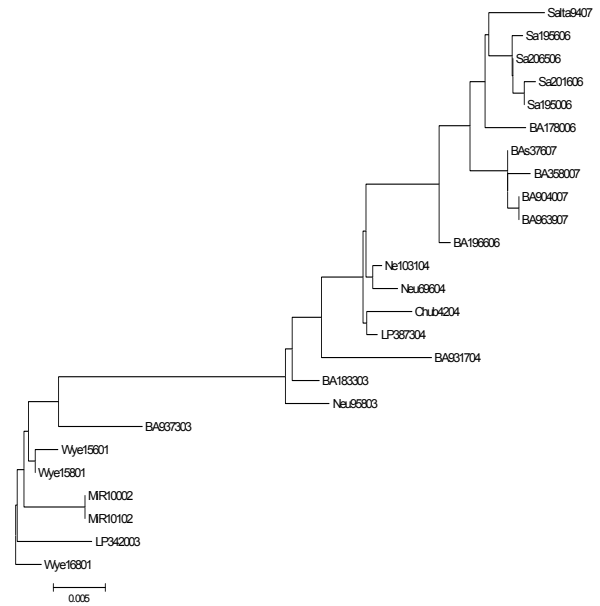
This study reveals an alarming increase in the incidence of amantadine-resistant influenza A H3 viruses since 2006 in Argentina and its circulation is still detected in 2007. Our results indicate that these drugs should not be used for the treatment or prophylaxis of influenza in Argentina until susceptibility to adamantanes has been reestablished among circulating influenza A isolates. The high proportion of influenza A viruses currently circulating in our country demonstrating adamantane resistance highlights the clinical importance of rapid surveillance for antiviral resistance. The phylogenetic analysis showed, as it was previously described, the existence of a distinct lineage (N-lineage) of adamantane resistant viruses isolated during 2006 and 2007 years. The N-lineage also contains an isolate that not possess the S31N mutation, this result could suggest a reassortment event occurred between co-circulating adamantane resistant and sensitive viruses. Taking into account that the N-lineage is characterized by 17 amino acid replacements across the complete genome, we

will need to work on not only HA1 segment but also on the complete genome to have a better understanding of the fitness and evolution characteristics of the resistant and sensitive strains circulating in the country. Testing of influenza isolates for resistance to antivirals will continue throughout the rest of 2007 influenza season.

Figure 1. Phylogenetic tree for the M2 protein (286 bp) for influenza A H3 viruses collected during the period 2001-2007 (n=55). Inside the red square are the resistant strains with the substitution S31N (n=20).



Figure 2. Phylogenetic tree for the HA1 protein (929 bp) for influenza A H3 viruses collected during the period 2001-2007 (n=25). Inside the red square are the members of the HA1 N-lineage.



Influenza Viruses Sensitivity to Antivirals in Russia

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Introduction

In Russia, like in other countries, rimantadine-resistant strains of flu A virus have been often isolated during the last seasons. However, the data received on stabilization of the process allow to continue to use Rimantadine for flu prevention. High sensitivity of flu viruses A to Arbidol has been demonstrated, including rimantadine-resistant strains. For flu viruses B, represented by different evolution lines, strain specificity towards arbidol has been demonstrated. Using combined schemes for specific drugs with different anti-flu activity allows to achieve effective inhibition on viral reproduction using lower concentrations of drugs. It will allow to prevent resistant mutants emergence against combined treatment schemes. Introduction: Along with vaccination that remains the leading means of flu prevention today, chemical drugs with specific anti flu viruses activity also play an important role in the fight against flu. Being much less dependent on the antigenic changes of flu virus is one of key advantages of chemotherapy and chemoprophylaxis, which makes it more important, especially in case of emergence and spread of a new pandemic flu A virus [1]. Two prescription medications that are active against influenza viruses are approved, commercially available and widely used in Russia: Rimantadine™ (blocks the function of the M2 protein) and Arbidol™ (inhibits the lipid membrane of influenza virus fusion with host cell) [2]. Our interest was to study the spectrum of epidemic influenza viruses sensitivity to these antivirals.

Materials and Methods

During five seasons (2002-2007) flu viruses were isolated from rhinopharyngeal swabs of patients with clinically diagnosed flu infections. For that MDCK cell tissue culture was used with future typing of haemagglutinin isolates in HI test. Influenza viruses drug sensitivity was studied using a few methods: by titration in HA-test and cell ELISA. 96-cells plates with MDCK monolayer were infected with epidemic influenza viruses with or without antivirals – Rimantadine (5 µg/ml) and Arbidol (10 µg/ml). In Rimantadine assays the drug was used at the same time with infecting cells, an Arbidol assays – two hours prior infections. Analysis of results was performed according to infections titers decrease to the value ≤ 2 lg in HI-test in 48 hours with human red blood cells of O(I) blood group and viral protein inhibition (NP and M1) in infected cells by 50% and more in cell ELISA after 18 hours.

Results

It should be noted that during studied period all three viruses were isolated – A(H3N2), A(H1N1) and B. A(H3N2) viruses were

the most active with continuous drift. Epidemic strains were similar to the following reference viruses: A/Fujian/411/03 (59% in 2002-2003 and 100% in 2003-2004), A/Kumamoto/102/02 (47% in 2004-2005) and A/California/7/04 (58% in 2005-2006 and 38% in 2006-2007). A(H1N1) viruses were the most active during 2006-2007 season with a higher rate (41%) than that of A(H3N2). During this season the population of flu A(H1N1) virus was heterogeneous and in part significantly different from the reference strain – A/New Caledonia/20/99/. Flu virus type B was represented by two evolutionary line references, moreover, flu viruses similar to reference strains B/Shanghai/361/02 were registered in 2004-2005 (49%) and B/Hong Kong/330/01 for 2005-2006 (30%). The high percent of resistance to Rimantadine for influenza A viruses (2002-2003) circulating in different regions in Russia was found (9.5%). Also our results were suggested by colleagues from CDC&P, Atlanta, USA for 2002-2004 epidemic seasons (8%). The tendency to its increasing was detected for the consecutive seasons and during 2005-2006 this index was equal to 38%. The comparable data was obtained for Moscow region as well (48%). Meantime the preliminary data for 2006-2007 epidemic season showed that situation was seemingly stable and we found that the population of resistant to Rimantadine mutants was 32% on the average for different regions of Russia and 44% for Moscow as well. Interesting data was obtained on flu virus type A specificity towards Rimantadine. During the five epidemic seasons (2002-2007) there was an increase in flu viruses A(H3N2) and then certain stability of resistant variants isolation for 2006-2007 (10%-14%-18%-48%-32%). For flu virus A(H1N1) significant increase in resistant mutants was only found in season 2005-2006 (25%) and its rate during last season was rather high, practically twice as high as the flu virus A(H3N2) rate (55%). Summarizes data of estimation the frequency of resistant mutants isolation during one epidemic season showed that the rates at the beginning, in the middle and at the end of the season did not reflect towards increase, and rather demonstrated some decrease in rates from 58% to 38%. Also relatively low rate was found in children (below 3 y.o.) and at the same time, in the age group ≥ 15 y.o. the rate was as high as 47%. Earlier we found, that Arbidol suppressed the plaque formation for all types of influenza viruses and its concentration for 50 percent of plaque formation decreasing in comparing with control monolayer was between 5 and 10 µg/ml. Our results showed that all influenza A viruses including Rimantadin-resistant mutants were sensitive to Arbidol (10 µg/ml). Meantime among influenza B viruses we found some differences, which may be caused by their strain specificity to Arbidol: only 57.0% among B/Shanghai/361/02-like epidemic viruses during 2004-2005 and all of B/Hong Kong/330/01-like epidemic viruses during 2005-2006 were sensitive to Arbidol. Investigations of effectiveness of combined application schemes of Arbidol and Rimantadine in model experiments confirmed enhanced influenza inhibitions effect in MDCK cell culture when drugs were used in lower concentration (Table 1).

Table 1. Combined rimantadine and arbidol application schemes activity towards flu viruses A(H3N2) reproduction in MDCK culture.

A(H3N2) epidemic strains	Viral reproduction inhibition rate (%) at drug concentration		
	Arbidol	Rimantadine	Arbidol+Rimatadine
A/Moscow/6/05	25.0 (2.5)	40.0 (0.001)	56.0
A/Rostov-on-Dono/57/05	47.0 (2.5)	34.0 (0.001)	68.0
A/Yaroslavl/12/05	41.0 (5.0)	33.0 (0.005)	57.0
A/Yaroslavl/11/05	34.0 (5.0)	33.0 (0.005)	76.0

Discussion

It is well known that during last epidemic seasons the continuing increase in adamantane resistance among influenza A viruses was noted in many countries [3,4]. The incidence of resistant mutants among influenza A(H3N2) viruses has dramatically increased from 1,0% before 2002 to 90,0% for 2005-2006 epidemic season in some countries in the world (USA, China, Canada). In Russia such investigations didn't make since the middle of 1990-th. We presented the last data concerning the situation with influenza virus sensitivity to Rimantadine and Arbidol in Russia for the last five seasons (2002-2007). We started our investigations since 2002-2003 epidemic season and found the high percent of resistance to Rimantadine for influenza A viruses (9.5%). The tendency to its increasing was detected for the consecutive seasons, but for the last one (2006-2007) we found the stabilization of the process. Also some differences in the forming of resistance to Rimantadine among A(H3N2) and A(H1N1) influenza viruses were detected, that may be explained by their activity in epidemic process during the last time. We received results that demonstrated high sensitivity of flu viruses A to Arbidol, including Rimantadine-resistant strains. For flu B viruses, represented by different evolution lines, strain specificity towards Arbidol has been demonstrated. Meantime combined schemes for specific drugs with different anti-flu activity allows to achieve effective inhibition on viral reproduction using lower concentrations of drugs. It will allow to prevent resistant mutants emergence against combined treatment schemes. Received results determinate the recommendations for antivirals using – Rimantadine and Arbidol in annual epidemic preparedness and allow to use both of them for influenza treatment and prophylaxis in Russia now.

Acknowledgements

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Long Term Expression of Small Interfering RNA Targeting M2 Gene Induces Effective Inhibition of Influenza A Virus Replication

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RNA interference (RNAi) provided a powerful new means to inhibit specific virus infection. Here, a lentiviral vector with H1- short hairpin RNA (shRNA) expression cassette and enhanced green fluorescence protein (EGFP) as surrogate marker is adopted to deliver small interfering RNAs (siRNAs) into mammalian cells. Upon the lentiviral integration property, we have successfully built up persistent cell lines to express siRNAs. Based on the established stable cell lines, the inhibition efficiency of the rationally designed siRNAs specific for conserved genome of influenza A virus has been well studied. The results showed that siRNA targeting influenza M2 gene (siM2) conferred more effective inhibition of virus replication compared with previously reported siRNA targeting NP gene (siNP). This high suppressive effect of siM2 was observed not only for H1N1 virus but also for highly pathogenic avian influenza H5N1 subtype. In conclusion, our study suggested M2 gene can be an optimal RNAi target for antiviral therapy. Those findings provide rational information for the development of siRNAs as prophylaxis and therapy for influenza virus infection in humans.

Introduction

Influenza A virus remains a scourge on human health. Its antigen drifts and shifts are an ever-changing challenge for available vaccines and drugs. Given the limitations of current anti-influenza A virus strategies, the need for novel strategies for prevention and treatment of influenza A virus is evident. RNAi is a form of posttranscriptional gene silencing mediated by short double-stranded RNA, known as siRNA. These siRNAs are capable of binding to a specific mRNA sequence and causing its degradation (Vaucheret, Beclin, and Fagard, 2001). siRNA and shRNA have been introduced into cells by transfection of chemically synthesized and RNA expression via plasmid cassettes utilizing RNA polymerase III transcription. The employment of siRNA/shRNA for gene knockout requires an efficient and stable transfection or transduction process. Until now, effective delivery is still a major issue. Lentiviruses, a subclass of retrovirus, can infect both dividing and nondividing cells due to their ability to get through the intact membrane of the nucleus of the target cells. After infection, the viral genome will be integrated into the target genome and viral genes will be expressed. Like other retroviral vectors, lentiviral

vectors are capable of producing high virus titers and inducing high-level gene expression in target cells (An et al., 2006). Therefore in our current study, the lentiviral vector developed by DIDIER TRONO group (<http://www.tronolab.unige.ch>) has been adopted. The HIV accessory genes *vif*, *vpu*, *nef*, and *tat* have been deleted from the packaging constructs, the necessary vector components are expressed from three distinct plasmids (LVTHM, psAX2 and pMD.2G), and the overlapping regions between these units have been minimized to limit the possibility of homologous recombination. Recent reports using siRNAs both in vitro and in vivo suggest that siRNAs hold great promise for treating influenza A virus infection (Ge et al., 2004; Tompkins et al., 2004). However, most of studies are focused on acidic polymerase (PA) or nucleoprotein (NP) gene. For other viral genes, the inhibition effect of RNAi is still unknown. Therefore, for RNAi to be effectively therapeutically, more effective viral targets and long term RNAi effects with a suitable delivery system still deserve further study.

Materials and Methods

Cell lines and viruses. MDCK cells were maintained in MEM (Invitrogene) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100U penicillin G mL⁻¹ and 100ug streptomycin mL⁻¹). Human embryonic kidney 293T cells were maintained in DMEM (Invitrogene) supplemented with 10% FBS and antibiotics. Influenza Virus strain A/New Caledonia/20/99-like (H1N1) and A/Hong Kong/483/97 (H5N1) were used in these experiments and stock virus were prepared on MDCK cells and virus titers were determined by TCID₅₀.

Plasmid construction. The sequences of siRNAs are as follows: M-950 (siM2), sense 5'-ACAGCAGAAUGCUGUGGAU-3', antisense 5'-AUCCACAGCAUUCUGCUGU-3'; NP-1496 (siNP), sense 5'-GGAUCUUAUUUCUUCGGAG-3', antisense 5'-CUCCGAAGAAAUAAGAUC-3' (Ge et al., 2003). Plasmid LVTHM allows for direct cloning of annealed shRNA into lentiviral vector. The H1-promoter-driven shRNA cassettes were made by annealing two primers (5'-CGCGTCCCC(N)₁₉TTCAAGAGA(N)₁₉TTTTTGAAAT-3', 3'-AGGGG(N)₁₉AAGTTCTCT(N)₁₉AAAACTTTA GC-5') containing the 19-nt sense and reverse complementary targeting sequences with a 9-nucleotide loop -TTCAAGAGA- and flanking MluI and ClaI cloning sites (highlighted in bold), and then cloned into the 3'-end of the H1 promoter in the LVTHM plasmid. The correct sequence and insertion were confirmed by DNA sequencing.

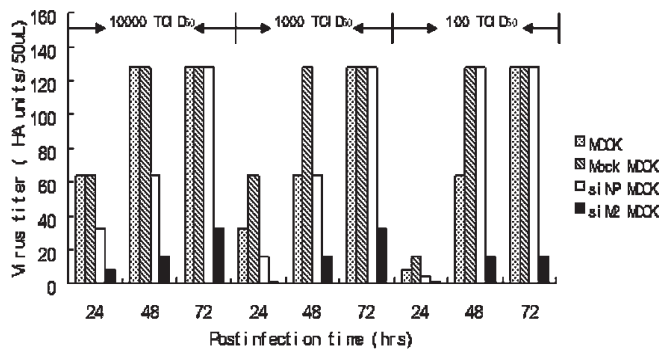
Lentiviral vector particle production. Lentiviral vectors were produced by calcium phosphate-mediated, three-plasmid transfection of 293T cells (An et al., 2003). Briefly, 293T cells (2.5×10⁶ cells in a 75T flask) were transfected with 20 µg LVTHM or LVTHM with shRNA insertion, 15 µg psAX2 and 6µg pMD.2G and cultured in DMEM supplemented with 10% FBS and antibiotics. Virus supernatants were collected on day 3 post-transfection, filtered through a 0.45µm pore-size filter, ultracentrifuged (40 000 g, 3h, 4°C) and resuspended in PBS. Virus stocks were titrated by infecting Hela cells with virus dilutions in DMEM supplemented with 10% calf serum and 8µg

polybrene (hexadimethrine bromide, Sigma) ml⁻¹ and analyzed for EGFP expression by flow cytometry. Titres of vector virus stocks were routinely 10⁷ ~10⁸ infectious units (TU) ml⁻¹.

Establishment of siRNA expressing stable cell lines. Lentiviral stocks were used to transduce MDCK cells. After 3 days of transduction, the medium containing the lentivirus was replaced with complete culture medium. The transduced MDCK was subcultured every 3-4 days for 4 weeks to get stable viral genome integration. Sorting of live GFP positive cells was performed using a FACStar+ instrument (Becton Dickinson) (Fish and Kruihof, 2004). Established stable cell lines are described as follows: Mock MDCK, MDCK cells transduced by lentivirus without shRNA insertion; siNP MDCK, MDCK cells transduced by lentivirus with shNP insertion (NP-1496); siM2 MDCK, MDCK cells transduced by lentivirus with shM2 insertion (M-950).

Influenza virus infection and viral load detection. MDCK and other established stable cell lines were grown in MEM containing 10% FBS and antibiotics. The culture medium was then removed and 100 ~ 10000TCID₅₀ influenza virus were added to each well of 24 well plate. After incubation for 1h, the infection medium was removed and MEM without FBS was added. Cell supernatants were collected at different time points. The viral load was detected by hemagglutination (HA) or plaque assays. The HA assay of virus samples was carried out in V-bottom 96 well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of a 0.5% suspension of turkey erythrocytes (Lampire Biologic Laboratories, Pipersville, USA) and incubated at room temperature for 45mins. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive. For plaque assay, serial 10-fold dilutions of virus sample were added onto a monolayer of MDCK cells in 1% semi solid agar. Three days after infection, plaques were visualized by staining with crystal violet.

Figure 1. Inhibition of influenza H5N1 virus production on stable cell lines. Cells were infected by H5N1 at different virus doses. Virus titer was measured at the indicated time after infection.



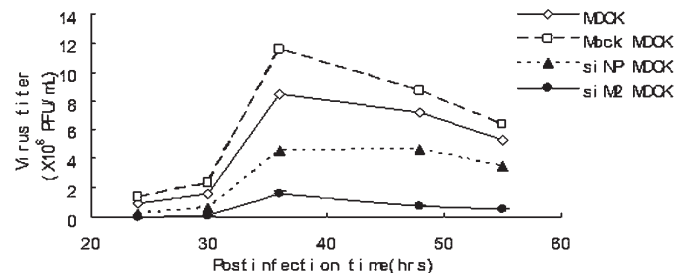
Results

Inhibition of H5N1 virus production on stable cell lines. To test whether siM2 inhibited influenza virus production, we directly

examined the effect on established stable cell lines. MDCK, Mock MDCK, siM2 MDCK and siNP MDCK were infected by H5N1 at 100TCID₅₀, 1000TCID₅₀ or 10000TCID₅₀. At different time after infection, culture supernatants were harvested to determine the virus titer by HA assay. As shown in Fig. 1, no matter how much virus infected, virus titers in the infected cultures increased over time, reaching peak values between 48-72hrs. Virus replication kinetics of Mock MDCK is very similar with that of MDCK, which means lentivirus integration doesn't influence virus replication. By comparison, virus production on siM2 MDCK is significantly inhibited regardless how much virus infected, such suppression effect can maintain up to 72hrs. However, siNP MDCK can only offer a minor inhibition effect at a very early stage of the virus infection.

Inhibition of H1N1 virus production on stable cell lines. Above results suggested siM2 provided a more potent protection against H5N1 compared with siNP. Given different virus subtypes possess different kinetics and tissue tropisms, we further tested the ability of siM2 to inhibit replication of H1N1 subtype. Using the very similar method for H5N1, we directly infected established stable cell lines by H1N1, and the culture supernatants were collected at different time points to determine the titers by plaque assay (Figure 2).

Figure 2. Inhibition of influenza H1N1 virus production on stable cell lines. Stable cell lines were infected by 100TCID₅₀ H1N1 virus. Virus titer was measured at the indicated time after infection.



The results showed siM2 conferred more effective inhibition compared with siNP. Almost 90% of H1N1 virus replication has been inhibited on siM2 MDCK. By contrast, siNP MDCK can only provide 60% inhibition compared with controls.

Discussion

The principal finding of our study is that rationally designed siRNA targeting influenza M2 gene conferred more effective inhibition compared with previously reported siRNA against NP gene (NP-1496). Such high suppressive effect is confirmed not only for H1N1 influenza A virus but also for highly pathogenic H5N1 subtype. Our results suggested siM2 provided a broad protection against influenza A virus and M2 gene is an optimal viral target for antiviral therapy of influenza virus infection. Depending on the stringency of siRNA-target base pairing, siRNA treatment may cause selection of siRNA-resistant

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viruses, and this has been shown with HIV (Das et al., 2004). For the development of siRNA therapeutic regimen to human influenza virus infection, this problem is inevitable. In our study, three kinds of stable cell lines are established through lentivirus transduction. Such stable cell lines can persistently express siRNA and provide a more convenient method to study long term inhibition effect of siRNAs and screen siRNA resistant mutants in quasispecies in vitro. Some work has been done about this problem in our study. The current data have shown no mutation appear on the targeted siRNA sequence after 25 passages' H5N1 virus culturing, therefore suggested siM2 possessed good long term inhibition effect for influenza virus replication.

Acknowledgements

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Antiviral Activity of Brazilian Propolis Against Influenza Virus In Vitro and In Vivo

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Introduction

Propolis has been used worldwide as a traditional dietary supplement to maintain and improve human health. We examined the anti-influenza virus activity of ethanol extracts of propolis in vitro and in vivo. Among 13 ethanol extracts, 4 extracts showed anti-influenza virus activity against A/PR/8/34 (H1N1) virus in vitro. In an intranasal influenza virus infection model in mice, one of the 4 ethanol extracts, AF-08, was significantly effective in reducing the body weight loss of infected mice after infection ($p < 0.05$). Thus, only AF-08 was found to be effective against influenza infection in vitro and in vivo. Further, AF-08 at 2 and 10 mg/kg significantly prolonged the survival times of infected mice for 10 days after infection but 0.4 mg/kg did not. AF-08 at 10 mg/kg was also effective in reducing virus yields in the bronchoalveolar lavage fluids (BALF) of lungs compared with the control at 1 to 4 days after infection ($p < 0.005$). The virus yields in the BALF were not statistically different from those in infected mice with administration of oseltamivir at 1 mg/kg. The oral administration of AF-08 at 10 mg/kg was not toxic in uninfected mice. The antiviral activity of AF-08 was confirmed to be effective against influenza B and oseltamivir-resistant viruses as well as influenza A virus in vitro. AF-08 did not inhibit virus adsorption to cells in vitro. Thus, AF-08, an ethanol extract of Brazilian propolis, was found to exhibit anti-influenza activity in vitro and in vivo and is a possible candidate for an anti-influenza dietary supplement.

Introduction: Influenza virus infects the respiratory tracts in humans and causes a variety of symptoms including fever, nasal secretions, cough, headache, muscle pain, and pneumonia. These clinical symptoms often become severe in high-risk groups such as the elderly and infants. Although two inhibitors of influenza virus neuraminidase (NA), zanamivir and oseltamivir, provide both antiviral effects and clinical benefits in humans, the appearance of resistant viruses to the NA inhibitors has been clinically reported. The development of compounds that reduce the appearance will be needed. Propolis has been used worldwide as a folk medicine since ca. 300 BC and as a dietary supplement to maintain or improve human health. It is a resinous material collected from various plants by honeybees and contains the various components derived from plants. Recently, some propolis, especially its aqueous extract, has been reported to exhibit various biological activities such

as antibacterial, antitumor, and immunostimulatory. Anti-influenza virus activity of propolis was also known in an in vitro study. However, the anti-influenza virus activity is not clear in vivo. In this study, we examined the anti-influenza virus activity of ethanol extracts of propolis in vitro and in vivo. An ethanol extract, AF-08, was found to exhibit therapeutic anti-influenza activity in infected mice. The activity was characterized in the comparison with oseltamivir.

Materials and Methods

Cell. Madin-Darby canine kidney (MDCK) cells were grown and maintained in Eagle's minimum essential medium supplemented with 8 % and 2 % heat-inactivated fetal calf serum, respectively.

Viruses. Influenza A/PR/8/34 (H1N1), A/Bangkok/94/03 (H1N1), A/Ishikawa/7/82 (H3N2), A/Fukushima/13/43 (H3N2), and B/Fukushima/15/93 viruses were propagated in MDCK cells. The oseltamivir-resistant virus was plaque-purified from influenza A/PR/8/34 (H1N1) virus-infected MDCK cell cultures in the presence of 30 µg/ml of oseltamivir in our laboratory. For in vivo assay, a mouse-adapted influenza A/PR/8/34 (H1N1) virus was used.

Propolis and Reagents. Thirteen propolis were collected by Africanized *Apis mellifera* bees in southern Brazil and supplied by AMAZONFOOD Ltd. The ethanol extracts of the collected propolis was dried and used for the in vitro and in vivo experiments. The ethanol extracts were dissolved in appropriate volume of ethanol and diluted with culture medium to make its various final concentrations for in vitro assay. The concentration of ethanol in each medium was less than 0.2 %. Oseltamivir was dissolved in distilled water, and diluted with culture medium. For in vivo assay, the ethanol extracts of propolis were dissolved in appropriate volume of ethanol, diluted with distilled water, and used for oral administration to mice. Oseltamivir was dissolved in distilled water and administered to mice.

Antiviral activity in vitro. Thirteen ethanol extracts of propolis and oseltamivir were examined for their antiviral activity against influenza A, B, or oseltamivir-resistant A virus by plaque reduction assay or MTT assay using MDCK cells. The cytotoxicity of the extracts was examined by trypan blue dye exclusion test or MTT assay. Anti-influenza activities were evaluated by the effective concentrations for 50% plaque reduction (EC_{50}) and cytotoxic concentrations for 50% cell viability (CC_{50}). To examine the effect of AF-08 on virus adsorption, influenza A/PR/8/34 (H1N1) virus was mixed with AF-08 at various concentrations and adsorbed to MDCK cells in plaque reduction assay.

Therapeutic efficacy in mice. Female DBA/2 Cr mice (weight, 17-19 g) were intranasally infected with influenza A virus at 1600 plaque forming units/25 µl of phosphate buffered saline under anesthesia. The ethanol extracts of propolis (0.4, 2, and 10 mg/kg) were orally administered once at 4 h before and 3 times daily for 7 successive days after infection. Ethanol solution at 1 % was used for a control. Oseltamivir at 1 mg/kg was orally administered to infected mice twice daily for 7 days after infection. The body weights of mice ($n = 6$) were measured daily for 4 days. The number of surviving mice ($n = 6$) were observed

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daily for 10 days after infection. The bronchoalveolar lavage fluids (BALF) of infected mice were prepared on 1 to 4 days after infection, and virus yields in the BALF were determined by the plaque assay using MDCK cells.

Results and Discussion

Screening of extracts with anti-influenza virus activity. The antiviral activities of 13 ethanol extracts to influenza A/PR/8/34 (H1N1) virus were screened by a plaque reduction assay. Of the 13 extracts, the EC_{50} values of the 4 were significantly lower than their CC_{50} values, indicating that the 4 extracts showed anti-influenza virus activity. Thus, the 4 extracts were selected as possible anti-influenza virus extracts for evaluating therapeutic anti-influenza activity in mice. In the murine infection model, an ethanol extract, AF-08 at 10 mg/kg was only effective in reducing the body weight loss of infected mice significantly for 4 days after infection ($p < 0.05$ by repeated measure two-way ANOVA). We used 10 mg/kg as a dose for mice corresponding to human use and no significant body weight loss was observed in uninfected mice for 10 days after oral administration. Thus, the anti-influenza virus activity of AF-08 was exhibited in vitro and its therapeutic anti-influenza activity was observed without toxicity in vivo. AF-08 was suggested to contain some component that exhibits anti-influenza virus activity in mice.

Confirmation of anti-influenza activity. We confirmed and characterized the anti-influenza activity of AF-08 in comparison with oseltamivir in mice. Oral administration of AF-08 at 10 mg/kg significantly prolonged survival times compared with the control for 10 days after infection ($p < 0.05$ by Kaplan-Meier method). The dose of 2 mg/kg also prolonged survival times for 10 days after infection significantly, but 0.4 mg/kg did not. The anti-influenza activity of AF-08 was observed in a dose-dependent manner. We confirmed the anti-influenza activity of AF-08 in mice. AF-08 at 10 mg/kg as well as oseltamivir at 1 mg/kg was significantly effective in reducing virus yields in the BALF of lungs of infected mice compared with the control for 1 to 4 days after infection ($p < 0.005$ by repeated measure two-way ANOVA). There was no significant difference between virus yields in the lungs of infected mice administered with AF-08 at 10 mg/kg and oseltamivir at 1 mg/kg. We used 10 mg/kg of AF-08 and 1 mg/kg of oseltamivir as a dose for mice corresponding to their human use in the infection model. Thus, AF-08 at 10 mg/kg may be effective to influenza virus infection in humans.

Possible anti-influenza action. Anti-influenza activity of AF-08 was examined using various influenza virus types and their subtypes. AF-08 was found to exhibit antiviral activity against influenza A (H1N1 and H3N2), B, and oseltamivir-resistant A (H1N1) viruses used in this study. Thus, there might be no type and subtype-specificity for the antiviral activity of AF-08 to influenza viruses. AF-08 also exhibited anti-influenza virus activity to oseltamivir-resistant virus. This suggests that the mechanism of anti-influenza virus action of AF-08 is different from that of oseltamivir. Furthermore, no AF-08 showed the inhibitory effect on virus adsorption to MDCK cells. The inhibition of the influenza virus growth by AF-08 probably occurs in the replication

process in the cells after adsorption. In conclusion, an ethanol extract of Brazilian propolis, AF-08, was found to possess the antiviral activity against influenza A, B, and oseltamivir-resistant A virus in vitro. The anti-influenza virus activity of AF-08 was verified in mice. This result is the first evidence demonstrating the anti-influenza activity of propolis. Propolis has been used worldwide as a dietary supplement. Thus, AF-08 is a possible candidate for anti-influenza dietary supplement. It also may be used supplementally for the treatment of known anti-influenza virus agents such as oseltamivir and zanamivir.

The NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit: Chemiluminescence Assay for Detection and Quantification Of Influenza Neuraminidase Activity

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Introduction

The NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit provides the NA-Star® 1,2-dioxetane chemiluminescent neuraminidase substrate, together with all necessary assay reagents and microplates, to measure the resistance level of influenza virus isolates to neuraminidase inhibitor antiviral therapeutics. NA-Star substrate provides highly sensitive detection of neuraminidase enzyme activity from influenza virus types A and B, including human, avian, porcine and equine viruses. Neuraminidase assays performed with the NA-Star 1,2-dioxetane chemiluminescent substrate provide approximately 50-fold higher sensitivity than the MUNANA fluorescence substrate. The chemiluminescent NA-Star assay provides linear results over 3-4 orders of magnitude of neuraminidase concentration compared to 1-2 orders of magnitude achieved with the fluorescent assay, providing a greater assay dynamic range. Virus dilutions are briefly incubated with neuraminidase inhibitor, and then the two-reagent detection assay is performed. The entire assay is completed in approximately one hour. Data analysis, using non-linear curve fitting dose response analysis software (not provided), is performed to determine the IC₅₀ value of the neuraminidase inhibitor with each viral isolate. The NA-Star chemiluminescence assay has been compared to MUNANA fluorescence assays performed with isolates of the major influenza types, H1N1, H3N2 and influenza B strains, including NI-sensitive and resistant strains. The NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit combines highly sensitive and rapid chemiluminescent quantitation of neuraminidase activity from flu virus isolates using supplied reagents and a simple assay protocol to provide a convenient method for use in research laboratories to monitor the resistance levels of both human and animal influenza virus isolates to neuraminidase inhibitors.

Materials and Methods

Influenza strains: Influenza Type A (VR-1469, H1N1, strain A/PR/8/34), and Influenza Type B (VR-1535, strain B/Lee/40) are from ATCC and were grown on MDCK cells. Additional strains are from the strain repository at the UK Health Protection Agency. Oseltamivir carboxylate was obtained from F. Hofmann-La Roche (Basel, Switzerland), and zanamivir

was from GlaxoSmithKline (UK). MUNANA was obtained from Fluka. NA-Star assays were performed with the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, P/N 4374422) according to the supplied kit protocol. Briefly, virus dilution is incubated with NI dilution for 15 min, then diluted NA-Star substrate is added and incubated for 30 min. NA-Star Accelerator is injected and light emission read immediately. MUNANA assays were performed according to standard published protocols. Data analysis (IC₅₀ determinations) was performed using non-linear curve fitting (GraphPad Prism 4.0) or Excel analysis using custom fitting algorithms.

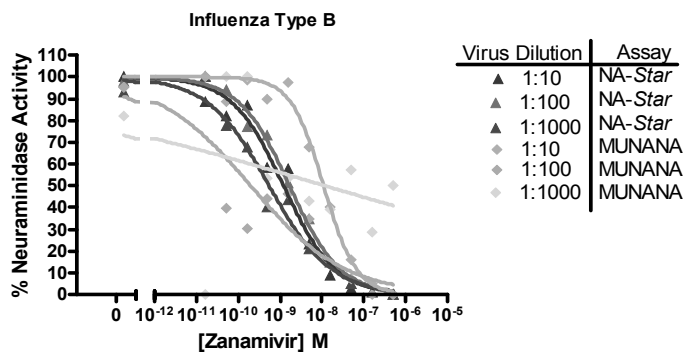
Results

Chemiluminescent reactions result in conversion of chemical energy to light energy, as light emission. The NA-Star substrate is a 1,2-dioxetane structure bearing a sialic acid cleavable group. To perform the NA-Star assay, virus dilutions (from cell culture supernatant) are first incubated in the presence of neuraminidase inhibitor. Then NA-Star substrate is added and incubated, under conditions optimal for neuraminidase activity, for 30 min for substrate cleavage to proceed. Finally, light emission is triggered immediately upon addition of an Accelerator (ideally performed with an on-board reagent injector), which provides required pH shift and a proprietary polymeric enhancer required for efficient light emission. Chemiluminescent assays are performed in solid white microplates, and light emission is measured in a luminometer. The NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit provides the NA-Star Substrate, NA-Star Assay Buffer (used for dilution of neuraminidase inhibitors, virus samples and NA-Star Substrate), NA-Star Accelerator, detection microplates and a user protocol. The NA-Star assay was initially compared to the MUNANA assay by quantitating neuraminidase activity in serial dilutions of both purified bacterial neuraminidases and virus culture supernatants. The chemiluminescent NA-Star assay provides linear results over 3-4 orders of magnitude of neuraminidase concentration compared to 1-2 orders of magnitude achieved with the fluorescent assay, providing a greater assay dynamic range. In addition, the sensitivity of the NA-Star assay is 15-50-fold higher than the MUNANA assay, enabling detection of lower concentrations of virus, and providing much higher signal-to-noise ratios (data not shown). So, with the NA-Star assay, virus culture supernatants can be used at higher dilutions for performing neuraminidase inhibitor quantitation assays, thus requiring much less of the virus culture supernatant. IC₅₀ values for zanamivir were determined with both the NA-Star and the MUNANA assay over a range of virus culture supernatant dilutions (Figure 1). The NA-Star assay provides a much higher signal/noise than the MUNANA assay at the same dilution. For the 1:10 virus dilution, the signal:noise value (virus dilution without drug:mock-infected media control) with NA-Star assay is 650:1, while that with the MUNANA assay is 5:1 (not shown). The IC₅₀ variability is considerably higher for the MUNANA

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assay at different virus concentrations, and at least for the type B virus above, only the highest concentration provides a good curve fit. With the NA-Star assay, accurate quantitation of IC_{50} s over a wider range of virus concentration minimizes the need for a virus pre-titration step prior to IC_{50} quantitation assay.

Figure 1. IC_{50} determination: NA-Star assay vs. MUNANA assay at multiple virus concentrations.



Several neuraminidase inhibitor-resistant mutant flu strains (and the corresponding wild-type sensitive strains) were assayed with both the NA-Star and MUNANA assays (Table 1). There is generally good correlation between the IC_{50} values determined with the NA-Star and MUNANA assays for the NI-sensitive strains. The IC_{50} values determined with the NA-Star assay tend to be slightly lower than values determined with MUNANA assay. For a few of the NI-resistant strains (H3N2 119V with oseltamivir; flu B 152K with zanamivir), the NA-Star IC_{50} values are significantly lower than those obtained with MUNANA, and ratio of neuraminidase activity between mutant and wild-type is not as great.

Table 1. NI-resistant viruses: NA-Star assay vs. MUNANA assay.

Virus Subtype	Reference Strain	Oseltamivir		Zanamivir	
		NA Star	Munana	NA Star	Munana
H1N1	274H WT	0.16	0.63	0.13	0.28
	274Y Mutant	86.55	349.60	0.22	0.46
	Ratio Mt/Wt	534.55	558.82	1.69	1.64
H3N2	119E WT	0.34	0.73	0.47	1.45
	119V Mutant	2.47	38.27	0.31	2.10
	Ratio Mt/Wt	7.30	52.53	0.66	1.45
	292R WT	0.28	0.37	0.60	1.00
	292K Mutant	1776.70	1560.32	4.86	12.00
	Ratio Mt/Wt	6352.63	4240.00	8.10	12.00
Flu B	152R WT	0.24	1.91	1.70	1.70
	152K Mutant	>3000	1560.32	13.00	115.00
	Ratio Mt/Wt	#VALUE!	815.38	7.65	67.65

Summary

The NA-Star[®] Influenza Neuraminidase Inhibitor Resistance Detection Kit provides higher sensitivity detection, wider assay dynamic range, improved ease-of-use compared to

fluorescent MUNANA assays. The complete reagent set supports “standardized” reaction conditions, enabling more accurate comparison of experimental results. Influenza NA assays using NA-Star substrate have been compared to MUNANA with a wide range of flu strains in public health labs globally, including the US, Canada, Australia, Europe, Japan and the UK [1-12]. NA-Star chemiluminescent substrate provides highly sensitive detection of neuraminidase activity from many flu viruses, including human types A and B, avian, equine and porcine viruses. This broad assay capability makes it an important new tool for researching the global spread of influenza drug resistance, as well as for additional applications: Identification and development of new NIs; NA detection in other pathogens; Influenza virus quantitation/neuraminidase standardization [13]; Quantitation of viral growth in culture for study of cellular infectivity/virus replication. The discrepancy in IC_{50} values obtained with the NA-Star and MUNANA assays has not been attributed to virus concentration or substrate concentration, but possibly could reflect differences in substrate cleavage efficiency by certain neuraminidases or assay protocol differences, and remains under investigation. Global monitoring of influenza strains for resistance to anti-viral inhibitors is essential for studying epidemiology of viral strains and mutations and for reliably understanding the efficacy of neuraminidase inhibitor therapeutics both in the event of a significant influenza outbreak and for seasonal influenza. The NA-Star kit provides highly sensitive detection with ease-of-use, standardized reagents and versatile assay capability. Ongoing substrate and assay development will provide improvements in light emission kinetics and new applications.

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Effect of Oral Gavage Treatment With ZnAL42 on Influenza A H5N1 and H1N1 Virus Infections in Mice

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Avian influenza H5N1 infections can cause severe, lethal human infections. The studies were done to evaluate the efficacy of a novel zinc formulation in two influenza A mouse models. Mice infected with influenza A/Duck/MN/1525/81 (H5N1) virus were treated orally 48 h before virus exposure and then twice daily for 13 days with ZnAL42. The optimal dosing regimen for ZnAL42 was achieved at 8.64 mg/kg 48 h prior to virus exposure, twice daily for seven days. The survival rate was 80% compared to 10% in the untreated control group and a 100% survival rate for oseltamivir (100 mg/kg, bid x 5 beg. 4 h beginning four hours before virus exposure). ZnAL42 treatment significantly lessened the decline in arterial oxygen saturation ($P < 0.001$). This regimen was also well tolerated by the mice. Mice were also infected with influenza A/NWS/33 (H1N1) virus and were treated using the same dosage regimen described above. The 8.64 mg/kg/day dose of ZnAL42 significantly prevented death. The data suggest that the prophylactic use of ZnAL42 is efficacious against avian influenza H5N1 or H1N1 virus infection in mice.

Introduction

The potential of zinc as a therapy for viral infections has been reported for the common cold virus [1], respiratory syncytial virus [2], cytomegalovirus [3], and herpes virus [4]. There are fewer reports on zinc and influenza virus. For example, the combination of zinc-containing superoxide dismutase and rimantadine hydrochloride was shown to significantly protect mice from a challenge with influenza A (H3N2) virus infection [5]. This report describes the results of both toxicity and antiviral efficacy evaluations of a zinc formulation, ZnAL42, against influenza A (H1N1) and avian influenza (H5N1) infections in mice.

Materials and Methods

Animals. Female pathogen-free 18-21 g BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). They were housed in polycarbonate cages with stainless steel tops, and provided tap water and standard rodent mouse chow *ad libitum*. The mouse chow contained an undisclosed concentration of zinc oxide.

Virus. Influenza A/Duck/MN/1525/81 (H5N1) was obtained from Dr. Robert Webster of St. Jude Hospital, Memphis, TN and was adapted to induce pneumonia-associated death in mice. Influenza A/NWS/33 (H1N1) virus was provided by Dr. Kenneth Cochran of the University of Michigan.

Compounds. ZnAL42 (Remedy Research Ltd., London, UK) was maintained at room temperature and drug was diluted in sterile distilled water or saline to the appropriate concentrations. Oseltamivir (TamiFlu™) was purchased from a local pharmacy and dissolved in sterile saline.

Arterial Oxygen Saturation (SaO₂) Determinations. SaO₂ was determined using the Ohmeda Biox 3800 pulse oximeter (Ohmeda, Louisville, OH). Use of an earlier Ohmeda Model (3740) for measuring effects of influenza virus on SaO₂ in mice has been previously described [6].

Preliminary Toxicity Determination. Groups of three mice were treated p.o. (gavage). Each animal was weighed before treatment and again 18 h after final treatment and observed daily for death for 16 days. After determining the maximum tolerated dose, similar dosage regimens using the maximum tolerated dose and dilute drug concentrations were evaluated as described below.

Prophylactic/Therapeutic Experiments. Mice were infected with a LD60 of the H5N1 strain of influenza virus or with a LD100 of the H1N1 strain. Mice were anesthetized with ketamine (100 µg/kg) and then instilled with 90 µl of virus in the nares. Ten mice each were treated by oral gavage with ZnAL42 at doses ranging from 0.24 to 8.24 mg/kg/day. Animals were treated with compounds twice a day (bid) for 13 days beginning 48 h before virus exposure. Oseltamivir (20 mg/kg/day) was administered per os twice daily for 5 days beginning 4 h post-virus exposure. Animals were observed for death for 21 days and SaO₂ levels measured daily from days 3 through 11. Three uninfected mice (toxicity controls) were treated in parallel with each dose of compound. These animals were observed as described above, and weighed prior to the first treatment and 18 h after the final zinc treatment. Three normal control mice were held in parallel; parameters measured were as above.

Statistical Analysis. Increases in total survivors were evaluated by chi square analysis with Yates' correction for small sample size. Increases in mean day to death and differences in mean SaO₂ values were analyzed by *t*-test.

Results

ZnAL42 treatment of H5N1 infection in mice. Treatment with ZnAL42, beginning 48 h prior to virus exposure and subsequently twice daily for 13 days significantly inhibited the virus infection at 8.64 mg/kg/day; 80% of the animals survived ($P < 0.05$), while 12 of 20 of the placebo control mice died (Table 1). SaO₂ levels in the animals treated with ZnAL42 were significantly higher ($P < 0.05$) than in infected placebo controls, although the mean day to death for both groups was similar. The lower dosages of ZnAL42 did not significantly inhibit the infection. Oseltamivir protected all the mice from lethal infection. SaO₂ values were at relatively high levels throughout the experiment. The toxicity control animals survived all doses, although the animals generally failed to gain weight at the same rate as the normal controls.

ZnAL42 treatment of H1N1 infection in mice. Mice were infected with influenza A/NWS/33 (H1N1) virus and beginning 48 h

before virus exposure, mice were treated by oral gavage with three dosages of ZnAL42 (8.64, 1.46, or 0.24 mg/kg/day) and bid for 12 days thereafter. All infected control mice died (mean day to death = 10.0 ± 1.7 days). The highest and lowest doses of ZnAL42 significantly inhibited death and promoted adequate arterial oxygen saturation (Table 2). All oseltamivir-treated (qd X 5 days, beginning 4 h post-virus exposure, 20 mg/kg/day) mice survived and arterial oxygen saturation levels in these mice were significantly higher than in placebo-treated animals. Both compounds were well tolerated by toxicity control mice.

Table 1. Effect of oral gavage treatment^a with ZnAL42 on influenza A (H5N1) virus infection in mice.

Compound	Dosage (mg/kg/day)	Toxicity Controls		Infected, Treated Mice		
		Survivor/ Total	Mean Host Weight Change ^b (g)	Survivor/ Total	Mean Day to Death ^c ± SD	Mean SaO ₂ Day 11 (% ± SD)
ZnAL42	8.64	3/3	0.2	8/10 ^a	8.0 ± 0.0	83.7 ± 4.9 ^a
	1.46	3/3	-0.5	1/10	7.8 ± 1.0	76.2 ± 7.3
	0.24	3/3	0.0	5/10	7.8 ± 0.8	81.3 ± 6.8
Osetamivir	20	3/3	0.6	10/10 ^{***}	>16.0 ± 0.0 ^{***}	87.0 ± 7.3 ^{***}
Saline	—	3/3	0.7	8/20	9.6 ± 3.2	78.8 ± 4.6
Normal Controls	—	3/3	1.6	—	>16.0 ± 0.0 ^{***}	90.0 ± 2.6

^aTreatment schedule: bid x 13 beg. 48 h pre-virus exposure (Osetamivir: bid x 5 beg. 4 h post-virus exposure).

^bDifference between initial weight and weight 18 h after final treatment.

^cMean day to death of mice dying prior to day 16.

^aP<0.05; ^bP<0.01; ^cP<0.001 compared to saline-treated controls.

Table 2. Effect of oral gavage treatment with ZnAL42 on influenza A (H1N1) virus infection in mice.

Compound	Dosage (mg/kg/day)	Toxicity Controls		Infected, Treated Mice		
		Survivor/ Total	Mean Host Weight Change ^a (g)	Survivor/ Total	Mean Day to Death ^b ± SD	Mean SaO ₂ Day 11 (% ± SD)
ZnAL42	8.64	3/3	0.8	3/10 ^a	11.0 ± 0.8 ^a	80.4 ± 5.1 ^{***}
	1.46	3/3	1.1	0/10	10.2 ± 1.3	76.1 ± 2.0
	0.24	3/3	1.0	4/10 ^{**}	9.3 ± 1.5	76.7 ± 2.2
Osetamivir	20	3/3	0.6	10/10 ^{***}	>21.0 ± 0.0 ^{***}	86.2 ± 3.3 ^{***}
Saline	—	—	—	0/20	10.0 ± 1.7	75.5 ± 1.0
Normal Controls	—	5/5	1.6	—	—	85.6 ± 3.2

^aDifference between initial weight and weight 18 h after final treatment.

^bMean day to death of mice dying prior to day 21.

*P<0.05; **P<0.01; ***P<0.001 compared to saline-treated control.

Discussion

Efficacy with ZnAL42 was achieved only with pretreatments, suggesting that pretreatment sufficiently disturbed the virus infection to allow the mouse immune system to prevent a lethal

infection. Thus, the ZnAL42 formulation may have not directly inhibited virus replication. This is supported by the finding that mice infected with virus and treated beginning 24 h after virus exposure with any dosage of ZnAL42 (data not shown) were not protected against lethal virus infection or decreased lung function as measured by SaO₂ measurements. The mechanism whereby ZnAL42 inhibited the influenza virus infection of mice in these studies may simply be one of coating the virion with zinc to prevent attachment and penetration as has been shown with herpes simplex virus [7] or to reduce virus neuraminidase activity as been shown in kinetic studies with purified influenza neuraminidase [8]. However, the zinc treatment may have ameliorated cell death events in the lung triggered by influenza infection. It has been shown that apoptosis may play a causative role in acute lung injury in part due to epithelial cell loss during influenza H5N1 infection [9]. It has also been reported that during inflammatory stress in the lung zinc supplementation inhibited apoptosis and paracellular leak caused by zinc deprivation [10]. Alternatively, zinc may have prevented oxygen free radical damage in the lung caused by the virus infection. Reactive oxygen species may be produced in the lung as a result of an influenza virus infection and cause an increase in local inflammation and thereby contribute to pulmonary tissue damage [11]. It has also been shown that superoxide free radical generation by alveolar phagocytic cells was significantly increased after influenza virus infection and remained at high levels throughout the infection [12]. Therefore, the ZnAL42 formulation, when administered prior to infection, could have caused the systemic zinc balance to be maintained despite the perturbation of the redox balance induced by the influenza virus infection [11], leading to a protection of lung cells from the destructive oxidative effects induced by influenza virus infection. In conclusion, the data suggest that the prophylactic use of ZnAL42 is effective against avian influenza H5N1 virus infection in mice and should be explored as an option for treating human influenza virus infections.

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Evaluation of Anti-Influenza Activity of Green Tea Extract In Vitro and In Vivo

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Green tea leaf extract and green tea byproduct extract were evaluated for their anti-influenza virus activity in vitro and in vivo. The 50% effective concentration (EC_{50}) of green tea leaf and byproduct extract for H1N1 influenza A virus were 6.36 and 6.72 $\mu\text{g}/\text{mL}$, respectively. Hexane and ethyl acetate soluble fractions of green tea byproduct extract showed anti-influenza virus activity more effectively than dichloromethane, n-butanol and water-soluble fractions. Oral administration with green tea byproduct extract significantly reduced H1N1 influenza A virus replication in lung in mice and H9N2 influenza A virus shedding in feces in chickens after influenza virus challenge. The green tea byproduct extract seemed to have comparable amount of anti-influenza viral compounds to the original green tea leaf extract. Ethyl acetate-soluble polyphenolic compound catechins as well as unidentified hexane-soluble active compounds in green tea byproduct extract seemed to bind to the influenza virus surface and act as virus entry inhibitor. Green tea byproduct extract are a promising resource of new antiviral compounds that prevent pandemic influenza.

Introduction

Influenza still poses a major threat to humans and several animal species. However, use of anti-viral drugs has been limited by emergences of drug resistant virus. Further, vaccine approach is difficult with regard to genetic re-assortment of influenza virus. Thus, clinically available alternative control measures are needed to prepare next pandemic influenza for preventive and therapeutic purpose. Green tea is a popular historic beverage of Asian populations and is produced from the leaves of the evergreen plant *Camellia sinensis*. The major active ingredients of green tea are polyphenolic compounds, known as catechins [1]. Antiviral effects of catechins have been reported for influenza virus. In recent antiviral effect study with catechins showed that these compounds could affect the infectivity of influenza virus not only by specific interaction with viral hemagglutinin (HA) protein, but altering the physical properties of viral membrane [2]. Green tea byproduct, which remain after processing in the commercial green tea beverages industry, still contain large amounts of proteins, carbohydrates, and phenolic compounds [3]. Several studies

have reported that the green tea byproduct have already been used as potential sources of natural anti-oxidants or functional materials. In this study we evaluated the anti-influenza activity of green tea byproduct and compared the activity of its extract with the original green tea leaf extract *in vitro* and *in vivo*.

Materials and Methods

Preparation and purification of green tea extract. Four types of green tea extract were prepared. 'Green tea leaf water-extract' was prepared by infusing the dried leaves with 90°C distilled water in the ratio of 1:10 (w/w). Used tea leaves were air-dried for preparation of the green tea byproduct. This green tea byproduct was extracted with 60% ethyl alcohol at 42°C for 4 hours to prepare 'green tea byproduct extract'. 'Green tea leaf extract' was prepared by direct extraction from the original leaves with 60% ethyl alcohol. 'Green tea byproduct MtOH-extract' was also prepared by extraction of the green tea byproduct with 100% methyl alcohol. These extracts were lyophilized for the examination. For identification active ingredient of green tea byproduct, the lyophilized green tea byproduct methanol extract power was dissolved in water and partitioned with hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and n-butanol (n-BuOH), consecutively. **Anti-influenza virus efficacy of green tea byproduct extract in MDCK cells.** To determine the anti-influenza virus activity and cytotoxicity of the green tea byproduct extract, MDCK cells were infected with influenza A/PR/8/34 (H1N1) virus ($10^{2.5}\text{CCID}_{50}/\text{well}$) and then various concentrations of test compounds were added in appropriate wells (0.1 ml/well). Cell viability was quantified with neutral red assay for MDCK cells [4, 5]. EC_{50} values were calculated in infected cells for the antiviral effect and CC_{50} values were calculated in un-infected cells for cytotoxicity.

Anti-influenza virus efficacy of green tea byproduct extract in vivo. **BALB/c mice.** Each group of ten mice (17~19g) were treated with various types of green tea byproduct extract (at dose of 100, 10, or 1 mg/kg/day) before 4 hours virus exposure (influenza A/NWS/33 (H1N1), $10^{3.0}\text{CCID}_{50}/90\mu\text{g}/\text{mouse}$, intranasally) as previously described [6]. For 5 days, the extracts were administered by oral gavages daily. Five mice per group were sacrificed on day 3 and day 6 for lung infection parameters. Lung from sacrificed mice were collected, weighed, given a severity score based upon lung consolidation and frozen for assay of infectious virus titer [7, 8]. **SPF chickens.** Each group of ten 3-weeks-old-SPF chickens were challenged with influenza A/HS/K5/01 (H9N2) virus at dose of $10^{6.0}\text{EID}_{50}/0.1\text{ mL}/\text{bird}$, intranasally. Treatment was begun 4 hours before virus exposure, then a diet supplemented with lyophilized green tea byproduct extract (1, 0.4, and 0.1% v/v) or drinking water containing green tea byproduct extract (10, 1, and 0.1 mg/mL) was administered for 5 days. On 5 days after virus challenge, the tracheas and cecal tonsils were collected for isolation of challenge virus [9].

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Results

In vitro anti-influenza virus efficacy. As shown in Table 1, the EC₅₀ of green tea leaf extract and green tea byproduct extract were similar (EC₅₀ was 6.72 µg/ml and 6.36 µg/ml, respectively; CC₅₀ was 52.88 µg/ml and 58.67 µg/ml, respectively) and about 6-fold more effective than green tea leaf water-extract (EC₅₀ 39.57 µg/ml; CC₅₀ 182.75 µg/ml). To identify effective fraction of green tea byproduct, anti-influenza virus activities of green tea byproduct MtOH-extract fractions were evaluated in MDCK cells. Hexane- soluble fraction (EC₅₀ 6.3µg/ml) and ethyl acetate-soluble fraction (EC₅₀ 6.69µg/ml) showed higher anti-viral activities than other fractions (dichloromethane fraction, EC₅₀ 24.44 µg/ml; water fraction, EC₅₀ 20.43 µg/ml; n-butanol fraction, not effective).

In vivo anti-influenza virus efficacy. Effect of green tea byproduct extract on lung parameters in BALB/c mice. Mice showed significant reduction of lung virus titer at dose of 100 mg/kg/day treatment of all three type of extracts (Table 1), although treatment with low concentration of test compounds (10 and 1 mg/kg/day) did not induce remarkably reduction of lung virus titer (data not shown).

Effect of administered green tea byproduct extract on influenza virus (H9N2) infection in SPF chickens. Green tea byproduct extract treated either by 1 % feed additives or by 1 mg/ml drinking water induced significantly decrease the number of birds shed AIV virus in cecal tonsil on 5 days after challenge (Table 2).

Table 1. Antiviral effects of oral administered various green tea extract against influenza infections in BALB/c mice.

Treatment (100 mg kg day)	Mean lung parameters					
	Day 3			Day 6		
	Score ^A ±S.D.	Lung weight (mg ± S.D.)	Virus Titer (log ₁₀ TCID ₅₀ /g ±S.D.)	Score ±S.D.	Lung weight (mg ± S.D.)	Virus Titer (log ₁₀ TCID ₅₀ /g ±S.D.)
Water infusion	0.7 ± 0.1	124 ± 5	5.4 ± 0.9*	2.5 ± 0.4	170 ± 45	6.3 ± 0.2
Byproduct	0.6 ± 0.1	120 ± 7	5.0 ± 0.8***	2.3 ± 0.3	164 ± 18	6.2 ± 0.6
Tea leaves	0.7 ± 0.1	120 ± 6	5.7 ± 1.1**	2.4 ± 0.1	182 ± 8	6.0 ± 0.3
H ₂ O	0.7 ± 0.1	128 ± 8	6.6 ± 0.4	2.9 ± 0.2	174 ± 39	6.5 ± 0.1
Normal control	0.0 ± 0.0	116 ± 5	0.0 ± 0.0	0.0 ± 0.0	128 ± 18	0.0 ± 0.0

^ALungs assigned a consolidation score ranging from 1 (normal) to 4 (maximal plum coloration).

p* < 0.05, *p* < 0.01, ****p* < 0.001 compared to saline-treated challenge controls by student's *t*-test.

As seen in Table 2, administration of green tea byproduct extract by feed additives and drinking water also significantly reduced the titers of challenge virus recovered from cecal tonsils (10^{0.35}-10^{3.8} EID₅₀/g) compared to non-treated control (10^{4.9} EID₅₀/g). Further, reduction of virus titers was better in high dose treatment groups (1% feed additives, 10mg/kg and 1mg/kg drinking water) than low dose treated groups.

Table 2. Reduction of viral shedding in Trachea and Cecal tonsils of SPF chickens treated with green tea byproduct extracts as feed additives and drinking water after challenge with H9N2 LPAI virus.

Treatment	Conc.	Excretion of challenge virus				
		Virus isolation ^A		Virus titer (log ₁₀ EID ₅₀ /g)		
		Trachea	Cecal tonsils	Trachea	Cecal tonsils	
Byproduct	Feed additive (%, v.v)	1	3:10	1:10 [§]	0.75 ± 1.30	0.35 ± 1.10***
		0.4	5:10	6:10	1.35 ± 1.67	2.10 ± 1.87**
		0.1	5:10	4:10	1.25 ± 1.40	2.30 ± 2.98*
	Drinking water (mg/ml)	10	3:10	4:10	0.55 ± 0.93	1.30 ± 2.06**
		1	3:10	3:10 ^{§§}	0.65 ± 1.18	1.15 ± 1.93**
		0.1	6:10	6:10	1.80 ± 1.60	3.80 ± 3.29
Control ^B		5:10	9:10	1.25 ± 1.48	4.95 ± 2.43	

^ANo. of birds reisolated / No. of birds inoculated, ^BControl groups were not treated after challenge.

[§]*p* < 0.05, ^{§§}*p* < 0.001 compared to non-treated control by Fisher's exact test.

p* < 0.05, *p* < 0.01, ****p* < 0.001 compared to non-treated control by student's *t*-test.

Discussion

According to the previous study, catechin compounds were highly concentrated in ethyl acetate-soluble fraction of green tea leaf extract [2]. In this study, ethyl acetate-soluble fraction of green tea byproduct extract showed effective influenza virus inhibitory effect and this result indicated that the anti-influenza activity of the ethyl acetate-soluble fraction seemed to be induced by catechin compounds. However, the anti-influenza activity of hexane-soluble fraction was as potent as that of acetyl acetate-soluble fraction, and suggested that green tea leaves may contain another active antiviral compounds except catechin. In mice, lung virus titer was only decreased in early phase of infection by treatment with green tea byproduct extract. These results are supported by previous report that catechins interact with HA protein leading to initial virus entry-inhibition [2]. In addition, affected organs in mice (lung) were different from chickens (intestine). We speculated that orally administered green tea byproduct extract interacted with influenza virus more easily in chicken intestine than in mouse lung, and thus anti-influenza virus effect in chickens was more obvious than in mice. Therefore, other application methods to deliver green tea byproduct extract into lung (e.g., aerosol treatment [10], nasal inoculation) may be more effective to prevent influenza infection in respiratory tract. With regards to the role of birds in avian influenza virus transmission, reduction of virus shedding in feces of chickens suggested that treatment with green tea byproduct extract could be used to minimize viral circulation among bird species. In conclusion, the green tea byproduct extract might provide a basis for developing new anti-influenza virus agents.

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The Emergence and Persistence of Adamantane Resistance in Influenza A(H1) Viruses in Australia and Regionally in 2006-7

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The adamantanes (amantadine and rimantadine) were the first antivirals licensed for use against influenza A viruses and have been used in some countries to control seasonal influenza. While high levels resistance to this class of drug have been reported recently for A(H3) viruses, only low levels of resistance have been seen with A(H1) viruses. In this study we analysed human influenza A viruses isolated in from 2005-7 that were referred to the WHO Collaborating Centre for Reference and Research in Melbourne, from Australia and the surrounding regions, for evidence of resistance to adamantanes. We found that the A(H1) viruses have also shown increased resistance to adamantanes with levels rising from 0% in 2005 to 22.3% in 2006 and to 33% in early 2007. By comparison, higher levels of resistance appeared earlier with A(H3) viruses with 43.1% of viruses isolated in 2005 from the region being resistant with these elevated levels of resistance persisting in 2006 (50.5%) and through early 2007 (47.8%).

Introduction

Two of the older antivirals used against influenza, amantadine and rimantadine, are still used in many parts of the world for the prevention and treatment of seasonal influenza, mainly due to their low cost. Amantadine and rimantadine (both adamantane derivatives) work by blocking the ion channel formed by the M2 protein of influenza A viruses which then inhibits the early stages of virus replication in cells [1]. Resistance to these drugs occurs when there are mutations in key residues in the M2 protein (at amino acid positions 26, 27, 30, 31 or 34) that lead to either the loss of binding of these drugs or changes in the structure of the ion channel which allow it to operate even in the presence of bound drug [1,2]. Until recently resistance to these drugs has been limited to patients under treatment or their close contacts without any consequent widespread outbreaks or persistence. Since 2003 however, there has been a marked increase in the resistance of influenza A(H3) viruses to these drugs, especially in China [3]. This trend has also been apparent in other countries including South East Asia and Oceania [4]. In contrast until very recently there was no similar increase in the resistance levels of A(H1) viruses to these drugs. To study these patterns further we have sequenced the matrix gene from both A(H3) and A(H1) viruses over recent years to determine the resistance levels in influenza viruses isolated in the Asia-Pacific region.

Materials and Methods

Viruses. Influenza A(H3) and A(H1) viruses were received at the Centre from WHO National Influenza Centres, WHO Influenza Collaborating Centres and other regional laboratories and hospitals from Australia, New Zealand, and the Asia/Pacific region. Viruses were received as isolates passaged in cell culture or as original clinical samples in which influenza A had been detected by immunofluorescence or by RT-PCR. After the samples were received, they were cultured in MDCK cells and monitored for growth by CPE and the presence of haemagglutination activity using turkey red blood cells (RBC's) as previously described [5]. Haemagglutination positive samples were typed using the haemagglutination inhibition assay (HAI) against a panel of known standard reference viruses and their homologous ferret antiserum [4, 6].

Sequencing and phylogenetic analysis. RNA extraction, RT-PCR and sequencing were performed on virus isolates as previously published [5]. Sequences were assembled using the Lasergene Seqman package IV (DNASTar 6) and phylogenetic relationships determined with PHYLIP V 3.5.77 [7] using the neighbour-joining method on ANGIS (Australian National Genomic Information Service) and dendograms were drawn using Treeview [8].

Adamantane sensitivity assay. A biological assay to determine virus susceptibility to both rimantadine and amantadine was performed following a previously published method [3], except that viruses were tested using three virus dilutions (1:2, 1:20, and 1:200) and plates were incubated at 35°C for 24 hours, rather than 37°C for 36 hours.

Neuraminidase enzyme inhibition assay. A fluorescence-based neuraminidase (NA) enzyme inhibition assay was used to assess susceptibility of viruses to the neuraminidase inhibitors zanamivir and oseltamivir carboxylate as described previously [9].

Results

From the viruses isolated in 2006, 103 A(H3) viruses and 103 A(H1) viruses were examined for the specific mutations known to correlate with resistance to the antiviral drugs amantadine and rimantadine (Table 1). Viruses from the first quarter of 2007 were also analysed (23 A(H3) and 12 A(H1) viruses. Viruses were selected on the basis of their country of origin and date of isolation. While it cannot be excluded, it is unlikely that many of these patients were taking amantadine or rimantadine at the time of sampling either because these drugs are not widely used in certain countries (e.g. Australia and New Zealand) or due to their relatively high cost (e.g. developing countries). For viruses isolated in 2006, 52/103 (50.5%) of influenza A(H3) viruses and 23/103 (22.3%) of A(H1) viruses had substitutions which would confer resistance to adamantanes while the early 2007 data showed this persisting with 11/24 (47.8%) A(H3) and 4/12 (33%) A(H1) viruses having a resistant genotype (Table 1). The highest frequency of resistant strains from the 2006 A(H1) viruses were those obtained from Macau (SAR) 9/11 (82%), Australia 38% (8/21) and South Africa 1/3 (33%), while a number of countries had low (e.g. Thailand 1/10 (10%), Singapore 1/10 (10%)) or no

apparent resistance (e.g. Malaysia 0/10 (0%), Philippines 1/15 (6.7%), New Caledonia 0/5 (0%)). In 2007 sufficient isolates of A(H1) had only been received from Australia to estimate resistance, which was 20% (1/5). For A(H3) viruses isolated in 2006, resistance levels were high in New Zealand 9/12 (75%), Australia 21/41 (51%), South Africa 10/10 (100%) and Taiwan 2/3 (66%). For 2007 100% (3/3) Macau A(H3) and 36% (4/11) Australian A(H3) viruses showed resistance (Table 1).

Table 1. Adamantane resistant A(H3) and A(H1) viruses in the region between 2005-7.

Year/ Country	A(H1)			A(H3)		
	2005	2006	2007	2005	2006	2007
Macau	0/3*	9/11	2/2	10/10	1/1	3/3
Taiwan	0/1	1/5	0/1	1/2	2/3	1/2
Thailand	0/2	1/10	0/3	3/13	6/12	0/3
Malaysia	0/3	0/10	1/1	8/10	2/14	2/2
Cambodia	-	0/3	-	2/2	-	-
Singapore	0/1	1/10	-	6/12	0/1	-
Philippines	-	1/15	-	2/10	1/1	0/1
Indonesia	0/1	-	-	0/3	-	-
Australia	0/23	8/21	1/5	7/22	21/41	4/11
New Zealand	0/2	1/7	-	3/12	9/12	1/1
New Caledonia	-	0/5	-	1/5	0/5	-
South Africa	0/1	1/3	-	-	10/10	-
Solomon Islands	-	0/3	-	-	-	-
Sri Lanka	-	-	-	1/1	0/2	-
French Polynesia	-	-	-	-	0/1	-
Resistant/Total % resistance	0/37 (0%)*	23/103 (22.3%)	4/12 (33%)	44/102 (43.1%)	52/103 (50.4%)	11/23 (47.8%)

*No. resistant viruses/ No. total viruses analysed, ** % of the viruses with a resistant genotype.

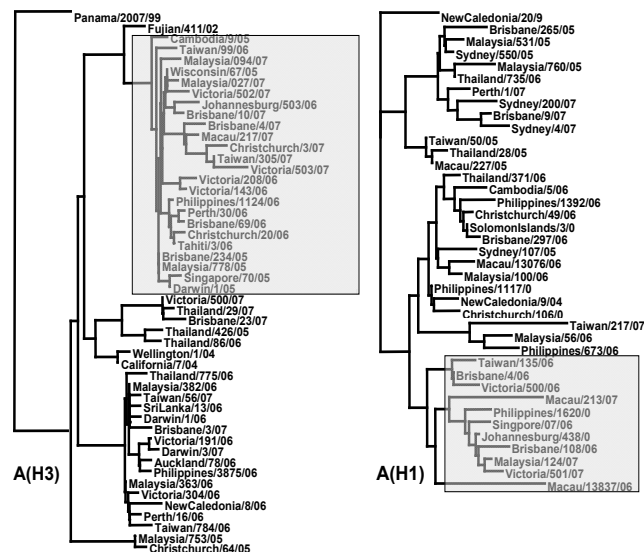
All of the 2006/7 A(H3) viruses had the same single nucleotide change (AGT to AAT) resulting in an S31N substitution in the M2 protein as did all of the A(H1) viruses except for a single isolate from Macau (SAR) (A/Macau/7334/2006 isolated on 21 February 2006) which had a V27A mutation. Six A(H1) adamantane-sensitive and 6 A(H1) adamantane-resistant viruses (as predicted by genotyping) that were isolated in 2006 were tested in a bioassay for sensitivity to rimantadine. The viruses predicted to be adamantane-sensitive all had their growth inhibited in MDCK cultures with 0.2ug/ml rimantadine while the viruses predicted to be adamantane-resistant were all able to grow well in 2 or 20ug/ml of rimantadine (data not shown).

Discussion

In 2006, influenza A(H1) viruses became more prevalent in many countries in the region such as the Philippines, Singapore and Macau (SAR) with moderate increases in Thailand but no increase in other countries like Australia. The majority of these viruses were similar to the reference and vaccine A(H1N1) strain A/New Caledonia/20/99 however a new group of variant viruses began to emerge in late 2006 which have since been designated as A/Solomon Islands/3/2006-like. This later group of viruses have to date had an adamantane-sensitive genotype and they appear to be slowly increasing in number in 2007. When the A(H1) matrix genes (full length) were compared phylogenetically the resistant viruses grouped together around

A/Philippines/1620/2006 (see Figure 1) while the sensitive viruses made up the rest of the evolutionary tree. Adamantane resistance for the A(H1) viruses seemed to be a relatively new phenomenon as no adamantane resistant influenza A(H1) viruses were identified in our laboratory in 2005 (0/37 isolates tested) or in previous years, while in 2006 the level of resistance had grown to 22.3% of the strains tested and this had increased slightly early in 2007 to 33%.

Figure 1. Phylogenetic analysis of the full length matrix genes from A(H3) and A(H1) viruses from 2005-7. Boxes indicate those viruses that had an adamantane-resistant genotype while non-shaded viruses had an adamantane-sensitive genotype.



The majority (50.5%) of influenza A(H3) strains isolated in 2006 were resistant to adamantanes and while this was higher than the levels seen in 2005 (43.1% resistant), this proportion was still lower than that seen in the USA in 2005-6 influenza season where 92.3% of strains were found to be resistant [3]. Phylogenetically the A(H3) matrix genes from resistant viruses grouped into one major group along the A/Wisconsin/67/2005 lineage while the sensitive viruses fell into a few other large subgroups (Figure 1). The A(H1) and A(H3) viruses were obtained from geographically distinct sites and generally represent all or a good representative proportion of the 2006/7 viruses from these sites available at the Centre for testing. A substitution in any one of 5 amino acids in the M2 protein in influenza A viruses can confer resistance to amantadine or rimantadine [1], but in this present study of influenza A viruses isolated in 2006, only one substitution (S31N) was evident in all of the influenza A(H3) viruses and for all of the A(H1) viruses except for one virus from Macau (SAR). This is consistent with our previous findings on A(H3) viruses [4] and with other reports [3, 10] and may indicate a common origin of these resistant subtypes. Prior to this report the most common mutation in A(H1) viruses conferring resistance to adamantanes reported was the V27A change that

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was present in 6/9 resistant viruses isolated from Japanese children in the winters of 1999-2000 and 2000-2001 [11]. In contrast recent studies on A(H5N1) viruses from SE Asia, found the S31N change was almost invariably associated with an additional L26I substitution [12, 13]. Analysis of the full matrix genes from A(H1) and A(H3) 2006/7 resistant viruses show that there are still distinct differences between the subtypes (there were conserved nucleotide differences at more than 58 sites in the matrix gene), indicating that the two resistant genotypes do not share the same genetic origin and remain subtype specific. None of the adamantane-resistant A(H3) or A(H1) viruses identified in this study that were tested, showed increased levels of resistance to the two licensed NI antivirals, oseltamivir or zanamivir as determined using a neuraminidase-inhibition assay (data not shown). Clearly influenza A(H1) and A(H3) viruses with the S31N substitution are capable of spreading within the community with no compromise to viral fitness and may now even have a selective advantage. As the adamantanes have proven useful in prevention of seasonal influenza A and may play a role if a pandemic eventuated, it would be ideal to reduce the frequency of resistance of human and avian influenza viruses to these drugs as soon as possible. This may be achievable if use of these drugs is limited for the next few years to allow resistant strains to be possibly replaced by non-resistant influenza A viruses which are co-circulating, such as with the A(H1) A/Solomon Islands/3/2006-like viruses. This would reduce the chance of an A(H5) virus reassorting with an A(H1) or A(H3) virus or the further proliferation of A(H5) adamantane resistant viruses, which have already been isolated in a number of countries [12, 13]. To monitor these changes, it will be important to assess the level of resistance for the M2 inhibitors in influenza A(H1), A(H3) and A(H5) viruses circulating in both human and avian species in the coming years.

Acknowledgements

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Investigation of the Effects of Verapamil on Influenza A Virus Replication: Impairment of PKC α -transmitted Raf/MEK/ERK Induction, Viral RDRP-Activity and Viral Protein Production

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Influenza Virus (IV) attacks mainly the upper respiratory tract. IV rapidly spreads around the world in seasonal epidemics and imposes a considerable economic burden in the form of hospital and other health care costs and lost productivity. IV activate the Raf/MEK/ERK-(MAPK) cascade late in their replication cycle. This is essential for efficient nuclear RNP-export and therefore for production of infectious IV. To characterize cellular factors involved in MAPK-activation in the context of the viral infection we recently analyzed the role of PKC α . The results so far indicate, that activation of the Ca²⁺ dependent PKC α is involved in the IV induced MAPK-signaling and that specific inhibition of this function using a Ca²⁺-channel blocker (Verapamil) at non toxic concentration, negatively affects IV propagation (Marjuki et al., 2006). We have now further analyzed the action of Verapamil for possible additional negative effects on IV replication. Therefore we have investigated viral protein production in IV infected human lung epithelial cells and found that PB1, NP, M1 and NS1 production is significantly reduced in IV infected and Verapamil treated A549 cells. Cell survival and cellular protein production does not seem to be affected arguing against a general effect of Verapamil on translation. Since PB1 is a functional important subunit of the viral polymerase the activity of the polymerase might be affected. Therefore we analyzed the polymerase activity in Verapamil treated cells for the production of different viral RNA species (vRNA, cRNA and mRNA) using primer extension analysis of a reporter transcript expressed by either a plasmid based replication system or in virus infected cells. Our current results indicate that viral mRNA production in the plasmid based replication system is not affected, while it seems to be reduced in virus infected cells. This indicates that Verapamil might alter viral transcription activity in virus infected cells. Taken together, we have first results indicating that Verapamil at non toxic concentrations inhibits IV replication via PKC α inhibition leading to reduced MAPK-activation and might also affect the activity of the viral polymerase thereby leading to reduced production of viral proteins without significant effect on cellular protein production and cell survival. Therefore Verapamil might be a useful in a potential anti-viral approach.

Introduction

Influenza A and B viruses are important worldwide pathogens for humans and animals and cause devastating epidemic and

pandemic outbreaks. During intracellular replication influenza viruses interact with many different cellular functions to promote their propagation (1, 2). Both influenza A and B viruses activate the cellular Raf/MEK/ERK(MAPK) signaling cascade. A block of this signaling pathway impairs RNP nuclear export and thereby virus replication without the emergence of resistant variants (3, 4). MAPK cascades are key regulators of cellular responses such as proliferation, differentiation, and apoptosis (5,6). The Raf/MEK/ERK cascade is the prototype of MAPK cascades. Growth factor-induced signals are transmitted by consecutive phosphorylation from the kinase Raf via MEK to ERK, which translocates to the nucleus to phosphorylate a variety of substrates (7, 8). The influenza A virus genome consists of eight RNA segments of negative polarity coding for at least 10 viral proteins. The three subunits (PB1, PB2, and PA) of the viral RNA-dependent RNA-polymerase and the nucleoprotein (NP) form the biological active ribonucleoprotein (RNP) complexes. Because influenza viruses pursue a nuclear replication strategy the RNPs must undergo bidirectional nuclear transport. Upon infection the RNPs are released into the cytoplasm and transported into the nucleus. The nuclear residency of the RNPs must be well coordinated to assure efficient viral genome replication and production of viral mRNAs/proteins, whereas late in the replication cycle newly formed RNPs have to be exported from the nucleus and transported to the cell membrane for virus assembly (9, 10). The virus-induced MAPK signaling is activated late in the viral replication cycle, and RNP nuclear export is dependent on this cellular function (3, 4). The MAPK-cascade is normally activated by membrane proximal events through activation of PKCs or through activation of Ras. Data in the literature so far indicates that PKC α and PKC β are classical PKCs and they are activated by Ca²⁺ and DAG. An increased Ca²⁺-influx occurs in FCoV-infected cells (11). A Ca²⁺-channel blocker inhibits influenza virus replication if added late in replication cycle. (12). PKC α and / or - β are activated when human polymorphonuclear leukocytes (PMNL) are incubated with Influenza virus (13). PKC-inhibitors reduce virus entry if added early in infection. PKC β II isoform is activated in early influenza viral replication but not PKC α . (14). Membrane accumulation of influenza virus hemagglutinin and "lipid-raft" association triggers RNP nuclear export via PKC alpha mediated ERK signaling (15). Calcium increase is blocked by L-type calcium channel blocker such as Verapamil. Verapamil blocks calcium channels in the endoplasmic reticulum therefore calcium influx from internal calcium store to cytoplasm is inhibited and our current data shows that calcium dependent PKC α induced Raf/MEK/ERK signaling in IV infected cells is negatively affected by Verapamil leading to reduced IV replication (15). To demonstrate the other possible additional negative effect of Verapamil on IV replication we further analyzed viral RDRP-activity and viral protein production.

Results

Analysis of the role of Ras activation and activity for virus induced MAPK-activation. Our current results suggest that receptor

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tyrosin kinase activity (that would activate the MAPK via Ras) is not significantly elevated in virus infected cells. By comparative virus titer analysis of cells transfected with a dominant negative version of PKC α and or Ras we were able to shown recently that PKC α activity is more important for virus propagation than Ras activity (40). These data argue against an important role of Ras for MAPK- activation in IV infected cells. (Data not shown)

Analysis of IV titers from infected A549 cells (+/-) Verapamil treatment. Verapamil at non toxic concentrations strongly reduces virus, titer which suggests that Verapamil has a negative impact on IV replication.

Analysis of the effect of Verapamil on cellular protein production. At different time points post Verapamil treatment whole cell lysate from equally distributed human lung epithelial cells was made. Analysis of relative protein concentration in the whole cell lysate (OD595 measurement) and ERK2 quantification in western blot analysis suggests that Verapamil has no negative effect on the general translation process and cellular protein synthesis. (Data not shown)

Analysis of the cell viability (+/-) Verapamil treatment. Verapamil in the concentration used for our study has no significant toxic effect on cell survival.

Analysis of the PKC α -and ERK-activation in virus infected A549 (+/-) Verapamil. Our Western blot result indicates that virus induced Raf/MEK/ERK-signal transmission is dependent on PKC α . (Data shown in Figure (A).)

Analysis of the RNP-export in virus infected A549 cells (+/-) Verapamil treatment. Our current results suggest that nuclear RNP-export is strongly impaired by Verapamil leading to inefficient formation of progeny virus. (Data shown in Figure (B).)

Primer extension analysis of the effect of Verapamil on viral mRNA production. Our most recent data indicates that in virus infected cells Verapamil seems to have a negative effect on viral transcription of a vRNA-like reporter Pol1-transcript. This could mean that viral mRNA production is affected by Verapamil. Whereas Verapamil seems not to affect mRNA production in a plasmid based replication system.

Analysis of viral protein production in virus infected A549 cells (+/-) Verapamil treatment. Western blot analysis of viral protein production suggests that Verapamil strongly reduces the amount of viral proteins (PB1, NP, M1 and NS1). (Data not shown here)

Figure A. PKC α -&ERK-Activation: IV-infected MDCK cells (MOI=1) either left untreated or treated with Verapamil (100 μ M, 2h p.i.) were analyzed for PKC α and ERK activation in Western blot using phospho-specific mAb.

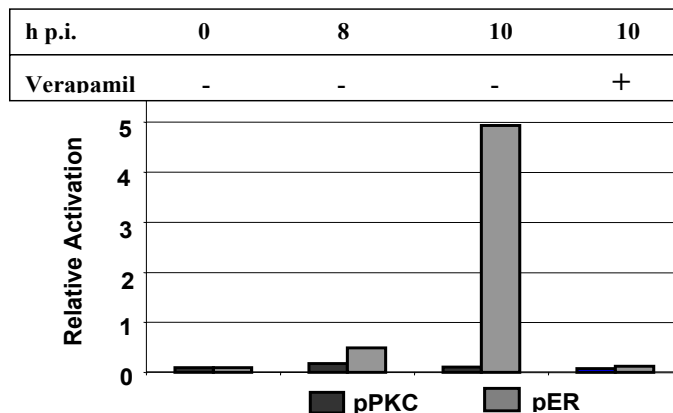
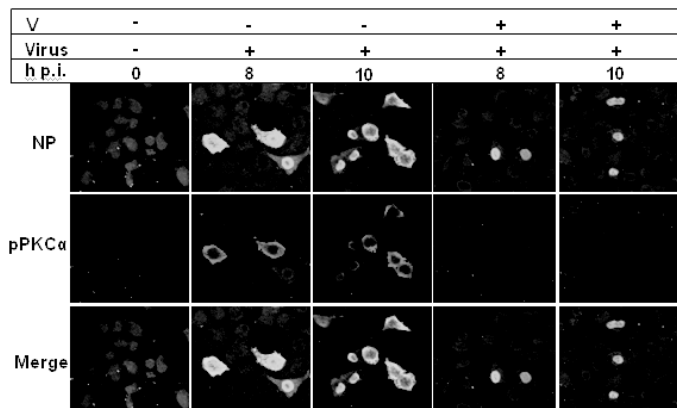


Figure B. Immunofluorescence analysis. PKC α (grey) is activated in IV infected (MOI=1 A549-cells and RNPs (white) are exported from the nucleus at late time points of virus replication. Verapamil (V) treatment blocks PKC α activation and RNP-export.



(B) Immunofluorescence analysis

PKC α (grey) is activated in IV infected (MOI=1) A549-cells and RNPs (white) are exported from the nucleus at late time points of virus replication. Verapamil (V) treatment blocks PKC α activation and RNP-export.

Discussion

To investigate the various possible targets of Verapamil on influenza A virus replication we analyzed the effect of Verapamil on (i) PKC α activated Raf/MEK/ERK signaling, which is important for the nuclear RNP export and efficient virus propagation, (ii) viral protein production and (iii) viral polymerase activity. In virus infected cells phosphorylation of tyrosine residues does not seem to be strongly enhanced, which suggests that receptor tyrosine kinase activity does not play an important role in virus induced activation of the Raf/MEK/ERK-signaling cascade via Ras. This idea is supported by the finding, that expression of Dn-PKC α expression in virus infected cells results in a stronger reduction of virus titers compared to expression of Dn- Ras. The fact that Verapamil is a potent inhibitor of virus induced ERK- and PKC α . activation as well as nuclear RNP export further

supports the idea, that IV induced MAPK-signaling is activated via calcium dependent PKCs. At the same time a negative effect of Verapamil on cellular protein synthesis and cell survival can be ruled out. The first results indicating that Verapamil seems to have a negative effect on viral mRNA production in virus infected cells, while it seems not to effect mRNA production in a plasmid based replicati on system suggest that a negative effect of Verapamil on viral polymerase activity depends on a natural infection by yet unknown mechanisms. Nevertheless Verapamil thereby reduces viral protein production leading to inefficient virus replication. Taken together, influenza virus induced calcium dependent PKC α plays an important role in transmitting signal to the Raf/MEK/ERK-cascade. As PKC α inhibition can be achieved at non-toxic Verapamil concentration, leading to strong reduction of virus titers Verapamil might be a potential anti-viral drug.

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Detection of Oseltamivir Resistance Mutation N294S in Humans With Influenza A H5N1 Prior to Treatment

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From Mar 2006 to Feb 2007 Egypt had 22 human cases of Influenza A H5N1 with 13 deaths. All patients were treated with oseltamivir using WHO guidelines. In Dec 2006 NAMRU-3 received 3 human specimens from the Ministry of Health that were reported positive for influenza H5N1. The cases included 2 females [age 35 and 16] and one male [age 26] from an extended family living in the Nile Delta. All patients participated in the slaughter of household ducks on Dec 13 and developed influenza like illness between Dec 15 and 19. On Dec 21, a throat swab was collected from each patient before beginning oseltamivir treatment. The patients were transferred to Abbassia Chest hospital in Cairo on Dec 23 for respiratory deterioration, and additional throat swabs were collected. All 3 patients died from pneumonia complicated by adult respiratory distress syndrome. The specimens were tested at NAMRU-3 using RRT-PCR. H5N1 positives were further analyzed by genomic sequencing. H5N1 was confirmed in 2 patients [16F and 26M], while the third remains under study. The NA gene sequence from specimens collected before and after antiviral therapy revealed a mutation [N294S] known to confer resistance to oseltamivir. The sequences were confirmed by the Influenza Division at the Centers for Disease Control. Phenotypic analysis, using the chemiluminescent NA inhibition assay, revealed a 12-15 fold reduction in oseltamivir susceptibility, but sensitivity to zanamivir. N294S is reported frequently in patients with seasonal influenza treated with oseltamivir, but has been reported in only one patient with H5N1. This patient was treated with a prophylactic dose of oseltamivir followed by a full course of therapy. N294S has not previously been described in humans before beginning oseltamivir treatment.

Introduction

Avian influenza virus [H5N1] is by far one of the most important public health concerns worldwide due to fears of eruption of a pandemic strain. Since late 2005, the virus has spread to many countries in Europe, Asia and Africa [1]. So far, there is no commercial vaccine for H5N1 for humans. In humans, the most common treatment for avian influenza is oseltamivir. Concerns about the emergence of a pandemic strain or strain that is resistant to oseltamivir are high. Previously, oseltamivir resistance was detected in H3N2 infected children under treatment [2]. A group of amino acid mutations were

responsible for variable degrees of resistance. Later on, resistance was monitored in H5N1 infected patients during oseltamivir treatment in Vietnam[3]. In collaboration with the Ministry of Agriculture In Egypt, first detection of the virus in poultry was confirmed in February 2006 at NAMRU-3. Egypt was affected with an H5N1 strain similar to the Qinghai strain detected in China in 2005 [1]. As a WHO reference laboratory for avian influenza, NAMRU-3 confirms human cases of H5N1 from Egypt. Infections were mainly due to exposure to sick household poultry. A clear decline in human infections was noticed during the hot spring/summer season [May – September] in Egypt. In October 2006, cases again began to be detected. A total of 38 human cases with 15 fatalities have been reported up to July 2007. Monitoring genetic changes in the HA and NA genes in Egypt is important to detect emerging pandemic strains or antiviral resistance. These cases were associated with exposure to household H5N1 infected chicken. In December, two deadly human cases of H5N1 were detected in a family in the Gharbiya governorate. Samples were collected both before and after initiation of treatment with oseltamivir. Sequencing of HA and NA genes from both cases was performed as part of our routine testing as a reference laboratory.

Materials and Methods

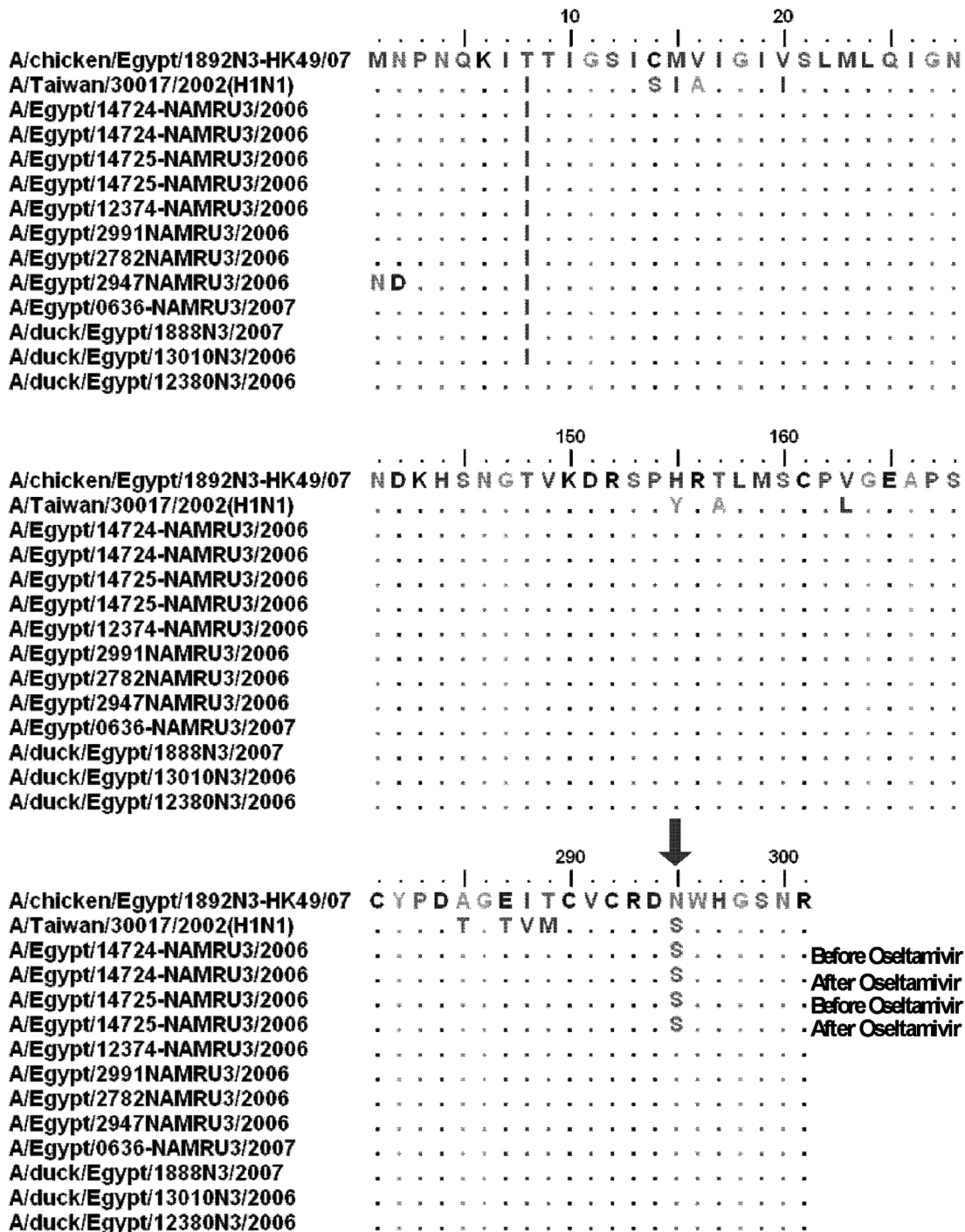
Throat swab samples collected on viral transport medium were received from the Central Public Health Laboratory [CPHL] of the Egyptian Ministry of Health. Samples were collected both before and after initiation of treatment with oseltamivir. Viral RNA extraction was performed using the Qiagen Viral RNA mini kit according to manufacturer procedure. CPHL positive results were confirmed at NAMRU-3 using specific real time PCR method for the detection of influenza A matrix and HA5 gene according to procedure by Spackman et al., 2002 [4]. NA1 gene specific real time PCR was performed using a procedure adopted from Payungyoung et al., 2005 [5]. PCR amplification of overlapping fragments of the HA and NA genes was performed using in-house designed specific primers. Sequencing of PCR amplicons was performed using the BigDye Terminator cycle sequencing reaction mix version 3.1 from Applied Bioystems, USA. Sequences were analyzed using Bioedit [6] and MEGA3.1 software [7]. The HA and NA genes sequenced of the human samples from this study were deposited in GenBank under accession numbers EF222322- EF222323 for the neuraminidase gene, EF200512- EF200513 for the hemagglutinin gene. Phenotypic analysis, using the chemiluminescent NA inhibition assay was performed at CDC, Atlanta.

Results

Both Gharbiya cases were confirmed by H5N1 specific real time PCR at NAMRU-3. Sequencing of HA gene from the studied cases revealed newly acquired amino acid mutations that were not seen in March-April cases, mainly M230I and V223I. M230I is a mammalian polymorphism at a receptor binding site. Phylogenetic analysis of the HA and NA genes revealed that studied strains are Qinghai-like viruses and cluster with

the previously sequenced H5N1 strains from human or avian species in Egypt. Bootstrap analyses suggested at least two main subclusters of strains circulating in Egypt, one in Upper Egypt and another in the Delta region. The NA gene carried a mutation "N294S" previously reported to be associated with oseltamivir resistance in N1 subtypes of influenza A viruses [Figure 1].

Figure 1. Oseltamivir resistance mutation N294S in two strains from Egypt.



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Resistance mutation was observed in samples collected before and after treatment. Phenotypic analysis, using the chemiluminescent NA inhibition assay, revealed a 12-15-fold reduction in oseltamivir susceptibility, but not to zanamivir.

Discussion

Avian influenza A virus H5N1 caused over 300 human infections since 1997[ref]. More than half of these were fatal cases. In Egypt, the number of human infections of H5N1 reached 38 cases till July, 2007, with 18 deaths. Epidemiological investigations of these cases indicated a previous contact with sick or infected household domestic poultry. Oseltamivir is the most common drug for treatment of avian influenza infection in humans. Previously reported oseltamivir resistance mutations were in cases under treatment and infected either with human or avian influenza viruses [2,3]. All these reports indicated the resistance to occur later after initiation of treatment. In this study, oseltamivir resistance mutation [Asn294Ser; N294S] was detected in an H5N1 strain from Egypt infecting humans before initiation of treatment. This is the first report to describe oseltamivir resistance in an H5N1 strain in humans prior to initiation of treatment. In addition, amino acid mutations with one in the receptor binding site [M230I], indicating that virus evolution was evident between 2006 and 2007 strains. Although no poultry were available for testing from the house of the studied cases, existence of resistance mutation prior to treatment in both cases strongly suggests that resistance mutation existed in strains infecting poultry in Gharbiya governorate, Egypt. These findings suggest that resistance mutations to oseltamivir could occur in avian influenza A viruses H5N1 before initiation of treatment. Since oseltamivir is the most widely stockpiled drug for a future possible influenza A virus pandemic, based on the findings of this study, the development and inclusion of alternative influenza A virus antiviral drugs in the treatment strategy need to be considered.

Acknowledgements

This work was supported by GEIS funds Work Unit No. E0018NAMRU3. 1999.0004. This study protocol was approved by the U.S. Naval Medical Research Unit No. 3 Institution Review Board [IRB DoD #: NAMRU-3. 1999.0004] in compliance with all Federal regulations governing the protection of human subjects. Conflict of interest: Authors hereby state that there is no conflict of interest of participation in the study.

Disclaimer

The views and opinions expressed herein do not necessarily represent those of the U.S. Army or U.S. Navy or the Department of Defense.

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Cinnamon Extract Inhibits Avian Influenza H9N2 Both In-Vitro and In-Vivo

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Influenza is one of the most prevalent and significant viral infections. The aim of the present study was to examine the ability of the Cinnamon Extract (CE) and its fraction CE_{ppt} to inhibit avian influenza H9N2, having previously demonstrated this antiviral activity against human influenza H1N1 and other viruses (Barak, I. and Ovadia, M., 2005, Gueta, K. and Ovadia, M., 2005). The ability of CE to neutralize the avian influenza virus was tested *in-vitro* on human erythrocytes by the hemolysis assay and *in-ovo* on chick embryos. In both cases virus activity was inhibited. A dose of 50-100 micrograms of the crude extract was sufficient to achieve total neutralization of 256 HAU of the virus within one minute. CE has a long shelf-life of at least two years whether in the refrigerator or at room temperature. It still retained its antiviral activity following dialysis in bags with a cut-off of 10 KD and after heating at various temperatures up to 121°C. The antiviral activity also remained stable at a wide range of pH (between 1 to 12). Moreover, the antiviral agent neutralized the virus after it had already attached onto the erythrocytes, and prevented its subsequent hemolytic activity. CE_{ppt} decreased the viral infectivity *in-ovo* by 5 Logs when injected together with the virus into the allantoic cavity of 11 days-old chick embryos. In conclusion, CE was shown to exhibit an effective antiviral activity against influenza virus both *in-vitro* and *in-ovo*. Cinnamon has been used in the human diet for thousands of years. Consequently, there should be no obstacle to introducing the isolated antiviral fraction for human and animal use. International Patent Application PCT/IL2004/001161.

Introduction

H9 influenza viruses have become endemic in land-based domestic poultry in Asia and have sporadically crossed to pigs and humans. Following the H5N1 influenza outbreaks in Hong Kong since 1997 and the isolation of avian H9N2 virus from humans, the current understanding envisages potential scenarios for the emergence of a new pandemic virus. Li et al (2003) reported that the H9N2 influenza viruses established in terrestrial poultry in southern China were probably transmitted back to domestic ducks, in which the viruses generated multiple reassortants. These novel H9N2 viruses are double or even triple reassortants that have amino acid signatures in their hemagglutinin, indicating their potential to directly infect humans. Some of them contain gene segments that are closely related to those of H5N1 (A/Hong Kong/156/97). More importantly, some of their internal genes are closely related to those of the novel H5N1 viruses isolated during the outbreak

in Hong Kong in 2001. Those H5N1 viruses were transmitted directly to humans from infected poultry. In the poultry markets in Hong Kong, both H5N1 and H9N2 influenza viruses have been co-circulating, raising the possibility of genetic reassortment. Such reassortants may directly or indirectly play a role in the emergence of the next pandemic virus. Effective antiviral drugs are a major component of public health countermeasures for the control of seasonal and pandemic influenza. Pending the availability of vaccines, antivirals will be the only medical intervention for use in a pandemic. Until recently, only two antiviral drugs, amantadine and its derivate rimantadine, were available for the treatment of influenza infection. Amino acid substitution(s) within the transmembrane domain of the viral M2 protein confers resistance to M2 inhibitors. In addition, these drugs may be associated with severe adverse effects including delirium and seizures, which occur mostly in elderly persons on higher doses (W.H.O. Fact Sheets, March 2003). New class of anti-influenza drugs, the neuraminidase (NA) inhibitors, zanamivir and oseltamivir, have been approved for use in humans (Gubareva et al, 2000). If administered within 48 hours of the first signs of the flu, they can shorten its course by 24 to 36 hours. An illness that normally lasts about eight to 10 days can therefore be reduced to only seven or eight days. However, these miracles of modern medicine come at a cost of \$45 to \$65 per prescription, and some health insurance companies do not cover their use. In addition, there are accumulating reports that demonstrate naturally occurring resistance to neuraminidase inhibitors (Hurt and Barr, 2007). These two drugs, zanamivir and oseltamivir, have fewer adverse side effects than the previous generation, although zanamivir may exacerbate asthma and other chronic lung diseases. In summary, these drugs should be administered within the first two days following the infection in order to be effective, they are expensive and currently not available for administration in many countries (W.H.O. Fact Sheet, March 2003; and W.H.O. Working Group, April 2004). In conclusion, this is a step forward, but not a giant one. Herbal remedies such as Echinacea and various types of tea are also used to ease the effect of cold and flu. Cinnamon has a long history both as a spice and as a medicine. Cinnamon's unique healing abilities come from the various components found in its bark. The low molecular weight essential oils (max. 4%) contain active components called cinnamaldehyde, eugenol, cinnamyl acetate, and cinnamyl alcohol, plus a wide range of other volatile substances such as safrole, coumarin [0.6%] and cinnamic acid esters. Cinnamon has unique healing abilities such as blood sugar control (Anderson et al, 2003., Khan et al 2003), anti-clotting actions, antioxidant (Murcia et al, 2004) and anti-microbial activity (Ouattara et al, 1997). In addition, we have recently demonstrated the antiviral activity of the cinnamon aqueous extract against human influenza H1N1 and other viruses (Barak, I. and Ovadia, M., 2005, Gueta, K. and Ovadia, M., 2005). The present study examines the ability of the cinnamon aqueous fraction to inhibit the H9N2 influenza virus both *in-vitro* and *in-ovo*. Cinnamon is not a commonly allergenic food and is not known to contain measurable amounts of

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goitrogens, oxalates, or purines. Anderson and his colleagues at the USDA Human Nutrition Research Center, who are studying the mechanisms by which cinnamon enhances insulin activity, found that potentially toxic compounds in cinnamon bark are found primarily in the lipid (fat) soluble fractions. They are present only at extremely low levels in water soluble cinnamon extracts, which are the ones with the insulin-enhancing compounds (Anderson et al, 2003). Cinnamon is considered extremely safe for consumption and is actually one of the oldest spices known.

Methods

Cinnamon extract. Cinnamon bark was ground into powder using a coffee grinder. The active neutralizing material was extracted from the powder into an aqueous phosphate buffer solution overnight and centrifuged. The supernatant was kept either in the refrigerator or at room temperature until use. CEppt fraction was then precipitated from the cinnamon extract (CE) by KCl 0.3M and dissolved in 0.02M phosphate buffer, pH 7. Assays for hemolysis and hemagglutination. The virus was propagated as described previously (Borkow and Ovadia, 1999) in the allantoic sac of 10-11-day-old embryonated chicken eggs and harvested 2-3 days following injection into the eggs. The hemolytic activity of the viruses (release of hemoglobin) was examined on human erythrocytes as follows: Human blood was obtained from the Blood Bank at Shiba Hospital, Ramat-Gan. Prior to use, erythrocytes were washed 5 times with Phosphate Buffered Saline (PBS), pH 7 and diluted to a concentration of 4%, with the same buffer. Hemolysis was tested as described previously (Rappaport et al., 1995; Gosh et al., 1997; Borkow and Ovadia, 1999). In brief, washed erythrocytes were mixed with the virus alone or with the virus preincubated with various amounts of the cinnamon extract at room temperature. Following the attachment, excess virus was removed by washing with PBS before adding 200 μ l of 0.1 M sodium citrate buffer at pH 4.5 for three min., in order to achieve fusion of the virus with the erythrocytes. The mixture was neutralized by Phosphate Buffer and washed before incubation in 0.8 ml PBS at 37°C for 3 hours followed by centrifugation to remove intact erythrocytes. 250 μ l aliquot were withdrawn from the supernatant of each sample into wells of an ELISA plate for measurement of the absorbance in an ELISA plate reader at 540 nm.

In-Ovo Assays. These experiments were performed in the ABIC Laboratories on SPF embryonated chicken eggs, 11-days-old. One ml containing 4.5 mg of CEppt and 10^7 EID₅₀ of influenza H9N2 was incubated for 20 minutes at room temperature before preparing 10-fold dilutions from this mixture. 0.1 ml of each dilution was injected into each allantoic cavity of 6 embryonated eggs. The same dilutions of the virus alone or CEppt alone were used as controls. The eggs were observed during the following week for vitality and viral hemagglutinating activity. Results: The ability of the cinnamon fraction CEppt to neutralize influenza virus H9N2 was examined both *in-vitro* using the assay for hemolysis and by *in-ovo* injection of the cinnamon fraction CEppt into SPF egg embryos. Figure 1 shows the hemolytic

activity of the virus to have been totally inhibited by 50 μ g of CEppt. Figure 2 shows that egg embryos survived high dose of 10^7 EID₅₀ of the virus when mixed with cinnamon fraction, the titer of the virus decreased by 5 Logs compared to non treated virus.

Figure 1. *In-vitro* Neutralization of the Virus. Various amount of CEppt were incubated with 0.1 ml containing 128 HAU of H9 virus in order to test the inhibition effect of the cinnamon fraction on the hemolytic activity of the virus. Virus alone CEppt alone and PBS were used as controls. The strong hemolytic activity of the virus was totally inhibited by 50 μ g of CEppt.

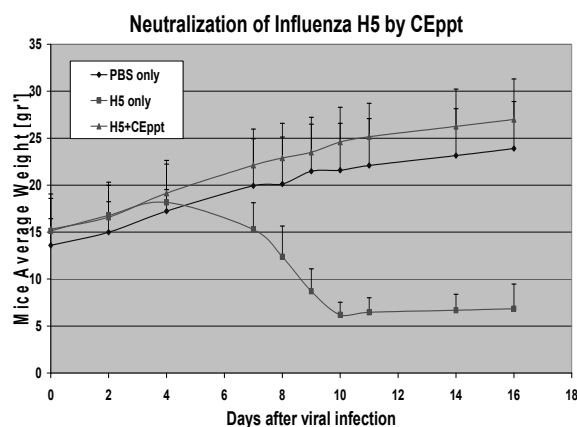
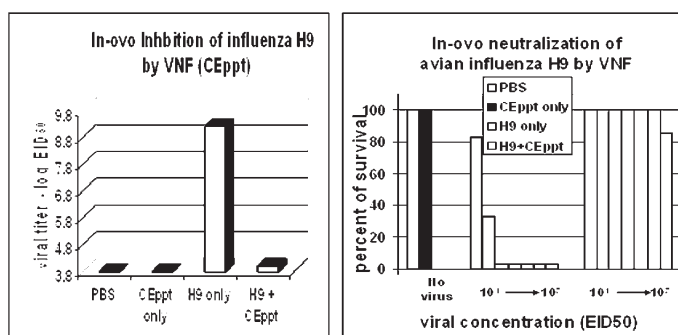


Figure 2. *In-Ovo* Experiments. One ml containing 4.5 mg of VNF and 10^7 EID₅₀ of influenza H9 was incubated for 20 minutes at room temp. before preparing 10 fold dilutions from this mixture. 0.1 ml of each dilution was injected into each allantoic cavity of 6 embryonated eggs (11 days old SPF). Same dilutions of the virus alone or CEppt were used as controls. The eggs were observed during the following week for vitality and viral hemagglutinating activity. CEppt decreased the viral infectivity by 5 logs.



Discussion

Today's highly mobile, interdependent and interconnected world provides myriad opportunities for the rapid global spread of infectious diseases, which are nowadays spreading faster than at any other time in history. An outbreak in any one part of the globe is only a few hours away from becoming an imminent threat somewhere else. The extensive spread of highly pathogenic avian influenza H5N1 viruses and the occurrence of human infections have raised concerns regarding their pandemic potential. Pending the availability of vaccines, antivirals will be the only medical intervention for use in a

pandemic. The emergence of resistance to oseltamivir, with a frequency of 15-20% in children, is of particular concern in relation to the extensive stockpiling of this drug in defence against a potential H5N1 pandemic. The isolates of H9N2 that contain PB1 and PB2 of H5N1 and the isolation of both viruses from the Hong Kong poultry market have raised the possibility of genetic reassortment between these viruses. The reported transmission of avian H9N2 to humans lends urgency to finding ways of neutralizing and controlling this virus. This study demonstrates the ability of the cinnamon fraction CEppt to neutralize influenza virus H9N2 was examined both *in-vitro* and *in-ovo*. In a parallel work (in preparation) we have found that the cinnamon fraction is able to neutralize H1N1 and H9N2 at three levels: free virions, after virus attachment onto the cells and by masking the cells with the neutralizing cinnamon fraction. This effective neutralization together with the encouraging results of the *in-ovo* experiments raises the possibility of using a mixture of the cinnamon fraction and the appropriate virus for immunizing embryos *in-ovo*. Such preliminary experiments have recently been carried out with encouraging results. In addition, a future work is being planned to examine the possibility of neutralizing influenza H5N1 by using the experimental H5N1/PR8 strain which will be provided by the CDC/USA.

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Similarity in the Pharmacokinetics of Oseltamivir and Oseltamivir Carboxylate in Japanese and Caucasian Children

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Introduction

Oseltamivir is an orally administered neuraminidase inhibitor that is widely used for the treatment and post-exposure prophylaxis of influenza in adults and children (aged >1 year) [1]. It is administered as a prodrug, oseltamivir, which is then rapidly metabolized to its active metabolite, oseltamivir carboxylate (OC), by hepatic esterases [2, 3]. To effectively curtail viral replication, therapeutic concentrations of OC must be rapidly achieved at all sites of infection; these concentrations must also be maintained for the duration of therapy. Individual patient characteristics, such as age and weight, may influence the attainment of this goal; the influence of ethnicity is also an important consideration. In a prior study, the PK profile of oseltamivir and OC were shown to be similar in Japanese and Caucasian adults; this finding formed the basis for the global use of the 75mg twice-daily treatment regimen [4]. There are currently no comparisons of PK data for Japanese and Caucasian children available. In the current assessment, we analyzed the serum concentrations of oseltamivir and OC obtained in four paediatric studies to establish whether their PK profiles were comparable across Japanese and Caucasian children.

Methods

Due to the different dosing regimens employed in these studies, serum concentrations were normalized to a 2mg/kg dose. Raw and body-weight normalized, time-scaled concentrations of oseltamivir and OC from four paediatric studies were compared via scatter-plot displays and summary statistics. Studies analyzed: *PP16351*. An open-label, PK study of single-dose oseltamivir in uninfected, otherwise healthy Caucasian children (n=24) aged 1–5 years. Children aged 1–<3 years (≤15kg) received a 30mg dose of oseltamivir, while those aged 3–5 years (>15–23kg) received 45mg. Up to seven blood samples were taken up to 24 hours post dose. *WV15758*. A multicentre, double-blind, randomized, placebo-controlled, phase III trial of otherwise healthy Caucasian children (n=452) aged 1–12 with confirmed influenza. Up to nine post-dose blood samples were taken within 12 hours of dosing from 99 patients aged 1–5, during 5 days' treatment with oseltamivir 2mg/kg twice daily. *NP15826*. An open-label, PK study of single-dose oseltamivir in uninfected, otherwise healthy Caucasian children aged 1–12 years (n=12) and adolescents aged ≥13 years (n=6). Up to 11 post-dose blood samples were taken within 24 hours following a single 2mg/kg dose. *JV16284*. A multicentre, open-label, phase II study in otherwise healthy

Japanese children (n=18) aged 1–12 years with confirmed influenza who received oseltamivir 2mg/kg for 5 days twice daily. Up to two post-dose samples of blood were taken within 24 hours of dosing.

Results

Mean doses of oseltamivir in these studies ranged from 41 to 74mg and the mean dose per kg body weight ranged from 1.80 to 2.89mg/kg. The normalized serum concentrations of oseltamivir (Figure 1) and OC (Figure 2) across Japanese and Caucasian children were comparable. After normalization, inter-quartile ranges were similar for both oseltamivir and OC concentrations across patient populations (Oseltamivir: 3.95–22.05 ng/mL for Japanese, 3.63 – 26.75 ng/mL for Caucasians, OC: 167–298 ng/mL for Japanese, 139 – 274 ng/mL for Caucasians). The current analysis was adequate to show similarity in oseltamivir and OC dose normalized serum concentrations between Japanese and Caucasian children. A more rigorous comparison would require the development of an oseltamivir/OC PK model and calculation of PK parameters according to a population PK approach, taking ethnicity (and other demographic variables) as covariates.

Conclusions

This analysis indicates that, as in adults, the PK profiles of oseltamivir and OC are similar in Japanese and Caucasian children; it also supports the use of identical dose regimens in both ethnic groups. This observation is of particular interest, as the weight-based unit dosing regimens of oseltamivir are used worldwide in children except for Japan. Instead, a fixed-dose regimen (2mg/kg) is approved in Japan that provides a lower systemic exposure; this has been identified as a potential contributing factor to the increased incidence of drug resistance observed in Japanese children [4,5].

Figure 1. Serum concentrations of oseltamivir in Japanese and Caucasian children across four studies (All concentrations normalized to 2mg/Kg dosing).

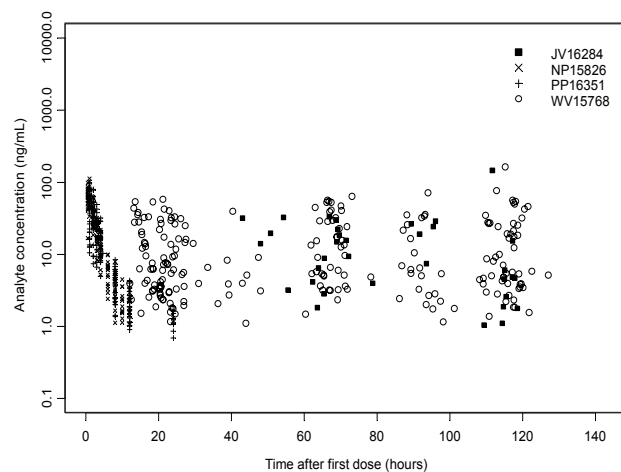
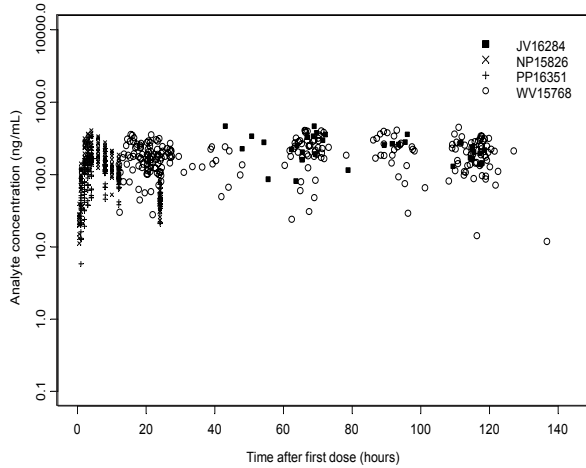


Figure 2. Serum concentrations of oseltamivir carboxylate in Japanese and Caucasian children across four studies (All concentrations normalized to 2mg/Kg dosing).



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HA-Pseudotyped Retroviral Vectors for Screening and Evaluation of Anti-Flu Inhibitors

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Influenza HA-pseudotyped retroviral vectors expressing a luciferase reporter were produced by co-transfecting 293T cells with plasmids for the expression of MuLV gag-pol, fire fly luciferase, HA (from A/Viet Nam/1194/2004 [H5N1]), and flu NA functions. Using the HA-pseudotyped vectors, we developed a cell-based assay to evaluate the flu HA-mediated entry using luciferase as a reporter. This assay was studied using A549, 293T, and H460 cells and validated by the inhibition using HA blockers such as specific HA antibodies, oligosaccharides of α -2,3-SialGal, and synthetic HA-binding peptide, EB. A natural product library consisting of fermentation broth and herbal extracts was used to screen for inhibitors for the HA-mediated entry in 384-well microplates. Specific HA inhibitors were identified that prevented the flu HA-mediated entry but were non-inhibitory to the VSV-G-mediated entry. In addition, for the preparation of high titer HA pseudotyped viral vectors, neuraminidase was needed, presumably to facilitate the release of pseudotyped viral vector from 293T cells. This requirement was used to measure the potencies of specific flu neuraminidase inhibitors (NAI) by the inhibition of the production of the pseudotype vectors.

Introduction

Only four drugs are currently approved for the prevention and treatment of flu infections. Many of the H5N1 flu strains are resistant to the M2 inhibitor drugs of Amantadine and Rimantadine (1). In addition, viruses resistant to neuraminidase inhibitors (NAIs) were also found that occurred naturally or being selected in drug treated patients (2). Thus, the discovery of new anti-flu therapies based on other antiviral targets is needed. Several attempts were made previously to discover and develop HA antagonists or fusion blockers as anti-flu therapies. These inhibitors were found as anti-flu leads that inhibited flu at the very early stage of the infection cycle. They were subsequently found to be either HA antagonists or inhibitors that block the HA-mediated fusion functions (3, 4). The alternative approach is to screen for flu entry blockers directly. However, safety considerations will be too high to be impractical for entry blocker screenings using H5N1 viruses. These problems can be avoided using the HA-pseudotype vector (PV) system that allows assessment of the entry event using a reporter without any concern on the infection by H5N1 influenza. This study reports the use of HA-PV for this purpose. In addition, we explored the indispensability of the

neuraminidase activity in PV production and used this property to develop a quantitative measurement for neuraminidase inhibitors (NAI).

Methods

For the preparation of HA-pseudotype vector (HA-PV), 293T cells were transfected with the following plasmids: pGagPol, pHA (Viet Nam1194), pNA, and pMuLV-FLuc (or pMuLV-GFP) (5). The plasmids pGagPol, pHA (VN1194), and pNA provided the MuLV Gag and Pol proteins, the HA, and the neuraminidase functions, respectively, for the formation of pseudotype vectors, and the plasmids pMuLV-FLuc (or pMuLV-GFP) served as the reporter for transduction and the quantification of the tiers of the pseudotype vectors. The pseudotyped vectors were used to transduce 293T, A549, MDCK, and H460 cells and expressed the GFP or the luciferase reporters successfully.

Results and Discussion

The transduction of these reporters required functional interaction of HA and cell receptors. This was validated by several studies. First, we showed that the HA-PV bound specifically to immobilized α -2,3-SialGal oligosaccharides but not to the α -2,6-SialGal oligosaccharides, consistent to the avian origin of the HA from A/Viet Nam1194/2004/H5N1. As a control, VSV glycoprotein pseudotyped vectors bound to neither oligosaccharide. The specific interaction to the α -2,3-SialGal receptor was further confirmed by inhibition of transduction to A594 cells using excessive α -2,3-SialGal oligosaccharides. In contrast, excessive α -2,6-SialGal oligosaccharides was non-inhibitory in a parallel study. Furthermore, the synthetic peptide, EB, that is inhibitory to HA-receptor binding (6) was also inhibitory to HA-PV transduction. In contrast, the control peptide, EBX, with scrambled sequences, was non-inhibitory at comparable concentrations. Finally, murine sera from mice immunized with inactivated NIBRG-14, a reassorted H5N1 influenza virus strain, were inhibitory to the pseudotype virus transduction to A549 cells (Figure 1) indicating that the interactions between HA and receptor were necessary for HA-PV transduction. The nature of the HA-receptor interaction prompted us to use the HA-PV to develop a simple and high throughput screening plate form for the identification of influenza entry inhibitors including HA antagonists and fusion inhibitors. Toward this objective, we tried different cells, optimized the cell numbers per well and transduction conditions. These optimization studies were done on 96-, 384- and 1536-wells. Acceptable Z' values (7) that evaluate the assay signal-to-noise qualities, were established (above 0.5) in assays conducted in 384 well plates using H460 cells. To test the screening protocol, we used a small natural product extract library of fermentation broth or herbal medicinal extracts for this study. Figure 2 shows a few of the non-cytotoxic entry inhibitors identified from the screening. There are two apparent classes of entry inhibitors. The first class of inhibitor inhibited the entry of both HA-PV and VSV-PV to similar extents, and the second class of inhibitors were

preferentially inhibitory to the entry of HA-PV. Twelve class two inhibitors were selected and used to study their inhibitory activities for the HA-mediated and the VSVG-mediated entry to H460 cells. One inhibitor, P7B3, was particularly potent and specific for the inhibition of the HA-mediated entry. After a ten thousand fold dilution, the diluted extract was inhibitory to the HA-mediated entry to much greater than 50%; while significant inhibition against the VSVG-mediated entry was not observed at the highest concentration of 0.3% used for the study (Figure 2). Our success in the identification of potent and specific inhibitors from the natural product extract library validated the use of the HA-PV as a simple method for high throughput screening for HA-mediated entry inhibitors. To the best of our knowledge, this is the first reported HTS method for flu entry inhibitors. The active component will be isolated from the hit extract and studied for possible development as anti-flu agents. We believe that the principle of entry inhibitor screening using HA-PV described in this report could be applicable to antiviral studies of other viruses.

Figure 1. Inhibition of the HA-PV transduction to A549 cells using anti-HA antiserum samples. Twenty thousand A549 cells were seeded per well in 384-well plates and later transduced with HA-PV in the presence of murine anti-HA serum samples. After an incubation of two days, cells were lysed and the luciferase activities determined. Inhibition of the HA-mediated entry by antisera was expressed as percent luciferase activities expressed of untreated controls.

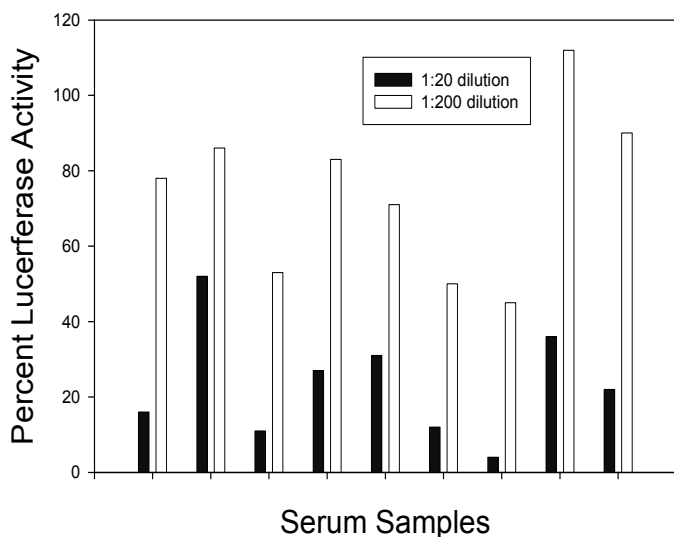
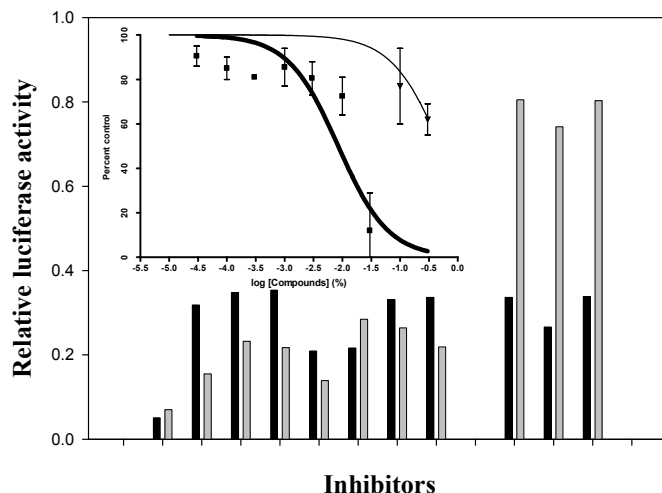


Figure 2. Screening and characterizations of entry inhibitors from a natural product extract library. The screening procedure used for entry inhibitor screening was similar to what described in Fig 1 except natural product samples at final concentrations of 0.5% were added to the H460 cells along with HA-PV. Hits identified from the screening using HA-PV were tested in parallel for the inhibition of the HA-PV mediated entry (dark bars) and the VSV-mediated entry (light bars) to H460 cells. The dose-response curves of a potent inhibitor (insert) show that P7B3 produced a greater inhibition of the HA-mediated entry (dark line) than the VSV-mediated entry (light line).



In addition to the potential use for the entry inhibitor screenings, the HA-pseudotyped vector could also be used for NAI studies. We found that neuraminidase activity is indispensable for pseudotype virus productions. The functional requirement of neuraminidase was employed to develop a quantitative assay for NAIs by measurement of the HA-PV titer versus NAI concentrations. The IC_{50} values of Oseltamivir and Zanamivir were determined by this method using wild type and NAI-resistant mutant influenza neuraminidase enzymes. This assay offered an opportunity to evaluate NAIs using physiological substrates under more relevant conditions without the need to encounter potential biohazards that the alternative approach of using infectious influenza virus might face. Thus, the HA-PV is a valuable tool for anti-flu drug discovery targeting at viral entry as well as at the budding steps.

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Evolution of Human Influenza A Virus Neuraminidase Gene and Evaluation of Susceptibility to Antivirals

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Introduction

Neuraminidase (NA) is the second major surface glycoprotein of Influenza virus and one of the two principal viral antigens, eliciting an immune response that reduces the pulmonary virus titre and the extent of lung lesions [1]. Moreover, this glycoprotein has a crucial role in the replication cycle of the virus since it is responsible for the cleavage of the terminal sialic acid moieties that are present in the host cell surface receptors, allowing virion release and preventing self aggregation of the newly synthesized virus. NA have also the capacity of cleaving the terminal sialic acid moieties that are present in the mucin existent on the surface of the respiratory tract, allowing the direct contact of the virus with the target epithelial cells [2, 3]. In spite of its antigenic and functional importance, the scientific interest and the amount of information available about NA have only start to grown up in the last years, especially since the development of neuraminidase inhibitors. With the clinical use of these inhibitors as 2nd generation antivirals has arisen too the concern about the emergence of resistant Influenza strains. Studies developed in vitro have revealed that resistance can arise due to mutations in either the NA, placed in or around the active site, or in the hemagglutinin (HA), lowering the affinity of HA for the cell receptors [4,5]. This study has two major objectives: (1) analyse the genetic variability of Influenza A virus N1 and N2 neuraminidase in order to achieve, in combination with all the information available concerning HA, a better knowledge of the epidemiology of Influenza A viruses; (2) evaluate the susceptibility of the strains isolated in Portugal to the neuraminidase inhibitors.

Materials and Methods

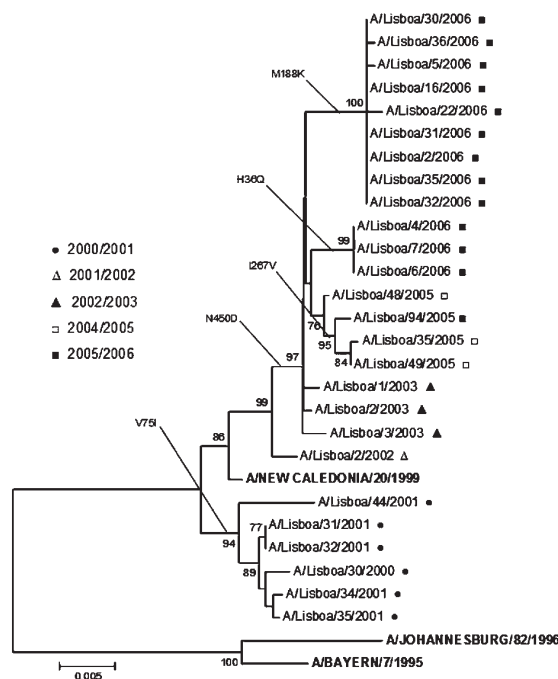
Sixty six strains of Influenza A viruses, 26 belonging to N1 neuraminidase subtype and 40 to N2 neuraminidase subtype, isolated in Portugal between the 2000/2001 and the 2005/2006 Winter seasons were analysed in this study. These strains were isolated from naso-pharyngeal swabs collected by physicians integrated in the National Influenza Surveillance System. Neuraminidase sequences were obtained using the dideoxynucleotide cycle sequencing method after RNA extraction by a protocol adapted from Boom et al., 1990 and RT-PCR amplification [6]. The sequences of each neuraminidase subtype were subsequently aligned by the Clustal method in the MegAlign programme (DNASstar package). The reference Influenza strains used in these alignments were obtained in the Influenza Sequence Database website, available on www.flu.lanl.gov. Phylogenetic trees were constructed in the MEGA 3.1 programme, using the neighbour-joining method and selecting

1000 replicates for the calculation of the bootstrap values. The presence of positive selective pressure (PSP) was tested by the maximum likelihood method using the codeml programme from PAML 3.15. The maximum likelihood estimates were obtained for the codon-substitution models M1a, M2a, M3, M7 and M8. Evaluation of susceptibility to neuraminidase inhibitors was only analysed by searching in all sequences the presence of the NA mutations that are already associated to a diminishing in the susceptibility to antivirals.

Results

Neuraminidase Evolution N1 Subtype. The NA sequences of the N1 neuraminidase subtype viruses shown to be similar to the NA sequences of the N1 viruses isolated in the previous season and to the NA sequence of the reference strain A/New Caledonia/20/1999 (Figure 1). Nine conserved asparagine-linked glycosylation sites were identified in all N1 viruses at positions: 44, 58, 63, 70, 88, 146, 235, 434 and 455 (N1 numbering). The 18 residues that constitute the NA active site were completely conserved over the study period [7]. The amino acid substitutions were observed at very low rates over the study period, with the highest value (1,1%) being obtained in the 2005/2006 Winter season. The N1 sites identified by the codeml programme as being under PSP can not be considered since the M2a/M1a and M8/M7 likelihood ratios were not statistically significant. In this way, it is not possible to admit the presence of positive selection on the N1 neuraminidase of the set of Influenza strains analysed.

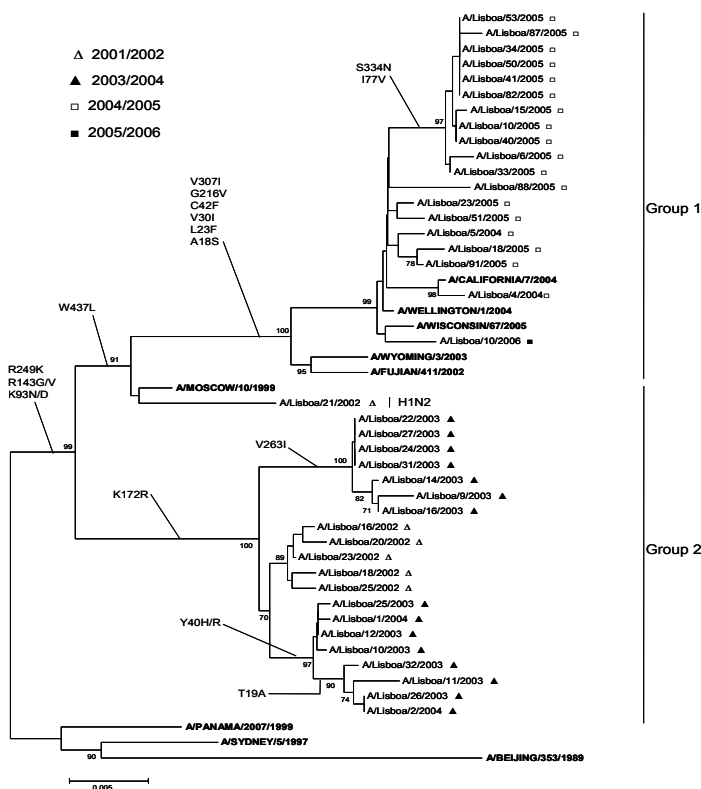
Figure 1. Phylogenetic tree of the amino acid sequences of N1 neuraminidase influenza strains isolated in Portugal between the 2000/2001 and the 2005/2006 Winter seasons. Influenza A(H1N1) reference strains are indicated in black and in bold and only the bootstrap values >70% are shown.



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N2 Subtype. The NA sequences of the N2 neuraminidase subtype viruses distinguished two phylogenetic groups (Figure 2).

Figure 2. Phylogenetic tree of the amino acid sequences of N2 neuraminidase Influenza strains isolated in Portugal between the 2000/2001 and the 2005/2006 Winter seasons. Influenza A(H3N2) reference strains are indicated in black and in bold and only the bootstrap values >70% are shown.



Group 1, represented by the reference strain A/Fujian/411/2002, is composed by the NA sequences of the N2 Influenza strains isolated in the 2004/2005 and in the 2005/2006 seasons. Within this first group the N2 viruses isolated in 2004/2005 shown to be similar to the reference strain A/California/7/2004 and the N2 virus isolated in 2005/2006 to the reference strain A/Winsconsin/67/2005. Group 2 is represented by the reference strain A/Moscow/10/1999 and contains the NA sequences of the Influenza strains isolated in the 2001/2002 and in the 2003/2004 Winter seasons. Nine asparagine-linked glycosylation sites were identified in N2 viruses at positions: 61, 70, 86, 93, 146, 200, 234, 329, 402 (N2 numbering). With exception of the N-glycosylation sites situated at positions 93 and 329, all the other ones are conserved in all N2 viruses. The 18 residues that constitute the NA active site were completely conserved in all the NA sequences of the N2 viruses [7]. The higher rate of amino acid substitution (7,12%) was verified in the 2004/2005 Winter season and of all the mutations identified (38), only 4 (10,5%) have occurred in potential N2 antigenic determinants [8]. Program codeml revealed the M2a and the M3 models as the codon-substitution models that better fitted

the N2 sequence data, with very similar likelihood estimates. In spite of M2a and M3 models considers the existence of 3 classes of sites, only two applied to this case: one large class (96,96%) of conservative sites and a small class (3,04%) of sites under PSP. The sites 199, 221 and 267 are recognized by the two models as the sites being under PSP (by Naïve Empirical Bayes). Of these 3 sites, only one (199) belongs to one of the potential antigenic NA determinants [8]. The likelihood ratio between models 7 and 8 revealed to be statistically significant ($p < 0,01$), which allowed to validate the existence of positive selective pressure on the neuraminidase of N2 viruses considered for this analysis. *Susceptibility to Neuraminidase Inhibitors.* The amino acid change H274Y (N2 numbering), located in the neuraminidase active site, is the only mutation associated with resistance to neuraminidase inhibitors that have been identified in N1 viruses and it is not present in any of the N1 sequences evolved in this study [9]. In relation to N2 viruses, there are already 3 mutations that are associated with resistance to neuraminidase inhibitors: E119V, R292K and N294S [9, 10] (N2 numbering). However, none is present in the N2 sequences that were analysed.

Discussion

The phylogenetic homogeneity observed between the NA sequences of all N1 neuraminidase subtype viruses was expected giving the lower incidence of H1N1 Influenza A viruses in the human population. This lower incidence explains too the conservation of the nine N1 N-glycosylation sites over the study period, the reduced annual rates of amino acid substitution that were obtained and the inexistence of N1 sites under PSP. The close relation verified between all N1 Influenza strains and A/New Caledonia/20/1999 is in conformity with the WHO information concerning the recommended composition of Influenza virus vaccines [11]. The phylogenetic heterogeneity exhibited by the NA sequences of the N2 neuraminidase subtype is a result of the higher circulation of H3N2 Influenza A viruses in the human population (comparing to subtype H1N1). The 2003/2004 Influenza A(H3N2) strains studied are reassortant strains that possesses a HA related to A/Fujian/411/2002 and a NA related to A/Moscow/10/1999, thus only partially agreeing with the vaccine strain recommended by the WHO for that season, A/Fujian/411/2002 [11]. The no conservation of the nine N2 N-glycosylation sites and the presence of N2 sites under PSP are in agreement with the phylogenetic diversity exhibit by N2 viruses over the study period. The neuraminidase active site shown to be completely conserved among all the Influenza A viruses analysed, which is in conformity with the fact that this site constitute the target of the neuraminidase inhibitors. Through sequence analysis it was possible to verify that none of the NA mutations that are already associated with the development of resistance to neuraminidase inhibitors are present in any of the NA sequences evolved in this study. However, sequence analysis is a poor predictor of drug-related phenotype of the virus and because of that more reliable assays based on the inhibition of NA activity (fluorescent and chemiluminescent assays) are already in progress.

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Additive Inhibition of H1N1 and H3N2 Influenza Viruses by Aurintricarboxylic Acid With Amantadine

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Introduction

The frequent drifting of influenza virus and uncertainty of the genetic identity of potential pandemic strains make vaccine development a daunting challenge. Clearly, there is a need to investigate therapeutic and preventive anti-influenza agents. In general, two families of approved anti-influenza drugs exist: M2 ion channel blockers and neuraminidase (NA) inhibitors. M2 ion channel blockers, (i.e. Amantadine and Rimantadine), target the hydrogen ion channel activity of the M2 viral protein, which is required for efficient viral uncoating [1,2]. NA inhibitors, (i.e. Oseltamivir and Zanamivir), block virus release from the cell [2]. Previous studies have also found that siRNA (short interfering RNA) specific for conserved regions of the influenza virus can inhibit viral replication [3]. Aurintricarboxylic acid (ATA) is a potent nucleases inhibitor [4]. It also inhibits many nucleic acid processing enzymes such as DNA and RNA polymerases, aminoacyl-tRNA synthetases and ribonucleotide reductases [4]. ATA has been found to have antiviral properties against several RNA viruses including HIV [4] and SARS-CoV, the causative agent of SARS (severe acute respiratory syndrome) [5]. When compared to untreated controls, ATA was demonstrated to inhibit SARS-CoV replication 10- and 100-fold more effectively than either interferon alpha or beta treatments, respectively [5]. These findings inspired us to investigate the potential antiviral properties of ATA against the Influenza strains A/Puerto rico/8, A/New Caledonia/20 (both H1N1 subtypes) and A/New York/55 (an H3N2 subtype).

Materials and Methods

1) *Virus and cell cultures.* MDCK (Madin-Darby Canine Kidney) cells were obtained from the American Type Culture Collection. Influenza A/Puerto Rico/8/34 (H1N1), Influenza A/New Caledonia/20 (H1N1) and Influenza A/New York/55 (H3N2) were kindly provided by Dr. Jim Robertson at the National Institute for Biological Standards and Control (England). MDCK cells were grown in minimum essential medium (MEM) with 2 mM L-glutamine and Earle's balanced salts adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 20 U/L penicillin and

0.02 mg/L streptomycin. The media for viral infections was MEM and for viral inhibition assays was MEM with 2 mM L-glutamine and Earle's balanced salts supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 25 mM HEPES buffer, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 2 µg/ml TPCK- treated trypsin. ATA and amantadine hydrochloride (AH) were obtained from Sigma (St. Louis, USA). Drug solutions were prepared as 100X stocks in dimethyl sulfoxide (DMSO).

2) *Infection of MDCK cells by influenza virus and analysis of antiviral activities of ATA and AH.* Confluent MDCK monolayers in 24-well plates were washed twice with PBS prior to addition of 200 µl of influenza inoculum at an MOI of 0.001 PFU/cell. Monolayers were incubated with virus, or with MEM alone in uninfected controls, at 37°C for 2 hr. After incubation, inoculums were removed and the media containing TPCK-trypsin, along with ATA and/or AH at various concentrations were added to the wells. In some cases, cells were pre-incubated with ATA to determine whether the compound might prophylactically protect against influenza infection.

3) *Cytotoxicity assays.* The cytotoxicity of ATA and the protection of cells from influenza infections by ATA and/or AH were measured with a neutral red (NR) assay kit (Sigma, St. Louis, USA) or an XTT kit (Roche, Mannheim, Germany). Neutral Red (NR) is a vital dye that is incorporated into the vacuoles of viable cells, and is detected spectrophotometrically. A NR dye uptake assay has previously been used to determine the *in vitro* efficacy and toxicity of antiviral compounds [1,2,6]. XTT is a yellow tetrazolium salt that is cleaved by metabolically active cells to form an orange formazan dye, the production of which is monitored spectrophotometrically [7].

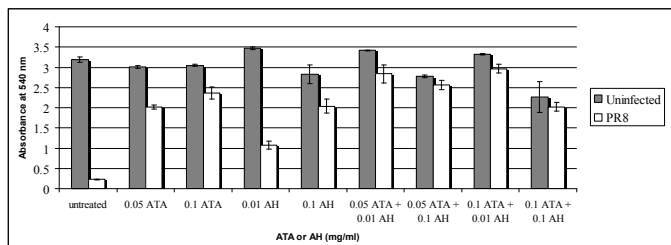
4) *ELISA.* An ELISA kit (Takara Bio Inc., Otsu, Shiga, Japan) detecting the whole virus was used to quantify influenza A viruses in cell cultures in the presence or absence of ATA and/or AH.

Results

The 50% cytotoxic concentration (CC_{50}) of ATA in MDCK cultures and the minimum effective dose of ATA required to protect 50% of MDCK cells from Influenza A/Puerto Rico/8 infection (ED_{50}) were determined by NR or XTT assay. The CC_{50} and ED_{50} were found to be 0.2 mg/mL and 0.005 mg/mL, respectively (data not shown). The therapeutic/selective index (SI) is a comparison of the amount of an agent that causes its therapeutic effect to the amount that causes toxic effects (CC_{50}/ED_{50}) and is one of the parameters used for the analyses of relative safety of the drug. An *in vitro* SI of 40 for ATA in MDCK cells suggests that ATA exerts its desired *in vitro* antiviral effect at a dose substantially below its cytotoxic dose. In subsequent viral inhibition studies, the concentrations of ATA were maintained below 0.2 mg/mL. As shown in Fig.1, 0.05 mg/mL ATA substantially protected cells from death induced by Influenza A/Puerto Rico/8 infections. This protection was significantly enhanced by concomitant treatment with 0.01 mg/mL AH. We obtained similar results when cells were infected with either Influenza A/New York/55 or Influenza A/New Caledonia/20 (data not shown), suggesting

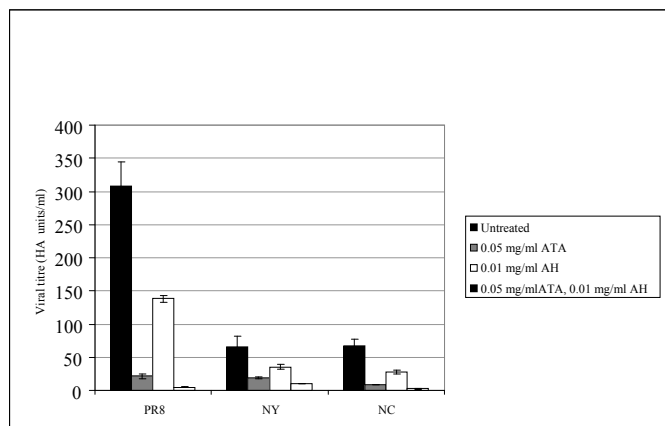
the cotreatment of ATA and AH can inhibit diverse influenza viral strains.

Figure 1. ATA at a concentration of 0.05 mg/mL protects MDCK cells from Influenza A and this protection is enhanced by 0.01 mg/mL AH. Incorporation of NR dye into viable cells was determined by measuring the absorbance at 540 nm. The concentrations of ATA or AH are indicated on the x-axis and are in mg/mL. The data represent the means of triplicates \pm SD.



The protective effects of ATA and AH were further confirmed by quantitatively detecting influenza viruses present in the culture supernatants by ELISA. Consistent with the cell viability assay, co-treatment with ATA and AH reduced the amount of detectable virus in the culture supernatants more than either drug treatment alone (Figure 2).

Figure 2. ATA and/or AH reduce the level of viruses released by infected MDCK cells. Viruses in culture supernatants were detected by ELISA. PR8 denotes infection by Influenza A/Puerto Rico/8/34 (H1N1); NY depicts New York/55 (H3N2); NC represents A/New Caledonia/20 (H1N1). The data represent the means of 6 replicates \pm SD.



To determine if ATA pretreatment can protect cells from influenza infection, MDCK cells were pre-incubated in the presence of ATA for 24 hr prior to viral infection in the presence of replenished ATA. The inhibitory effect of ATA was no different than that observed in samples that were not pretreated (data not shown). This suggests that the inhibitory effect of ATA may occur after viral entry into the cell.

Discussion

Because of its low toxicity in cell culture [5] and animals [8], ATA has been evaluated for its antiviral potential against RNA viruses including human immunodeficiency virus type I (HIV-1) [4]. We recently reported that ATA could selectively inhibit SARS-CoV, the causative agent of severe acute respiratory system (SARS) [5]. Though ATA has been found to exhibit diverse biological activities, including inhibition of protein synthesis, prevention of the attachment of mRNA to ribosomes in cell-free systems and suppression of enzymes involved in polynucleotide metabolism [4,5], the mechanism underlying the antiviral effects of ATA remains to be elucidated. ATA was found to enhance the inhibitory effects of AH on influenza virus replication. This additive effect suggests ATA may inhibit influenza replication by a mechanism distinct from M2 blocking. Our preliminary results obtained from electron microscopy studies showed that ATA may affect the release of viruses from cells into the culture medium. We are currently conducting in-depth analyses of the life cycle of influenza viruses in the presence of ATA to better understand its antiviral mechanism. Given that influenza viruses mutate frequently and strains resistant to currently-approved antivirals have been isolated in patients, the search for new anti-influenza drugs must be relentlessly pursued. Here, we report for the first time that ATA is a potent anti-influenza compound with low toxicity in tissue culture and should be further explored in animal studies.

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Proceedings Topic #10

Innate and Adaptive Immunity

Poster Presentations

Immune Response to Experimental Influenza Infection and Vaccination in Mouse Nasal-Associated Lymphoid Tissue (NALT)

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Introduction

The local immune responses of mucous membranes of an organism are the first and most significant barriers preventing many viral infections, including influenza. The barrier against influenza infection is the mucosal-associated lymphoid tissue of the upper airways. It is considered that rodent nasopharyngeal-associated lymphoid tissue (NALT) is equivalent to the lymphoid tissue of Valdeyer's ring in humans [1]. Russian Live Influenza Vaccine (LIV) is prepared by genetic reassortment of currently circulating wild-type influenza virus with cold-adapted (*ca*) attenuated influenza virus strain known as the master donor strain. Reassortants which inherit 2 genes encoding surface antigens from the wild-type virus and 6 internal genes from the master donor strain virus are selected. By traditional reassortment technology and are known as 6/2 reassortants. Post-infection immunity provides a standard of immunogenicity for influenza vaccines. To estimate the immunogenic properties of vaccines, it is necessary to compare the quantitative and qualitative parameters of immune responses induced by the vaccine strain with those induced by the virulent or parental strain from which it was derived. For ethical reasons such studies are best performed in animal models. This investigation is the first attempt to analyze and compare the development of cellular and antibody immune responses in the NALT in a mouse model of influenza infection and vaccination using an attenuated reassortant influenza A (H1N1) virus and its parental strains. Specifically, the aims of this study are to estimate *in vivo* parameters of local (antibody and cellular) immune responses after inoculation of 6/2 reassortant strain and its parental strains compared with systemic antibody and cellular responses. In addition, we evaluated the degree of apoptosis in local and system lymphocyte populations after influenza inoculation with the 6/2 vaccine strain or either parental strain.

Material and Methods

Mice and viruses. Groups of CBA mice were inoculated with 10^4 50% egg infectious dose (EID_{50}) of pathogenic A/PR/8/34 (H1N1) – a model of uncomplicated influenza infection, 10^6 EID_{50} of the master donor strain A/Leningrad/134/47/57 (H2N2) or 10^6 EID_{50} of the attenuated 6/2 reassortant strain containing HA and NA from A/PR/8/34 and all the internal genes from cold-adapted master donor strain A/Leningrad/134/47/57 (H2N2) – a model LIV strain. An additional group of uninfected mice served as controls.

Detection of immune responses. Serum or nasal wash antibodies

were measured using an enzyme-linked immunosorbent assay (ELISA) to detect levels of virus-specific IgG, IgM and IgA. Single cell suspensions were recovered from the NALT using the procedure described by Asanuma et al. [2]. Proliferation of lymphocytes from the NALT and spleen was estimated using an MTT assay and cytokines were measured by flow cytometry was performed on lymphocyte populations from the NALT and spleen using antibodies to detect CD4⁺, CD8⁺, CD19⁺ cells or Annexin V and propidium iodide to detect lymphocyte apoptosis.

Results

Experimental influenza infection and vaccination. A/PR/8/34 replicated efficiently in the upper airways, whereas levels of the attenuated strains (attenuated 6/2 reassortant strain and master donor strain) were significantly reduced (data not shown). The 6/2 attenuated reassortant strain did not replicate at all in the lower airways (lungs). These results indicated that the 6/2 reassortant had properties in mice consistent with the parental donor strain and the attenuated phenotype necessary for *ca* vaccine strains.

Antibody immune responses. Serum and local nasal wash antibodies were detected by ELISA. Inoculation of each virus results in high-titered virus-specific IgG, IgM and IgA serum antibody responses. The wildtype parental A/PR/8/34 virus induced higher levels of serum IgG antibody compared with the attenuated strains. All viruses induced nasal wash antibodies. The geometric mean titers of total IgG+IgM+IgA reached 1:64 following infection with either the wildtype A/PR/8/34 or attenuated strains. Compared with A/PR/8/34 virus, the attenuated strains were less capable of inducing antibody responses in lungs. There were no substantial differences in the kinetics of the IgG, IgM and IgA responses among the three viruses. Therefore, the 6/2 *ca* vaccine reassortant induced local antibody responses at the primary site of infection (upper respiratory tract) similar to that seen with the virulent virus, but induced substantially lower levels of serum antibody.

Cellular immune responses. Lymphoproliferative response. All viruses induced a similar level of proliferation in NALT lymphocytes (Table 1). Significant increases in the stimulation indices (SI) after infection and vaccination were observed on day 3 post-infection ($p < 0.05 - 0.01$). SI values after inoculation of pathogenic parental virus and attenuated 6/2 reassortant strain were not significantly different ($p > 0.05$). Peak SI values in the spleen were observed on day 7 post-infection. Lymphocyte proliferation kinetics were similar following inoculation with each virus. Interestingly, a short-term immunosuppression was detected in both NALT and spleen within the first 24 hours after inoculation of viruses (data not shown).

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Table 1. Cellular proliferative response and lymphocyte phenotypes in NALT and spleen of virus infected mice.

	Virus	Lymphoproliferative response (peak SI*)	Cells levels after virus inoculation (%)**		
			CD4 ⁺	CD8 ⁺	CD19 ⁺
NALT (day 3)	Pathogenic virus A/PR/8/34 (H1N1)	1.8	50	29	69
	Attenuated 6/2 reassortant strain	2.2	54	29	70
	Master donor strain A/Leningrad/134/47/57 (H2N2)	2.2	40	26	40
	Control	1.0	24	19	10
Spleen (day 7)	Pathogenic virus A/PR/8/34 (H1N1)	1.7	32	47	38
	Attenuated 6/2 reassortant strain	1.7	38	24	29
	Master donor strain A/Leningrad/134/47/57 (H2N2)	1.6	26	24	31
	Control	1.0	19	12	13

*SI = Sample optical density / Control optical density. Optical density was measured in supernatants after 3 days of lymphocyte culture with homologous virus using the MTT-assay [3].

**The percentage of cells expressing the indicated markers were determined by flow cytometry.

CD4⁺, CD8⁺ and CD19⁺ cells levels after virus inoculation. The relative frequency of CD4⁺, CD8⁺ and CD19⁺ lymphocytes in the NALT on day 3 and in the spleen on day 7, the peak time points for lymphoproliferation, were measured by flow cytometry (Table 1). Infection with the attenuated 6/2 reassortant strain elicited an accumulation of CD4⁺, CD8⁺ and CD 19⁺ lymphocytes in both NALT that was similar to the parental wildtype A/PR/8/34 virus. However, in the spleen the frequency of CD8⁺ cells following infection of mice with the 6/2 reassortant was lower than that detected following infection with the virulent A/PR/8/34 virus.

Secretion of Th1/Th2-cytokines. A significant increase in IFN- γ and IL-6 production was observed in NALT after inoculation with any of the viruses. Peak cytokine production of IFN- γ and IL-6 was detected in the NALT on days 7 on 14, respectively (data not shown). In the spleen, increased levels of IFN- γ but not IL-6 were detected. A/PR/8/34 virus induced substantially more IFN- γ in the spleen compared with the attenuated strains. The highest concentration of IFN- γ in spleen was observed on day 17 for attenuated viruses and on day 7 for wildtype A/PR/8/34virus. No IL-4 production was detected in the NALT or spleen.

Lymphocyte apoptosis and proliferation after virus inoculation. We next examined the extent of apoptosis in lymphocytes isolated from the NALT or spleens of inoculated mice using Annexin V staining and flow cytometry (Table 2). Significant overall differences in apoptosis intensity were observed in splenocytes and NALT lymphocytes (20 - 30 % and 6 - 9 %, respectively). After the first or primary inoculation, there was a short-term enhancement of apoptosis intensity both in NALT and in spleen detected within the first 24 hours after virus inoculation. By 48 hours post-inoculation, lymphocyte apoptosis levels had decreased to baseline levels. As indicated above, the lymphoproliferative activity exhibited a transient immunosuppression (decrease of NALT and spleen lymphocyte SI to below control levels) within the same time period (i.e. within the first 24 hours after inoculation). The

second inoculation with any of the viruses, did not result in the rapid increase in lymphocyte apoptosis detected after the first inoculation of A/PR/8/34 virus or either attenuated strain. Similarly, immunosuppression of lymphoproliferation following the secondary response was not detected.

Table 2. Lymphocyte apoptosis in the NALT and spleen following intranasal inoculation of mice.

	Virus	% An ⁺ PI ⁺ cells								Control
		First inoculation				Second inoculation				
		12 h*	24 h*	48 h*	day 7*	12 h*	24 h*	48 h*	day 7*	
NALT	Pathogenic virus A/PR/8/34 (H1N1)	10.1	52.4	5.2	6.4	6.5	8.6	7.4	5.4	7.5
	Attenuated 6/2 reassortant strain	8.1	66.5	7.3	7.0	5.1	6.4	6.0	7.6	
	Master donor strain A/Leningrad/134/47/57 (H2N2)	9.2	69.4	6.1	8.4	7.2	8.4	4.2	9.4	
Spleen	Pathogenic virus A/PR/8/34 (H1N1)	19.7	76.6	26.4	24.4	24.7	20.2	22.7	24.5	24.0
	Attenuated 6/2 reassortant strain	22.4	79.8	28.4	20.4	24.9	25.5	23.6	28.4	
	Master donor strain A/Leningrad/134/47/57 (H2N2)	16.6	66.4	24.9	19.0	20.0	25.1	23.3	24.5	

*Time points after virus inoculation.

Conclusion

This study has shown that the *ca* 6/2 reassortant H1N1 vaccine strain inherited the ability to induce local immune responses in the NALT, the primary immunological site in the upper airways of mice. Compared with the more virulent parental strain, the attenuated 6/2 reassortant virus was less efficient in the induction of CD8⁺ cells, IFN- γ production in the spleen and serum IgG. While the primary inoculation with wildtype or attenuated strains led to a transient increase in lymphocyte apoptosis, secondary inoculation did not result in a change in the apoptotic status of lymphocytes in either the NALT or spleen.

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Influenza Virus Infection Enhances Expression of Epithelial Defensins and Surfactant Proteins in Murine Airway

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Introduction

Human influenza A virus (IAV) is a major cause of morbidity and life-threatening respiratory tract disease worldwide. During natural transmission, IAV first infects epithelial cells lining the airway that include nasal, pharyngeal, laryngeal and bronchial mucosa. Although these mucosal tissues are important sites of initial virus-host encounter, relatively little is known about the nature of early mucosal infection and the associated innate defenses. In a newly infected host, IAV multiplies quickly with a replication cycle lasting only a few hours, and thus early innate immunity could influence the outcome of infection. Innate immune mechanisms become particularly important in immunologically naïve persons who lack specific adaptive defenses against IAV. Other than providing a protective anatomic barrier equipped with the mucociliary clearance apparatus, the airway also releases soluble factors such as the secretory immunoglobulin A (IgA), complement, fibronectin, surfactant proteins and antimicrobial peptide such as defensins. In addition to acting as first-line of defense against invading organisms, defensins and surfactant proteins also trigger antigen-specific adaptive immune responses [1, 2]. Surfactant protein A (SP-A) and SP-D are members of a family of proteins known as collectins that have collagen-like amino (N)-terminal regions and C-type (calcium dependent) carbohydrate-recognition domains. In the lung, SP-A and SP-D are secreted by type II pneumocytes in alveolar walls and Clara cells in bronchiolar epithelium. More recently, these surfactant proteins have also been found in nasal derived tissues [3, 4]. SP-A and SP-D have been shown to bind to various bacteria, viruses and apoptotic cells resulting in enhanced phagocytosis, antigen presentation and oxidative killing by neutrophils. By interaction with various immune cells, these surfactants also serve to modulate the production of cytokines and inflammatory mediators [2]. The protective role of SP-A and SP-D has been reported for several important respiratory viruses including influenza, parainfluenza and respiratory syncytial viruses [5, 6, 7]. Other investigators have shown that deficiency in SP-A and SP-D were associated with greater susceptibility to both bacterial and viral infections in mice and in neonatal baboons [8, 9]. Mammalian α and β defensins are produced by both leukocytes and epithelial cells and are cysteine-rich, cationic peptides that display broad-spectrum antimicrobial activity [10, 11]. Human α -defensins (HD1, -2, -3) are predominantly found in azurophil granules of neutrophils but β -defensins (hBDs) including hBD-1, hBD-2, and hBD-3

are widely expressed in epithelial cells [1, 11]. Several murine β -defensins have also been described [12, 13]. Epithelial β -defensins are detectable in most cutaneous and mucosal sites including the normal airway and oral mucosa and are believed to be key mediators of innate mucosal defense system [14, 15]. For examples, both constitutively expressed hBD-1 and induced hBD-2 and hBD-3 have been shown to be microbicidal to a variety of bacterial and fungal pathogens [10, 11], and more recently, shown to inactivate viruses such as human immunodeficiency virus type 1 (HIV-1) and adenovirus [16, 17]. In addition to their antimicrobial activity, hBD-2 also links innate and adaptive immunity by attracting memory T cells and recruiting immature dendritic cells through chemokine receptor CCR6 [18]. Dendritic cells have been shown to form networks within airway mucosa and thus play important role in the sampling and presentation of airway antigens [19]. Hence, the demonstrated role of dendritic cells in antigen presentation together with the influence of hBDs on their recruitment makes it important to better understand the role of epithelial defensins in influenza virus infections. Recently, α -defensins have been shown to be inhibitory to IAV replication in vitro [20]. It probably plays an important role in vivo as an effector molecule associated with neutrophils infiltration in IAV infected lungs. However, unlike α -defensins, the in vivo role of epithelial β -defensins during IAV infection is not known. Here we investigated the in vivo expression of several mucosal components of the innate immune system including a surfactant protein (SP-D) and epithelial defensins (murine β -defensins MBD-1, MBD-2, MBD-3, MBD-4) in IAV infected mice.

Materials and Methods

We infected mice with either influenza A/PR/8/34 (H1N1) or A/Hong Kong/8/68 (H3N2) using small droplets (10- μ l) of inocula that are thought to be more similar to natural infection and collected tissues at various time points after infection for viral plaque titer determination in Madin-Darby canine kidney cells (MDCK). Separately, trachea and micro-dissected nasal and paranasal sinus mucosa were analyzed for SP-D and β -defensins mRNA expression. Total RNA from tissue samples were isolated using TRI Reagent procedure and cDNA of SP-D, MBD-1, MBD-2, MBD-3, MBD-4 and the housekeeping gene β -actin were generated by reverse transcription using IScript cDNA synthesis kit (BioRad). Quantification of mRNA by real-time RT-PCR was performed using specific primer pairs and SYBR-green PCR Supermix (BioRad) as we previously reported [21].

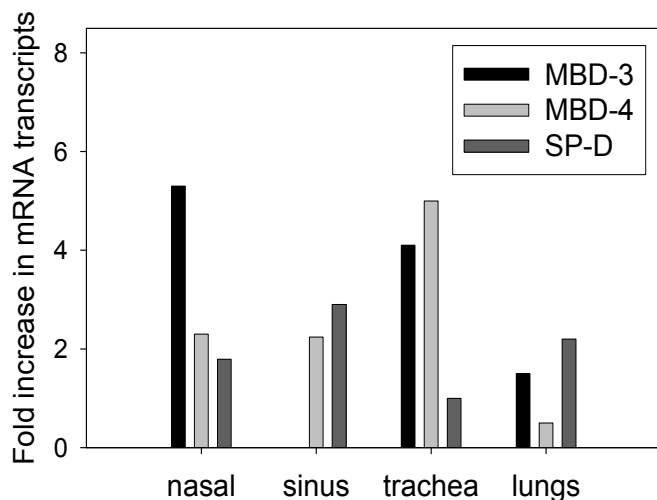
Results

In our mouse model, both the H1N1 and H3N2 IAV strains replicated to high titer of $\sim 5 \times 10^6$ pfu/g and $\sim 5 \times 10^5$ pfu/g tissue 24 hours post infection in the nasal and paranasal sinus mucosa, respectively. These levels persisted for the 7-day study period. Although trachea also achieved peak virus titer early in infection, viral level declined rapidly beyond day 5 to $\sim 3 \times 10^2$ pfu/g by day 7 post infection. Unlike the upper

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airway and trachea, small volume intranasal inoculation of IAV only lead to low level infection in the lung with peak titer of $\sim 2 \times 10^3$ pfu/g tissue. Our results showed varying levels of MBD-1 and MBD-2 mRNA expression in nasal and tracheal mucosa. Importantly, the levels of MBD-3 and MBD-4, which are the murine homologs of human β -defensin 2 and 3, were enhanced 2-6 fold in the trachea, nasal and paranasal sinus mucosa. SP-D expression was readily detected in normal airway mucosa and the levels were enhanced 2-6 fold at various times post-infection. Unlike SP-D and MBD-4, MBD-3 was expressed at very low or undetectable levels in uninfected nasal and sinus mucosa but was upregulated in IAV infected nasal mucosa. Immunostaining were performed using commercial primary antibody preparations against influenza virus (Virostat Inc.), defensins (Santa Cruz Biotechnology Inc.) and SP-D (Millipore Corp.). MBD-3, MBD-4 and SP-D proteins were localized to the epithelial cells of airway mucosa (data not shown). Lastly, the extent of induction of SP-D and MBDs were similar for the two IAV strains studied.

Figure 1. Expression of murine β -defensins (MBD-3, MBD-4) and surfactant protein-d (SP-D) mRNAs in airway mucosa 3 days post infection with A/Hong Kong/8/68 (H3N2). Bars represent mRNA expression normalized to B-actin and relative to mock-infected mucosa using the $2^{-\Delta\Delta CT}$ analysis.



Conclusion

β -defensins are upregulated in the conducting airway mucosa particularly in nasal and tracheal mucosa and might contribute to innate and adaptive immune responses targeted against IAV infection of the respiratory tract. SP-D are upregulated in the trachea, nasal and paranasal sinus mucosa suggesting that SP-D plays a role in IAV infection of the upper airway in complement to its previously reported role in lung infection.

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Antibodies Induced By the Light Chain of Influenza A Hemagglutinin Improve the Course of *In Vivo* Influenza Infection

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We followed the effect of polyclonal antibodies induced by the light chain of influenza A virus hemagglutinin (HA2 gp) on the course of *in vivo* infection. BALB/c mice were immunized with recombinant vaccinia viruses expressing chimeric molecule of hemagglutinin, composed from HA1 and HA2 gp of influenza A virus of different subtypes, originated from A/PR8/34(H1N1) and A/NT/60/68(H3N2) viruses. IgG fraction from sera of immunized mice at defined concentration of specific anti-HA2 antibodies was passively transferred to BALB/c mice which were subsequently infected with influenza A virus. We showed that the antibodies induced by two-step immunization with recombinant vaccinia virus expressing the HA2 gp of homologous subtype (and HA1 gp of heterologous subtype) to the challenge influenza virus A/Mississippi/1/85(H3N2), lowered the mortality of infection with dose 2LD₅₀ by 36% in comparison to the control group of mice. Control mice were immunized with wild type vaccinia virus which did not expressed any influenza virus protein. The protection effect mediated by specific anti-HA2 antibodies was confirmed also by the lower levels of infectious virus in lungs and by earlier elimination of virus and vRNA from lungs of immunized mice than in the control. Though this way of immunization stimulates the complex immune response, the passive transfer of IgG fraction containing the specific antibodies showed that antibodies induced by expressed HA2 gp moderate influenza A infection and cause earlier recovery from the lethal infection.

Introduction

Heavy (HA1gp) and light (HA2gp) glycopolypeptides of influenza A virus hemagglutinin (HA) are strong inducers of immune response after the natural infection [5, 10]. HA2gp, in contrast to the HA1 gp, represents the conserved part of HA [7]. It comprises the common epitopes not only within one subtype, but some epitopes on HA2 gp are shared also among different subtypes. Consequently, as it was also shown, antibodies (polyclonal or monoclonal) induced by HA2 gp are broadly crossreactive within given subtype, but some of them also among various subtypes [1, 8, 9, 13]. We showed in our previous studies that HA2-specific monoclonal antibodies can inhibit the fusion activity of influenza HA [14] and reduce *in vitro* replication of influenza virus [15]. Here we followed the contribution of HA2-specific antibodies induced by immunization with vaccinia virus recombinants (VVR), expressing chimeric HA composed from HA1gp and HA2gp of different subtypes [3], to the protective immunity against lethal influenza A virus infection of

mice. This model allowed us to follow separately the effect of HA1 and HA2 gp. We followed the course of influenza infection of mice after the intravenous application of purified antibodies specific to the HA2 gp induced in mice by recombinant vaccinia virus.

Materials and Methods

Six-week old female BALB/c mice were immunized intraperitoneally with two identical doses (in two-weeks intervals) of one from two vaccinia virus recombinants (VVR) (1x10⁶ PFU/0.5ml/mouse) expressing HA1 gp and HA2 gp of influenza A virus of different subtypes, originated from A/PR8/34(H1N1) and A/NT/60/68(H3N2) viruses (kind gift from Dr. J.W.Yewdell, NIH, USA). The titer of specific anti-HA1 and anti-HA2 antibodies was estimated after each immunization dose by Western blott method using purified viruses A/PR8/34(H1N1), A/NT68/60(H3N2) and A/Mississippi/1/85(H3N2). Viruses originated from NIMR, London,UK. Fourteen days after the second immunization dose mice were bled and IgG were purified from immune sera by affinity chromatography on protein A Sepharose. IgG of defined titers were intravenously applied to BALB/c mice (200µg/200µl/mouse). Two hours after the passive immunization mice were intranasally infected with dose 2LD₅₀ of mouse adapted influenza virus A/Mississippi/1/85 (H3N2) in a light ether narcosis. On days 2, 4, 6, 8 and 10 post infection, infectious virus and vRNA in lungs of mice from each group was estimated. The titer of infectious virus in lungs was determined from a suspension of lungs cell homogenate by a rapid culture assay on MDCK cells. The replicated virus was detected 18 hours after the infection of MDCK cell monolayer with supernatants from lungs cells homogenates and fixation cells with cold methanol, using MAAb 107L [12], specific for NP of influenza virus and SWAM-Px. Infected cells (red stained) were scored in an inverted light microscope after adding the substrate (amino-ethylcarbazole with 0.03% H₂O₂) [11]. Total RNA was isolated from lung's cell suspension using phenol-chlorophorm method after the extraction of RNA with Instapure (Eurogentec). vRNA was estimated by polymerase chain reaction after the reverse transcription (RT-PCR) of RNA to cDNA using primers specific to influenza A virus nucleoprotein as described before [16]. PCR products were analyzed by electrophoresis in 2% agarose gel containing ethidium bromide.

Results and Discussion

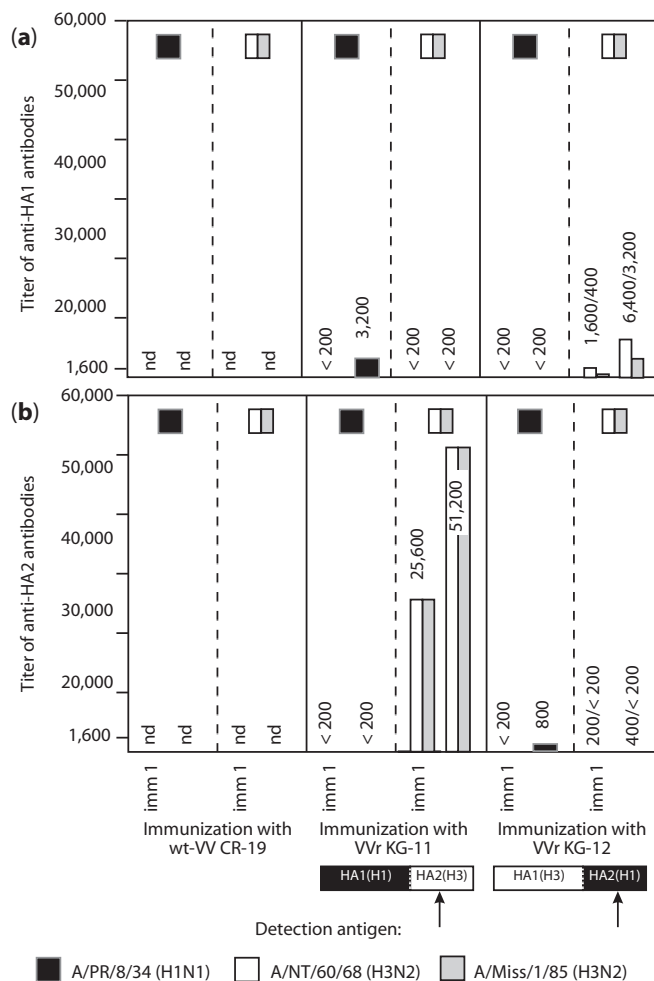
Induction of specific antibody response in mice by vaccinia virus recombinants expressing chimeric influenza A hemagglutinin. BALB/c mice immunized with 2 successive doses of VVR raised specific antibodies to the HA1 and HA2 gp expressed by corresponding vaccinia virus recombinants KG-11 or KG-12. KG-11 [HA1 gp from A/PR8/34(H3N2); HA2 gp from A/NT/60/68 (H3N2)], or KG-12 [HA1 gp from A/NT/60/68 (H3N2); HA2 gp from A/PR8/34(H3N2)]. Control group of mice received the wild type of vaccinia virus (wt-VV) CR-19 not expressing influenza virus protein. Intraperitoneal application of vaccinia virus recombinants to mice led to the induction of specific anti-HA1 and anti-HA2 antibodies already

after the first immunization dose (Figure 1). Their titer however increased significantly 14 days after the second immunization dose. Their potential to protect mice against the lethal influenza A infection was further followed. *The course of the influenza A infection of mice after the passive transfer of antibodies induced by vaccinia virus recombinants expressing chimeric molecule of influenza HA.* Purified antibodies induced as above were applied intravenously to BALB/c mice which were subsequently infected with influenza virus A/Mississippi/1/85(H3N2). The survival of infected mice and the presence of infectious virus and viral RNA in mouse lungs were followed 14 days after the infection. In the control group of mice (n=12) which received before the infection IgG from serum of mice immunized with wt-VV CR19, all mice died (survival 0%) (Figure 2a). In the group of mice (n=11), which received IgG from mice immunized with VVr KG-12 expressing HA1 gp of homologous subtype (H3) to the challenge virus, survived 45.5% of mice (p=0.0137). It should be stressed that also in the group of mice which were immunized by IgG from mouse serum obtained by immunization with VVr KG-11 (n=11) expressing HA2 gp of homologous subtype to the challenge virus A/Mississippi/1/85(H3N2), survival was improved to 36.4% (p=0.0373). Passive transfer of IgG induced by VVr KG-11 or KG-12 to mice before the infection caused also two-day earlier clearance of infectious virus and vRNA from lungs of infected mice in comparison to the control group which received IgG from serum of mice immunized with wt-VV CR-19, not expressing influenza HA (Figure 2b). Antibodies induced by VVr KG-12 expressing HA1 gp of A/NT/68/60(H3N2), which was of homologous subtype to the challenge virus A/Mississippi/1/85(H3N2), did not confer 100% protection to mice, because the challenge virus was not identical with the HA1 gp expressed by VVr. The antigenic drift between these two strains was the reason of only partial protection of mice, which was comparable to that obtained by antibodies induced with HA2 gp of homologous subtype to the challenge virus. However, mechanism of protection mediated by HA2-specific antibodies is different from that of HA1-specific. While the most effective HA1-specific antibodies block the binding of virus to the target cells [2], we suppose that HA2-specific antibodies are effective in the second stage of replication cycle and block the fusion of viral and endosomal membranes. The possible contribution of ADCC mechanism however can not be excluded. From above results we conclude that besides antibodies specific to HA1gp, also antibodies elicited by HA2 gp of homologous subtype to the challenge virus significantly contributed to the protection of mice against the influenza infection. The new and most important result from the above experiments is that HA2 gp is able to actively induce a protective antibody response. These studies confirmed our previous observation that intravenously applied HA2-specific monoclonal antibodies contribute to the protection of mice against lethal influenza A infection [4]. Thus HA2 gp, a highly conserved part of influenza HA, could be a suitable antigen in the preparation of a vaccine with a broader spectrum of protection.

Acknowledgements

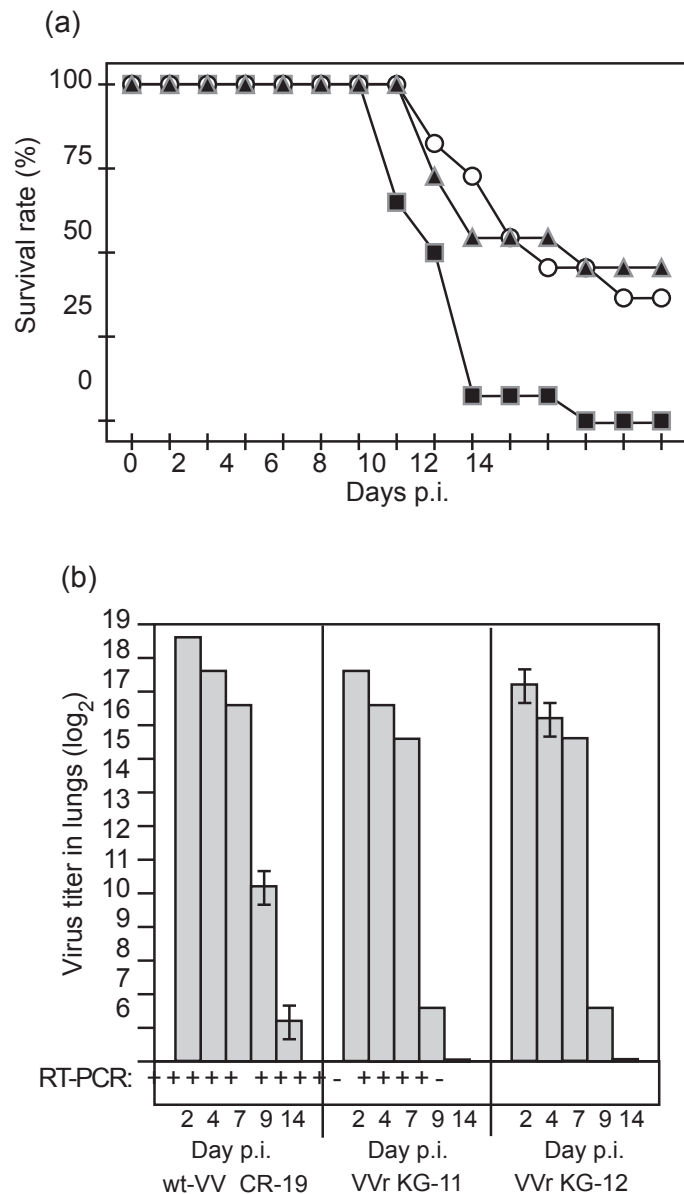
We thank Elena Ulicna and Jana Mesarosova for their excellent technical assistance. This work was supported by VEGA, Slovak Republic, grants 2/6077/27, 2/7065/27, and by APVV, grant APVV-51-021605.

Figure 1. Western-blott analysis of anti-HA1 (a) and anti-HA2 (b) antibody response of mice immunized with wt-VV CR-19 and VVr KG-11 or KG-12 expressing HA composed from HA1 gp and HA2 gp of various subtypes. Titers of antibodies specific to the HA1 gp and HA2 gp of influenza HA were detected with purified influenza viruses, which were blotted to the nitrocellulose membrane after the separation of viral proteins in 12% SDS-PAGE under the reducing conditions. Titer was determined as the reciprocal value of the highest dilution of sera, which gave the colour band of the corresponding molecular weight.



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Figure 2. Survival of mice infected with influenza virus A/Mississippi/1/85 (H3N2) (a) and kinetics of viral infection (b) after the passive transfer of immunoglobulins induced by VVr.



IgG were purified from sera of mice immunized with wt-VV CR-19 not-expressing, and VVr KG-11 or VVr KG-12, expressing the chimeric influenza hemagglutinin composed from HA1 and HA2 of various subtypes of hemagglutinins. Significance of survival was evaluated by Fisher exact test. Kinetics of viral infection was followed by the assessment of titer of infectious virus in mice lungs by rapid culture method on MDCK cell monolayer and by the detection of vRNA in lungs of mice by RT-PCR using primers specific for influenza A nucleoprotein.

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Inhibition of Influenza Viruses By Proteins of the Innate Immune System

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Introduction

It has long been established that human and animal sera contain a variety of non-specific inhibitors of influenza virus that neutralize virus infectivity and/or inhibit haemagglutination by the virus (for reviews see [1, 2]). The term non-specific inhibitor refers to inhibition of virus haemagglutination and/or infectivity by a mechanism distinct to that of antibody. Historically, non-specific inhibitors have been classified as α , β and γ inhibitors based on their chemical composition and properties. α and γ inhibitors are heat stable, sialylated glycoproteins that act by competing with sialylated cell surface receptors for binding to the viral haemagglutinin (HA). The so-called Francis or α inhibitors were the first described serum inhibitors of influenza virus and are active against both type A and B viruses. While acting as potent inhibitors of haemagglutinating activity, α inhibitors do not neutralize virus infectivity and so are thought to play a limited role in innate host defence. γ type inhibitors are present in a range of animal sera and share many properties with α inhibitors, however they also mediate potent inhibition of virus infectivity. The high molecular-weight glycoprotein α 2-macroglobulin is the major inhibitor present in non-immune horse, guinea pig and pig sera and the characteristics of equine α 2-macroglobulin in particular have been well characterized. Horse and guinea pig α 2-macroglobulin contains the sialic acid *O*-acetyl-*N*-acetyl-neuraminic acid, which resists hydrolysis by viral neuraminidase [3, 4]. Growth of human H3 influenza viruses in the presence of horse serum resulted in selection of horse serum resistant mutants with an altered receptor binding specificity from preferential recognition of sialic acid in an α 2,6 linkage to galactose to sialic acid linked α 2,3 to galactose [5, 6]. Thus, selection of mutants in the presence of non-specific inhibitors can have profound effects on the receptor specificity of the HA molecule. β inhibitors, on the other hand, are Ca^{2+} -dependent, non-sialylated and generally heat-labile. They are particularly abundant in bovine serum, but are also present in mouse, guinea pig and human serum. For many years, the mode of action of these inhibitors was unclear until studies by Anders *et al.* demonstrated that the β inhibitors in bovine and mouse serum were mannose-binding lectins [7]. Further characterization of the β inhibitors of bovine and mouse serum demonstrated that they were distinct but related mannose-binding lectins. The bovine inhibitor was identified as conglutinin, whereas the inhibitors in mouse and guinea pig serum are related to the serum mannose-binding lectin (MBL) [8, 9]. Both conglutinin and MBL belong to a family of

Ca^{2+} -dependent mammalian lectins known as the collectins. *Collectins - innate inhibitors of influenza virus.* Identification of serum β inhibitors as members of the collectin family led to intense interest in the ability of these and other collectins to function in antiviral host defence against influenza viruses. Collectins are a group of collagenous lectins that are present as large, multimeric proteins in serum and body fluids [10, 11]. Members of this family known to bind to influenza virus and display antiviral activity *in vitro* include the serum proteins MBL, conglutinin and collectin-43 (CL-43) and lung surfactant proteins A (SP-A) and D (SP-D). SP-D, MBL, conglutinin and CL-43 act as classic β -inhibitors and bind in a Ca^{2+} -dependent manner to oligosaccharides present on the HA and the NA glycoproteins of influenza viruses [7, 8, 12]. As such, the degree or pattern of glycosylation is an important factor in determining sensitivity of a particular virus strain to inhibition by these collectins. In contrast, SP-A inhibits influenza viruses in a Ca^{2+} -independent manner by binding of viral HA to sialic acid expressed on the carbohydrate moiety of the SP-A molecule, thereby blocking the ability of HA to interact with cellular receptors [12, 13]. Thus, SP-A functions as a γ inhibitor, acting against virus in a manner similar to the serum inhibitor α 2-macroglobulin. More recent studies have formally addressed the *in vivo* roles for SP-A and SP-D in innate defence against influenza virus through the use of knockout mice. Following intranasal infection, enhancement of viral replication and inflammation was observed in the lungs of SP-D^{-/-} mice compared to wild-type controls [14]. SP-A-deficient mice also displayed enhanced susceptibility to influenza infection, however the effect was less pronounced than that observed in SP-D^{-/-} mice [15]. Furthermore, in the mouse model SP-D levels in lavage fluids increased markedly following infection of mice with influenza viruses, consistent with a role for this molecule in early host defence [16].

Pentraxins - a possible role in innate host defence against influenza? Like the collectins, pentraxins are a group of proteins highly conserved during evolution and characterized by multimeric, usually pentameric structures (reviewed by [17]). The classic short pentraxins, C-reactive protein (CRP) and serum amyloid P component (SAP), are produced by the liver in response to inflammatory signals, and in particular to interleukin (IL)-6. PTX3, the prototype of the long pentraxins, shares similarities with short pentraxins but differs in the presence of an unrelated N-terminal domain as well as in gene organization, cellular source and ligands recognized. PTX3 is produced by macrophages, dendritic cells and other cell types and plays a complex, non-redundant role *in vivo*, recognizing a diverse range of pathogens, modulating complement activity by binding C1q and facilitating pathogen recognition by macrophages and DCs [18]. Despite its well-documented role in innate defence against certain bacteria and fungi [19], few studies have addressed the antiviral activities of PTX3 although human PTX3 was recently shown to bind and inhibit the infectivity of human and murine cytomegaloviruses [20]. Furthermore, the short pentraxin SAP has been shown

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to act as a β inhibitor against influenza viruses, binding in a Ca^{2+} -dependent manner to glycans on the viral HA to inhibit both haemagglutination and viral infectivity [21, 22]. The antiviral activity of PTX3 against influenza viruses has yet to be determined.

The pentraxins PTX3, SAP and CRP differ in their ability to bind influenza virus. Pentraxins are a family of pattern recognition receptors that may play a role in innate host defence during influenza infections. To gain insight into their mechanisms of action, we compared the ability of human pentraxins PTX3, SAP and CRP to inhibit the haemagglutinating activity of influenza virus strain X-31. As seen in Figure 1, SAP inhibited the ability of X-31 to agglutinate erythrocytes in a Ca^{2+} -dependent manner while the antiviral activity of PTX3 was unaffected in the presence or absence of Ca^{2+} . In contrast, CRP was unable to inhibit the agglutinating activity of X-31, even at concentrations of $>10 \mu\text{g/ml}$. Together, these data suggest that the pentraxins PTX3, SAP and CRP differ in the manner in which they bind to influenza virus strain X-31.

Figure 1. Inhibition of virus-induced agglutination by pentraxins. Dilutions of the human pentraxins PTX3, SAP, or CRP were prepared in tris-buffered saline (TBS) CONTAINING 10 mM CaCl_2 or 5 mM EDTA. Results show concentrations of each inhibitor required to inhibit haemagglutinating by 4 haemagglutinating units (HAU) if influenza virus strain X-31. Haemagglutination = ●. Inhibition of haemagglutination = ○.

10	5	2.5	1.2	0.6	0.3	Pentraxin ($\mu\text{g/ml}$)
○	○	○	○	○	○	PTX3 + 10 mM Ca^{2+}
○	○	○	○	○	○	PTX3 + 5 mM EDTA
○	○	○	○	○	○	SAP + 10 mM Ca^{2+}
○	○	○	○	○	○	SAP + 5 mM EDTA
●	●	●	●	●	●	CRP + 10 mM Ca^{2+}
●	●	●	●	●	●	CRP + 5 mM EDTA

Conclusions and Future Directions

Naturally occurring inhibitors of influenza virus have been known to exist in serum of different species (including humans) for many years. In addition, respiratory secretions contain a complex mixture of innate immune proteins with the capacity to act against influenza viruses. The ability of collectins to act as innate inhibitors of influenza virus has been well described, however additional proteins, including members of the pentraxin family, may also play an important role in antiviral host defence. In this study, we demonstrate that pentraxins PTX3 and SAP mediate antiviral activity against influenza viruses. Furthermore, while both PTX3 and SAP act as innate inhibitors of influenza virus they appear to do so by different mechanisms. Current studies are aimed at (i) characterizing the antiviral activity of pentraxins against a range of influenza viruses *in vitro*, (ii) selecting pentraxin-resistant mutants to determine the binding site of each pentraxin on different influenza virus strains, and (iii) assessing the therapeutic

potential of pentraxins following intranasal infection of mice with pentraxin sensitive and resistant virus strains.

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Influenza Infection and Vaccination With Live Attenuated Influenza Vaccine in the Experimental Model of Allergic Bronchial Asthma

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Introduction

Allergic bronchial asthma is a chronic inflammatory lung disease characterized by allergen-specific activation of Th2-cells secreting IL-4, IL-5, and IL-13 associated with airway hyperresponsiveness against inhaled allergens [1]. Secretion of IL-5 and IL-13 result in infiltration of the lung tissues with inflammatory cells, mainly eosinophils, and IL-4 is a most important factor to switch B-cells into production of allergen-specific IgE [2, 3]. Influenza virus continues to be an important contributor to exacerbations of asthma and elevates morbidity and mortality in all age categories of asthmatic subjects during epidemics [4, 5]. Prevention of influenza infection in asthmatics is based on the safety and effectiveness of current influenza vaccines. It has been shown that inactivated influenza vaccine is safe in this high-risk population [6, 7], but the safety of live attenuated reassortant influenza vaccine (LAIV) is still being discussed. The purpose of this study was to compare the allerge-immunological consequences of influenza infection and immunization with LAIV in murine model of allergic bronchial asthma. Experimental protocols were designed to investigate two critical issues of infection and vaccination on the asthma; 1) How virus itself influences allergic inflammatory responses when infection or vaccination is performed at different stages of allergic bronchial asthma and 2) how infection or vaccination at the asthma interferes in the subsequent exposure to allergen.

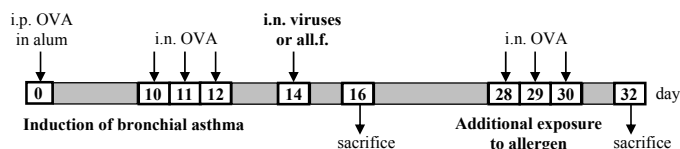
The model of ovalbumine (OVA)-induced bronchial asthma. Five to 7 week old CBA mice were used for experiments. We modified the model of OVA-induced bronchial asthma described by Tsitoura D.C. et al. [8]. OVA-sensitization was performed by intraperitoneal (i.p.) injection of 100 µg of OVA precipitated on 2 mg of aluminum hydroxide on day 0. Ten days later mice were exposed three times to intranasal (i.n.) 50 µl of PBS containing 50 µg of OVA one day apart (Figure 1). To confirm the development of allergic bronchial asthma the total and OVA-specific serum IgE levels in ELISA and airway inflammatory changes in histological analysis were measured. Lung sections were performed and stained with hematoxylin and eosin using standard histological protocols.

Two phases of OVA-induced bronchial asthma. Two pathogenetic phases of allergic respiratory tract inflammation were defined: the acute asthma phase (48 hours after the last administration of i.n. OVA) and the asthma remission phase (10 days after the last administration of i.n. OVA). These phases were characterized by increased levels of total serum IgE (28.27±5.13 and 27.55±4.07

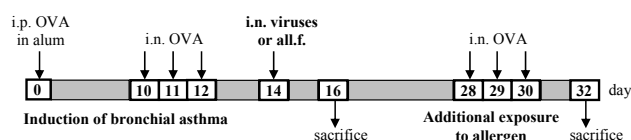
µg/ml at the acute asthma and remission respectively) and OVA-specific serum IgE (241.08±20.18 and 165.60±10.12 ng/ml at the acute asthma and asthma remission respectively). Moreover different inflammatory changes were observed in lungs of OVA-sensitized mice at the acute and remission phases of asthma. 48 hours after the last administration of i.n. OVA the emphysema and marked inflammation were present in the lungs, with dense peribronchiolar and perivascular infiltrates composed of eosinophils and neutrophils. However 10 days after the last administration of i.n. OVA areas of emphysema were decreased and minimal peribronchiolar and perivascular infiltrates consisting predominantly of mononuclear cells were observed. All the immunological and histological features of allergic bronchial asthma were absent in serum and lungs of non-sensitized mice treated with i.p. and i.n. PBS instead of OVA (15.17±2.09 µg/ml and <2.0 ng/ml of total and OVA-specific serum IgE respectively and normal lung parenchyma structure without any inflammation).

Influenza infection and vaccination. Mice were immunized i.n. with 50 µl of PBS containing: (i) 4 log EID₅₀/0.1 ml of pathogenic influenza virus A/PR/8/34 H1N1 – the infection group; (ii) 6 log EID₅₀/0.1 ml of mouse-adapted attenuated reassortant (A/Leningrad/134/47/57 x A/PR/8/34) H1N1 virus which is an experimental analog of LAIV strain – the vaccination group; (iii) allantoic fluid (all.f.) diluted 1:100 in PBS – the control group. Experimental protocols. Two experimental protocols were designed: immunization with viruses at the acute asthma phase and at the remission phase (Figure 1). Mice with OVA-induced bronchial asthma 48 hours or 10 days after the last administration of i.n. OVA were exposed to pathogenic or attenuated virus. 48 hours later some animals were bled and sacrificed. OVA-specific serum IgE and inflammatory changes in lungs were measured to consider how virus itself influences the allergic inflammatory responses when infection or vaccination is performed at the acute asthma phase or the asthma remission phase. Then residual mice were exposed three times to i.n. 50 µg of OVA again. Additional exposure to OVA was performed 14 days after viral immunization when viruses were not detectable in the lungs. 48 hours later animals were bled and sacrificed. OVA-specific serum IgE, OVA-specific production of IL-4 by spleen and lung lymphocytes and inflammatory changes in lungs were measured to consider how infection or vaccination at the asthma phase interferes in the subsequent exposure to allergen.

Figure 1. Experimental protocols. On day 0 CBA mice were sensitized to OVA by i.p. injection of OVA precipitated on alum adjuvant. To obtain allergic bronchial asthma 10 days later mice were exposed three times to i.n. resolving doses of OVA. 48 hours (1. – at the acute asthma) or 10 days (2. – at the remission of asthma) after the last administration of i.n. OVA mice were immunized with pathogenic or attenuated reassortant influenza virus or were sham inoculated with all.f. Fourteen days later mice were additionally exposed three times to i.n. OVA. For the evaluation of virus- and OVA-specific immune responses mice were sacrificed 48 hours after virus inoculation and additional exposure to OVA.



1. Immunization with viruses at the acute asthma phase.



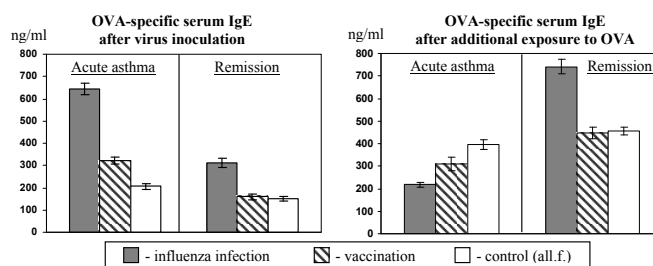
2. Immunization with viruses at the asthma remission phase.

Virus-specific immune responses. The virus-specific serum IgG were measured in OVA-sensitized mice immunized with viruses and then additionally exposed to OVA (i.e. 17 days after virus inoculation – 32 or 40 day of experiment at the acute asthma or at asthma remission respectively). In same animals the spleens were removed to assess the virus-specific proliferation of spleen lymphocytes after in vitro stimulation with heat-inactivated homologous influenza virus. Immunization with pathogenic or attenuated reassortant viruses both at the acute asthma and remission phases resulted in significant increase of virus-specific serum IgG levels and vigorous proliferation of spleen lymphocytes in comparison to control mice treated with i.n. all.f. instead of viruses (data not shown). These data indicate that vaccination at the asthma phase with attenuated reassortant virus (similarly influenza infection) is capable of inducing effective antibody and cell-mediated virus-specific immune responses, similar to pathogenic influenza infection.

Allergen-specific immune responses and lung histology after virus inoculation. Mice with OVA-induced allergic bronchial asthma were immunized with pathogenic or attenuated reassortant influenza virus at the acute asthma phase (48 hours after the last administration of i.n. OVA) or at the asthma remission phase (10 days after the last administration of i.n. OVA). To investigate how virus phase influences allergic inflammatory responses when infection or vaccination is performed at different stages of allergic bronchial asthma the allergen (OVA)-specific immune responses and lung histology of these mice were evaluated. Influenza infection resulted in significantly increased levels of OVA-specific IgE when pathogenic virus was administered both at the acute phase (of asthma) and at the remission phase (Figure 2). Significant airway inflammation with dense peribronchiolar and perivascular eosinophilic infiltration and

large areas of emphysema were observed in lungs of these mice. Attenuated reassortant virus administered at the acute asthma phase also elevated the production of IgE but to a considerably smaller extent than the pathogenic virus (Figure 2). However, in contrast to influenza infection, vaccination during asthma remission did not affect the allergen-specific IgE-responses. Moreover the marked eosinophilic and neutrophilic inflammation was present only in lungs of mice vaccinated at the acute phase of asthma. The extent of airway inflammation of mice immunized with attenuated reassortant at the remission phase did not markedly differ from the control mice treated with all.

Figure 2. Allergen-specific immune responses after virus inoculation and after additional exposure to allergen. Mice with OVA-induced bronchial asthma were immunized with pathogenic or attenuated reassortant influenza virus or sham inoculated with all.f. at the acute asthma (48 hours after induction of asthma) or at the remission (10 days after induction of asthma). 14 days after virus inoculation when viruses were not detectable in the lungs mice were additionally exposed three times to i.n. allergen (OVA).



Allergen-specific immune responses and lung histology after additional exposure to allergen (OVA). Mice with OVA-induced allergic bronchial asthma were immunized with pathogenic or attenuated reassortant influenza virus at the acute asthma or asthma remission phase and then were additionally exposed to i.n. allergen (OVA). To investigate how infection or vaccination in the asthmatic interferes in the subsequent exposure to allergen, the allergen-(OVA)-specific immune responses and lung histology of these mice were evaluated. Additional exposure to OVA led to unexpected decrease of OVA-specific IgE levels in mice exposed to pathogenic influenza virus at the acute asthma phase and significant increase of IgE-responses in mice infected during asthma remission (Figure 2). These findings however agree with OVA-specific production of IL-4 by spleen lymphocytes (data not shown). Furthermore considerably increased secretion of IL-4 by lung lymphocytes of mice exposed to pathogenic influenza virus both during the acute and remission asthma phases coincides with substantial inflammatory changes observed in the lungs of these mice. These data indicate that the additional exposure to allergen after influenza infection may result in the recurrent exacerbation of bronchial asthma independently of a stage of this disease. In contrast to infected animals, mice vaccinated with attenuated reassortant virus and subsequently exposed

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to OVA had remarkably little evidence of allergen-specific IgE-responses (Figure 2) and secretion of IL-4 (data not shown). All these immunological parameters of allergic asthma were equivalent to the same characteristics observed in the control group. Histological analysis illustrated the minimal inflammatory infiltration in lungs of mice vaccinated both at the acute asthma and remission and subsequently exposed to OVA. Most part of airways practically represented the absence of allergic inflammation and normal parenchyma structure similar to those observed in lungs of control mice treated with all.f. instead of viruses.

Conclusion

Influenza infection provokes asthma exacerbation both at the acute phase and remission of allergic airway inflammation. In contrast to infection, vaccination of mice with attenuated reassortant influenza virus at the remission of asthma does not enhance the allergen-specific immune responses and inflammatory changes in lungs. Thus our results indicate that it is possible to find the optimal approach to administration of LAIV to subjects with respiratory allergy.

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Analysis of the Cellular and Humoral Immune Response in Mice Following Intranasal Administration of Cold-Adapted Live Attenuated Influenza A Virus Reassortants

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Introduction

Influenza viruses are frequent causes of medically attended respiratory disease due to their ability to evade the host immune defenses. Vaccination of adults and children is the most efficient control measure for the prevention of transmission. Cold-adapted (*ca*) live attenuated influenza vaccines (LAIVs) have been introduced as alternatives to existing inactivated vaccines and have been shown to be both safe and highly efficacious [1-3]. *Ca* LAIVs should be suitably attenuated, non-transmissible, genetically stable, possess suitable *ca* and *ts* markers and induce local, systemic humoral and CMI immune responses. The ease of administration (intranasal) safety and high efficacy make this vaccine suitable for the prevention of influenza virus infections in all age groups [4, 5]. The influenza A components of FDA-approved *ca* LAIVs have common internal NP, M1, P and NS genes derived from the donor strain *A/Ann Arbor/6/60-ca* and HA and NA surface genes derived from current wild-type epidemic strains. Previous studies in outbred mice have shown that reassortants of *A/Ann Arbor/6/60-ca* are less immunogenic than *wt* parental strains with the same surface antigens gene, and the HA protein is a major determinant of immunogenicity for all reassortants. What is not known is the role of the HA and NA viral surface antigens in the induction of CMI by the NP, M1, P, and NS non-surface antigens on innate and adaptive immune responses to *ca* LAIVs. In the present study, the relative immunogenicity of *ca* LAIV reassortants with the same internal proteins, but different surface proteins, was investigated in two different inbred mouse strains, BALB/c (H-2d) and C57BL/6 (H-2b). CMI responses following vaccination were assessed by quantitative of lung virus-specific-CTLs and -ASCs using tetramer staining and ELISPOT assays. The results obtained were compared in parallel with serum antibody responses.

Materials and Methods

Influenza viruses. Two reassortants, *A/QLD/6/72-ca* (H3N2) and *A/HKCHECK-ca* (H1N1), and their donor strain *A/AA/6/60-ca* (H2N2) were used in the study. All viruses were grown in 10-day-old embryonated eggs for 2 days at 34°C. Stock viruses had been titrated for HA and infectivity (plaque titre). Their cold-adapted (*ca*) and temperature sensitive (*ts*) phenotypes of all viruses and their gene compositions were confirmed. Influenza

viruses were purified using density gradients centrifugation (30-60% sucrose) at 36,000 rpm in a SW41 rotor for 90 min. Following subsequent ultra-centrifugation, purified viruses were used to coat plates for enumeration of influenza-specific antibody secreting B cells (ASCs) using an ELISPOT assay. *Tetramer*. The tetramer containing peptide from *A/PR/8/34* NP₃₆₆₋₃₇₄ was used for the identification of influenza *A/AA/6/60 ca*-specific cytotoxic T lymphocytes (CTLs) by flow cytometry. *Mice*. Groups of 3 ~ 6 C57BL/6 (H-2D^b) and BALB/c (H-2D^d) mice were inoculated intranasally with 10^{5.3} TCID₅₀ of each virus reassortants.

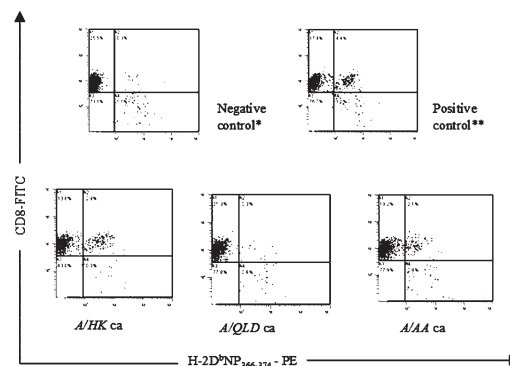
Lung virus titres. Three days after primary inoculation, lungs were dissected and homogenized in 2 ml of PBS. Viral titres in the lungs of infected mice were determined following virus isolation and plaque assay on MDCK cells.

Immune Assays. Seven days after the first and second inoculation, mice were sacrificed. Lungs and spleens were removed and single cell suspensions prepared for ELISPOT assay and flow cytometry assay. Serum anti-influenza antibody levels were measured by haemagglutination inhibition (HAI) assays. Geometric mean titres (GMT) for each group were calculated.

Results

MHC class I tetramer H-2Db containing *A/PR/8/34* nucleoprotein (NP₃₆₆₋₃₇₄) peptide stained *A/AA/6/60* specific CTLs. In addition, the strongest CMI responses were observed in the lung where infections occur. All three *ca* LAIV viruses elicited a significantly greater local CMI response in C57BL/6 (H-2D^b) mice than in BALB/c (H-2D^d) mice 7 days after the second i.n. inoculation. By contrast, C57BL/6 mice showed similar or much weaker humoral immune responses than BALB/c mice. The level of serum antibody and local virus-specific CMI responses showed no direct correlation. In the case of *A/HK-ca*, grew to low titre *in vivo* compared with the other reassortants; it induced the strongest CMI responses but the lowest humoral immune responses in C57BL/6 mice.

Figure 1. Lung virus specific-CTL responses (7 days after second intranasal inoculation).



** Positive control group: 1st dose *A/HKx31* (H3N2), possessing the same internal genes as *A/PR8*. 2nd dose *A/PR8* (H1N1) 3-weeks later.

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Table 1. Immune responses to *ca* LAIVs in C57BL/6 mice.

Viruses		Primary inoculation		Secondary inoculation		
Name	surface antigens	Viral titre pfu/ml	Serum HAI GMT	CTL % in lung	IgA ASCs per 10 ⁶ lung lymphocytes	Serum HAI GMT
A/HK- <i>ca</i>	H1N1	8	<10	2.58	336	10
A/QLD- <i>ca</i>	H3N2	203	<10	0.00	329	80
A/AA- <i>ca</i>	H2N2	97,133	40	1.75	385	80

Discussion

From this study, it is concluded that levels of antibody can influence both T_h and T_c cell function in the development of both innate and adaptive immune responses. Similar findings from large clinical trial by Belshe *et al* [8], indicated that a serum antibody response to influenza A/H3N2 occurred in 92% of initially seronegative children after the first dose of vaccine, compared with only 18% of initially seropositive children. Thus, the humoral immune responses may influence CMI responses might through changes to the efficiency of formation of the antigen-MHC complexes. In addition, different strains of mice showed different immune responses to the same virus. We have demonstrated, for the first time, that the balance between the humoral and CMI, local and systemic immune responses is a key factor for the evaluation *ca* LAIVs. In addition to serum antibody levels, the roles of T and B effector cells in animal models could be assessed to evaluate the efficacy of live vaccines. To further explore regulation between the humoral and CMI responses to *ca* LAIVs *in vivo*, cytokines secreted by T_c and T_h cells, such as IL-2, IL-4, IL-6, IL-12 and INF- γ , should be tested. These studies will help better understanding of the immune response to *ca* LAIVs and the role of the internal/external viral proteins in humoral/CMI responses. Together, information from these types of study will help in the design of better candidate live vaccines.

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Proceedings Topic #11

Mathematical Modeling

**Poster
Presentations**

Aerosol Generation Model for Cough Simulations

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The airborne transmission of disease is of great concern to the public health community because of the pandemic potential of newly emerging diseases like avian influenza. The possible spread of infectious disease by aerosols is of particular concern among health-care workers and emergency responders, who face a much greater risk of exposure to these hazards than does the general public. Influenza is believed to spread by dissemination and inhalation of aerosols of relatively small droplets that are produced by coughing and remain airborne for an extended time but the actual mechanisms of transmission are not well understood. For that reason a better understanding of the processes which lead to generation of aerosols is important. The goal of this study is to investigate the air-flow dynamics and the aerosol generation during coughing. A fairly simple model is developed for simulation of the flow inside the upper respiratory tract, focusing on the larynx and its vicinity, and to predict the number and size distribution of the aerosols generated during coughing. The aerosol generation and entrainment model (AGEM) is composed of droplet entrainment, generation and the break up models. The flow model solves for the velocity shear stress and pressure distribution, as well as the turbulent kinetic energy. These, in turn, are used as input parameters in AGEM to calculate the aerosol formations during a cough. The size distribution of the aerosol droplets after coughing is calculated and compared with the experimental results. The model is shown to be capable of calculating the size distribution of aerosols consistent with the experimental findings.

Theoretical Formulations. In the current pseudo two-dimensional study, a simplifying assumption has been made for the three-dimensional flow within the larynx to calculate the average velocity. The flow is governed by the one-dimensional continuity and momentum equations. For the numerical solution of the momentum equation the pressure-correction method is employed as proposed by MacCormack [1] and implemented by Tatli [2] and Celik et al. [3] and Ersahin [4]. *Droplet Entrainment Model:* The role of droplet entrainment model (DEM) is to calculate the total amount of mucus to be entrained into the system. The wall of the respiratory tract is assumed to be partially covered with mucus at a certain thickness depending on age, gender and health condition of the person. The thickness of the mucus and the fraction of the area covered with mucus are two of the unknowns at this stage of the study and need to be obtained from experiments. The Laursen formula for sediment discharge, which is used to calculate the total discharge in sediment transportation

[5, 6], is modified to calculate the entrainment during the cough. The entrainment rate is taken to be proportional to diameter of the respiratory tract (D), turbulent kinetic energy (k), which represents the entrainment velocity, the wall shear stress (τ_w) and the critical shear stress (τ_{cr}). Rearranging these variables and introducing two model constants (C_ε and n) and a coefficient (ε) which defines the fraction of area covered with mucus to the inner surface area of the upper respiratory tract, the entrainment rate can be expressed as;

$$\frac{dE}{dt} = C_\varepsilon \varepsilon A \frac{D^2}{2} \sqrt{k_{\max}} \left(\frac{\tau_w}{\tau_{cr}} - 1 \right)^n \quad (1)$$

Selection of the model coefficients and the unknown physical properties, such as, the fraction of the area covered with mucus, is done in a way that the total amount of the entrainment matches the total amount of the aerosol generated during a cough measured experimentally. This is done only for one case; thereafter these values are retained.

Droplet Generation Model. Droplet generation model (DGM) takes the turbulence into consideration and determines the sizes of the drops formed. The maximum diameter of the droplets formed is a function of surface tension and the thickness of the mucus, the dissipation rate of the turbulent kinetic energy and turbulent length scale as given in [7];

$$D_{\max} = \min \left\{ C_B \left(\frac{\sigma}{\delta} \right)^{\frac{3}{5}} \varepsilon^{-\frac{2}{5}}, \ell_{tur} \right\} \quad (2)$$

If the total amount of mucus entrained, E , and the maximum drop size, D_{\max} are known, then the total number of droplets, n , can be calculated from the following relation.

$$n = \left[\frac{E}{\frac{4}{3} \pi \left(\frac{D_{\max}}{2} \right)^3} \right] \quad (3)$$

Droplet Breakup Model. Although the droplet breakup can occur in different ways, it is common to assume a binary droplet breakup process and has been shown that it agrees well with the experimental measurements [8, 9]. During a binary breakup process the mother droplet divides into two daughter droplets. The sizes of the daughter droplets are less likely to be the same size. Therefore, the size of one of the daughter droplet is first calculated and the complementary daughter size is then obtained in such a way that the total mass is conserved. The sizes of the daughter droplets cannot be larger than the maximum drop size and smaller than the minimum drop size. Minimum drop size is a function of physical properties of the fluid making the droplet and the flow parameters and it is calculated from;

$$D_{\min} = \left(\frac{12\sigma}{\beta\rho D} \right)^{\frac{3}{2}} \varepsilon^{-1} \quad (4)$$

where ε is the turbulent dissipation rate of the air flow, σ and ρ are surface tension and density of the mucus fluid, respectively, D is the diameter of the mother droplet, and β is an empirical coefficient which is obtained experimentally to be 8.2 [10]. A random function is utilized in order to estimate the diameter of the first droplet. A pseudo-random number is generated within the range of $[D_{\min} - D_{\text{mother}}]$ and this number is assigned as the diameter of the first daughter droplet. Then the complementary daughter drop size is calculated.

Droplet Breakup Frequency. Droplet breakup frequency depends on a characteristic length and a characteristic velocity. Among other factors, the droplet diameter is selected as the characteristic length and the relative velocity is written as a function of the dissipation rate and the droplet diameter by following Kolmogorov's universal theory [11], which leads to the frequency time scale equation given by;

$$f_{\text{breakup}} = K_g \sqrt{\beta (\varepsilon d_0)^{\frac{2}{3}} - 12 \frac{\sigma}{\rho d_0}} / d_0 \quad (5)$$

where K_g is found to be 0.25, experimentally, and $\beta = 8.2$ [12]. The value of d_0 is equal to the diameter of the mother droplet.

Turbulent Kinetic Energy Equation. Integration of the aerosol generation and the entrainment model into the one-dimensional flow field requires information about dissipation rate and turbulent kinetic energy from the main flow solver. This information is passed to the model through the solution of a one-dimensional integral turbulent kinetic energy equation. The solution of turbulent kinetic energy equation and passing the results to the model calculations is the most important interaction between the one-dimensional flow solver and the droplet models. The derivation of the integral turbulent kinetic energy equation can be found elsewhere [13, 14]. Here, the final form of the integral turbulent kinetic energy equation is given;

$$\frac{\partial}{\partial t} (\rho A \Delta x k) = \left(\rho u A k - \Gamma_k \rho A \frac{\partial k}{\partial x} \right)_w - \left(\rho u A k - \Gamma_k \rho A \frac{\partial k}{\partial x} \right)_e + G - D \quad (6)$$

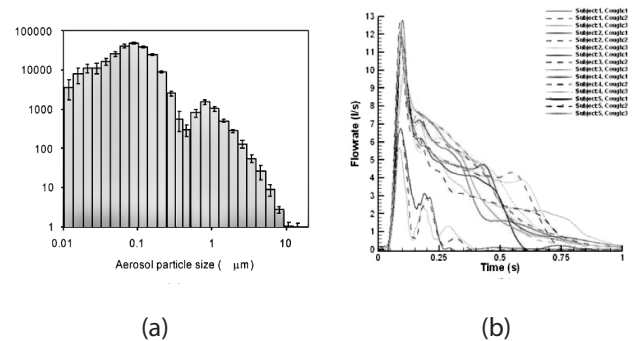
where k represents the turbulent kinetic energy, ρ and u are density and average flow velocity, respectively, A is the cross-sectional area normal to the flow direction and Γ_k is the effective diffusivity. G and D denote turbulent kinetic energy generation and dissipation, respectively. Equations 1-6 constitute the mathematical model that is used to predict size and distribution produced from coughing.

Results and Discussion

The model constants in the turbulent kinetic energy equation are tuned by comparison with a multi-dimensional,

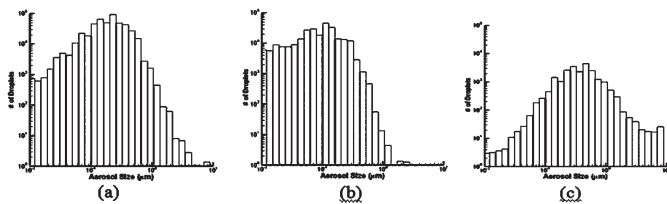
computational fluid dynamics model, namely FLUENT [15]. The tuning of the model coefficients in the aerosol generation and entrainment model are done in comparison to one set of experimental data available in hand [15]. The experimental data and the aerosol size distribution obtained by the proposed model is given in Figures 1 and 2a, respectively. It can be seen from the figures that, the model can produce similar results to the experimental measurements. The aerosol size distribution obtained by the model given in Figure 2a is the average of twelve different aerosol distributions for twelve different cough signals. In order to see the effects of different cough signals, aerosol size distribution for different cough signals are calculated. Since these cough signals were approximately equal to each other, it was expected to obtain similar results for each cough signal with some discrepancies. The random function, used in the binary droplet breakup mechanism, also has an effect on this similarity but not on the exact size distribution pattern. It is noted that, for the same coughing signal it is possible to obtain slightly different aerosol distribution every time the simulation is done. This reflects in a way the random nature of aerosol generation process. The effect of the physical properties of the mucus is also investigated and it is found that, the effect of the surface tension is the largest among the other physical properties. The density, viscosity and surface tension has been varied in the simulations and it has been noted that the effect of density and viscosity is much less than that of surface tension. In Figure 2, the aerosol size distribution is given for three different values of the surface tension. When the surface tension decreases, the number of aerosols increases since it is easier for them to break, and the size distribution shifts to left, with smaller aerosol size; when the surface tension increases, the opposite effect is observed. For more detailed study see [15].

Figure 1. a) Measured averaged aerosol size distribution during cough (The vertical axis is the concentration of aerosol); b) Coughing data obtained from NIOSH.



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Figure 2. (a) Averaged aerosol size distribution during a cough; Effect of surface tension on the distribution (b) 0.1σ ; and (c) 10σ .



Conclusion

In this study a numerical model was developed in order to predict the aerosol size distribution during a cough. The model consists of sub-models, which make up the complete aerosol generation model that predicts the aerosol size distribution during a cough. This model is integrated into a one-dimensional flow solver, which simulates the flow in the upper respiratory tract and provides necessary information for the aerosol generation model through the solution of turbulent kinetic energy. Different coughing conditions have been simulated and an averaged aerosol size distribution was obtained. It was shown that the predicted aerosol size distribution is in good agreement with the experimental measurements. After tuning the model coefficients and predicting aerosol size distributions, which agrees with the experimental measurements, a parametrical study was carried out. The parametrical study has shown that the surface tension of the mucus affects the aerosol size distribution. As the surface tension increases, the size distribution shifts to the left producing larger aerosols. The present model shows that it is capable of predicting the aerosol size distribution during coughing, and it may be used in further studies to predict the physical properties of the mucus lining in the upper respiratory tract, and can be included as a source term in predicting the aerosol dispersions in confined areas.

Acknowledgements

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Scenarios of Diffusion and Control of an Influenza Pandemic in Italy

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To investigate the spread of a pandemic strain of influenza virus through Italy and the impact of control measures, we introduce a SEIR deterministic model, with a stochastic simulation component. We modelled the impact of control measures as vaccination, antiviral prophylaxis and social distancing measures. In absence of control measures, the epidemic will be reached approximately 4 months after the importation of first cases, and the epidemic will be over in 7 months. The considered interventions, when independently implemented, reduce the cumulative attack rate to approximately 10%, at their best. In accordance with international findings, our results highlight the need of respond to pandemic with various preventive measures.

Introduction

Following the emergence in 1997 of a new strain of avian influenza, A(H5N1), capable of infecting humans [1] and its spread to Europe in 2005 [2], concerns were raised over the occurrence of a pandemic caused by this or a closely related strain [3;4]. Consequently, countries have been urged to strengthen their preparedness for an influenza pandemic [5], an important aspect of which is predicting the spread of infection. According to the predictive models used to date [6-12], influenza would spread worldwide over a period of 2 to 6 months, depending on the basic reproductive number (R_0), and reducing transmission would entail combining control measures, specifically, reducing contacts and performing both antiviral prophylaxis (AVP) and vaccination [7-9;11;13]. We developed an SEIR (susceptible – exposed, but not yet infectious - infectious - recovered, and no longer susceptible) deterministic model with a stochastic simulation component to predict the spread of pandemic influenza in Italy and to evaluate the impact of vaccination, AVP and social distancing measures.

Material and Methods

We developed an SEIR model in which the population is structured according to age and region of residence. National demographic data were obtained from the 2001 Census [14]. In the model, the national population (56,995,744 inhabitants) was divided by age class and was distributed in Italy's 20 regions. The contact matrices were defined

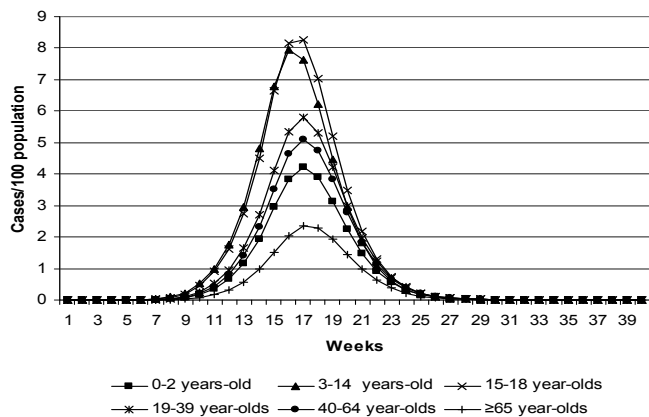
considering separately household, school/work-place and random contacts, and using data on households composition, school attendance, employment status. We also introduced a stochastic component that takes into account all of the random effects that exceed the deterministic approximation during the pandemic's initial and final stages, when the number of infected individuals would be low. In each simulation, the pandemic began with the introduction of 5 infected adults in the Lazio Region, where Rome's intercontinental airport is located. Based on published studies [15], we fixed the R_0 at 1.8, which corresponded to a cumulative infected attack rate (AR) of 35%. Based on the literature [6;8;16], in the model we assumed an incubation period of 1 day and an infectious period of 3.9 days. The results were obtained by averaging over 200 simulations for each scenario. We considered both single and combined control measures, most of which are included in the National Preparedness Plan [17]. We assumed that two doses of vaccine would be administered, one month apart. The target population was divided into 4 categories: i) personnel providing essential services (15% of the 25-60-year-old working population) [14]; ii) elderly persons (≥ 65 years); iii) children and adolescents aged 2-18 years; and iv) adults aged 40-64 years. We assumed a vaccination coverage of 60% of the target population [18]. Vaccine Effectiveness (VE) was assumed to be 70%, beginning 15 days after the second dose. We considered different scenarios of vaccine availability, assuming that adequate VE be reached at 4 and 5 months after the first world case. An adequate VE at 4 months would be feasible only if the first dose contained an avian virus precursor of the pandemic strain [3], followed by a dose of pandemic vaccine; the actual VE of this regimen was assumed to be equal to that of two doses of the pandemic vaccine. The AVP for uninfected individuals was assumed to reduce susceptibility by 30% and infectiousness by 70% [8]. We assumed that AVP be provided to all household contacts of 80% of clinical cases, limiting the use of AVP to the first 2, 4 or 8 weeks of the pandemic. We considered the nationwide closing of all schools, public offices, and public gathering places (e.g., cinemas, theatres, and churches). We simulated school closure for 3 weeks, public-office closure for 4 weeks, and public-gathering-place closure for 8 weeks. We assumed that these measures would be introduced simultaneously at different times (i.e., 2, 4 or 8 weeks after the epidemic started).

Results

In the absence of control measures, the epidemic peak would be reached approximately 4 months after identification of the first case, with a total of 3 million cases during the peak week. The epidemic would be over in 7 months, with a cumulative AR of 35% (approximately 20 million cases). The dynamics of the epidemic were similar in all age-groups, whereas the cumulative AR varied markedly by age-group. The incidence would be particularly high among 15-18 year-olds, with a cumulative AR of 54% (Figure 1).

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Figure 1. Weekly attack rate by age group, with no control measures.



The impact of single control measures is shown in Table 1. Vaccination seems to be the most effective measure, especially when VE is reached at 4 months. Vaccinating three of the four target categories (i.e., personnel providing essential services; elderly persons; 2-18 year-olds) would reduce the cumulative AR from 35% to 25%. Vaccinating also the fourth target category (i.e., 40-64-year-olds) would not induce a further relevant reduction in the cumulative AR (Table 1).

Table 1. Effectiveness of single and combined control measures on the dynamics of a pandemic with an $R_0=1.8$ and with an AR of 35%.

Interventions	Attack rate (%)	Avoided cases	Treated individuals With vaccine	With antiviral
Single containment strategies				
<i>Protective Vaccine Efficacy at 4 months</i>				
Category I and II	28.9	3,323,574	12,076,619	-
Category I, II, III	25.3	5,386,482	17,279,633	-
Category I, II, III, IV	24.4	5,902,209	25,814,908	-
<i>Antiviral</i>				
2 weeks	34.7	0	-	50
4 weeks	34.7	0	-	355
8 weeks	34.7	0	-	12,389
<i>Social distancing measures</i>				
From 2 weeks	34.7	0	-	-
From 4 weeks	34.7	0	-	-
From 8 weeks	34.1	343,818	-	-
Combined containment strategies with protective vaccine efficacy at 4 months				
Social distancing measures from 2 weeks and antiviral for 2 weeks	12.6	12,663,963	25,837,928	55
Social distancing measures from 2 weeks and antiviral for 4 weeks	11.9	13,065,084	25,837,928	182
Social distancing measures from 2 weeks and antiviral for 8 weeks	10.9	13,638,114	25,837,928	717
Social distancing measures from 4 weeks and antiviral for 2 weeks	12.0	13,007,781	25,837,928	51
Social distancing measures from 4 weeks and antiviral for 4 weeks	11.5	13,294,296	25,837,928	373
Social distancing measures from 4 weeks and antiviral for 8 weeks	10.1	14,096,538	25,837,928	1,690

If protective VE were reached at 5 months (2 doses of pandemic vaccine), vaccinating all four categories, the cumulative AR would be 32.5%. Social distancing measures and AVP were not effective in reducing the AR. However, AVP for 8 weeks and social distancing measures starting at week 4 or 8 would delay the epidemic peak by one or three weeks, respectively. The combination of control measures would be relevantly

more effective than single measures. The highest reduction (from 35% to 10%) would be obtained by starting social distancing measures at week 4, providing AVP for 8 weeks, and performing vaccination to all categories with VE at 4 months. The cumulative AR would be slightly higher (20%) if VE were reached at 5 months.

Discussion

Our results confirmed the need to combine different control measures [7-9]; in fact, none of the single measures was shown to be effective in containing the pandemic, with the cumulative AR decreasing at most from 35% to 24%. Combining measures would be much more effective, especially if using the pre-pandemic vaccine (reaching VE at 4 months), in which case the cumulative AR would be 10%, which is similar to that observed during severe seasonal epidemics [19]. To the best of our knowledge, most of the SEIR models used to simulate a pandemic do not consider the stochastic factors that can strongly influence the dynamics of the pandemic in its early phases. Another important finding is that the decrease in the AR depended on which target groups were vaccinated. If a pandemic were to occur, vaccine supplies would be limited and the target groups would have to be prioritized (i.e., personnel of essential services, elderly and persons with chronic disease, children and young adults, and healthy adults) [17]. Our results showed that the vaccination of children and young adults would considerably reduce the incidence also in other age groups (i.e., resulting in “herd immunity”), probably because of the important role of children and adolescents in the spread of influenza, as also observed in inter-pandemic periods [20]. Our simulations show that appropriate and prompt measures, when combined, could be effective in containing an influenza pandemic. Implementing such measures, however, would entail organizing a variety of both medical and non-medical resources, and some measures, such as the closing of schools, would also have a social impact.

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Collection Efficiency of a Personal Cyclone Sampler

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Public exposure to viral agents, especially influenza, is of great concern in workplaces such as schools, office buildings and hospitals. The assessment of personal exposure risk factors in such environments requires efficient and accurate sampling methods valid for both short- and long-term, area and/or personal samples. The National Institute of Occupational Safety and Health (NIOSH) recently developed prototypes for single and two-stage personal aerosol samplers that operate on the principles of a cyclone. The innovative design makes use of commercially available microcentrifuge tubes of various designs that can be attached to the sampler. Since the collection of bio-aerosols takes place in these microcentrifuge tubes, the design has the added advantage of zero transfer losses, hence eliminating the sample transfer step. In the current study Computational Fluid Dynamic (CFD) simulations are performed on both the single and two-stage designs to assess the collection efficiency of the cyclone samplers for various particle sizes and flow rates. Initial validation studies were conducted with particle diameters ranging from 0.5 to 16 μm for the single stage sampler and 0.5 to 6 μm for the two-stage sampler. The inlet flow rates of 2 and 4 LPM for the single stage sampler and 2 and 3.5 LPM for the two-stage sampler were adopted in order to match the experimental conditions. Results showed good agreement with the experimental data for both the single stage and two-stage samplers, thus opening the way to use CFD for virtual sampling in field studies.

Introduction

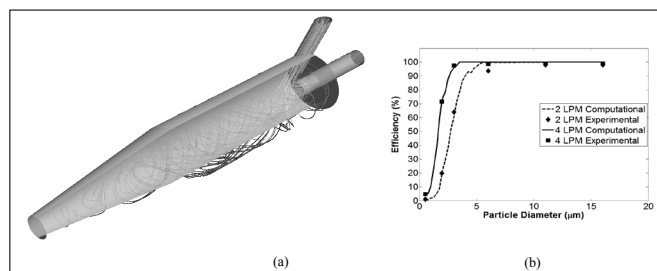
Exposure to air-borne microbiological agents can lead to various health problems including allergies, asthma, and most notably, influenza. Because of these health risks, it is becoming increasingly important to investigate human exposure to such bio aerosol agents. In order to accurately assess the risk of personal exposure a sampling method is required that is capable of collecting both short and long term samples over a large area. Unfortunately, most current methods collect only short-term area samples and are not indicative of personal exposure. Current methods for analyzing collected samples involve culturing, where a collected sample is introduced into a culture medium and the resulting growth is analyzed to determine the number of micro-organisms in the sample. Culturing techniques are both difficult and time consuming. A faster technique involves the analysis of collected samples via modern molecular and immunological techniques which use centrifuge machines. Recently the National Institute for Occupational Safety and Health (NIOSH) developed a prototype personal aerosol sampler (Chen *et al.*, 2004 and

Lindsley *et al.*, 2006) that employs the principles of cyclone to extract biological particles from the air and deposit them into commercially available micro-centrifuge tubes. The cyclone sampler works by taking in air at an angle to the wall of the micro-centrifuge tube thus creating centrifugal force and pushing the denser particles into the walls of the tube which eventually settle at the bottom of the tube. This personal sampler is compact enough to be worn during the course of a full day, allowing assessment of total individual exposure. In addition to allowing for spatially variable samples, the NIOSH design totally eliminates transfer losses during analysis because the particle collection occurs in the micro-centrifuge tubes. A two-stage sampler that allows for collection of size-segregated samples was also recently developed at NIOSH. This design makes it possible to separate different microbiological species based on their individual aerodynamic properties. In the current study we apply Computational Fluid Dynamics (CFD) techniques to characterize the flow through both the single and two-stage cyclone samplers and assess collection efficiencies for a range of particle sizes at different inlet volume flow rates. It is the goal of this study to develop and validate virtual CFD samplers. We also demonstrate the advantages of CFD which allows for fast and inexpensive testing of sampler prototypes at different flow conditions and particle sizes, and most importantly, identify new design parameters for future cyclone samplers.

Methodology

GAMBIT (Fluent Inc. Lebanon, NH) was used to build a grid to geometric specifications for each of the prototype samplers. The single stage sampler (Chen *et al.*, 2004) consisted of a cylindrical section 24.48 mm long and 8.27 mm in diameter, a conical section 19.09 mm in length with a tip diameter of 2.99 mm. The tangential inlet tube is 1.99 mm in diameter and the outlet tube has a diameter of 2.24 mm. The computational grid used for single stage sampler contained 781000 cells (Figure 1).

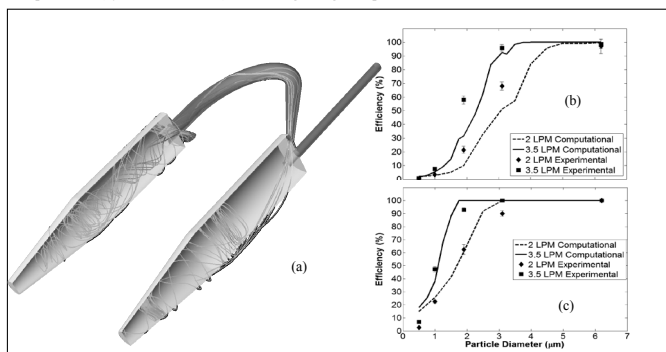
Figure 1. (a) particle trajectories for 3 μm particle at 2LPM and (b) computed collection efficiency compared to experiments (by Chen *et al.*, 2004) for the single-stage sampler.



The 3-D, steady, incompressible flow was solved using FLUENT (Fluent Inc. Lebanon, NH) for inlet volumetric flow rates of 2 LPM and 4 LPM. The fluid was modeled as air at 1 atm and 287.5 K (Standard conditions). The flow was considered laminar for

both the 2 LPM and the 4 LPM cases based on inlet Reynolds numbers, 1460 and 2920 respectively. Once the continuous phase was resolved, the Discrete Phase Model (DPM) was used to solve for the trajectories of particles ranging in size from 0.5 μm to 16 μm . Because all of the particles being considered in this study were in the micron range, their effect on the fluid is neglected during calculation of particle trajectories. The two-stage sampler (Lindsley *et al.*, 2006) had the same dimensions for the cylindrical and conical sections but the inlet and outlet tube diameters are different. The inlet tube to the first stage has a diameter of 2 mm. The outlet of the first stage has a diameter of 3 mm (this larger diameter limited the undesirable collection of biological material in the connecting tube) that tapers down to 1.3 mm at the inlet to the second stage (see Figure 2). The final outlet has a diameter of 1.5 mm. The grid used for the

Figure 2. (a) particle trajectories for 2 μm particle at 3.5LPM and computed collection efficiency of (b) first stage and (c) second stage compared to experiments (by Lindsley *et al.*, 2006) for the two-stage sampler.



two-stage sampler contained 1.5 million cells. For the two-stage sampler inlet volumetric flow rates of 2 LPM and 3.5 LPM were used. The flow is considered to be laminar for both the 2 LPM and the 3.5 LPM cases based on their inlet Reynolds numbers, 1453 and 2544 respectively. The Discrete Phase Model was used to solve for the particle trajectories, with particle sizes ranging from 0.5 μm to 6 μm .

Collection Efficiencies. To find the number of particles collected in each of the samplers, wall boundary conditions were applied to the sampler (cylindrical and conical sections) walls, the inlet tube walls, the outlet tube walls, and the connecting pipe walls (only for the two-stage sampler). The DPM will cause the termination of the particle trajectory when it is in contact with any of these walls in the sampler. Once the particle trajectories are calculated for a certain particle, the collection efficiency of the sampler for that particle and flow rate is calculated by dividing the number of particles collected in the specific tube by the number of particles collected there and after in the sampler.

Results and Discussion

The two most important design parameters for sampler are the collection efficiency distributions for different particle sizes and the d_{50} cut-off diameter (particle diameter which results in a 50% collection efficiency). The computed collection efficiency of the single stage sampler for various particle diameters and flow rates agree very well with the experiments by Chen *et al.* (2004). The d_{50} cut-off for the 2 LPM and 4 LPM cases were found to be 2.7 μm and 1.6 μm respectively, which is in good agreement with the 2.5 μm and 1.5 μm values reported by Chen *et al.* (2004). Figure 2 shows the computed collection efficiencies for the 1st stage collection tube and the 2nd stage collection tube of the two-stage sampler compared to the experimental values reported by Lindsley *et al.* (2006). For the 1st stage collection tube the d_{50} cut-off diameters for the 2 LPM and 3.5 LPM cases were calculated to be 3.1 μm and 2.3 μm respectively. These computed values are slightly higher than the 2.6 μm and 1.8 μm values for the 1st tube reported by Lindsley *et al.* (2006). For the 2nd stage collection tube d_{50} cut-off diameters for the 2 LPM and 3.5 LPM flow rates were calculated to be 1.7 μm and 1.1 μm respectively. These values agree well with the 1.6 μm and 1.0 μm values identified by Lindsley *et al.* (2006) for the 2nd stage collection tube. The relatively small discrepancy between the experiments and the computations are believed to be due to inherent modeling errors (i.e. approximations in governing equations of motion). The difference between three different DPM's employed was in the order of the error between the experiments and the computations. Conclusions: The results of the current study show that CFD can be used to accurately predict collection efficiencies for a wide range of particle sizes, as well as the d_{50} cut-off diameters for different inlet volumetric flow rates. The ability of the two-stage sampler to collect size segregated samples can also be accurately modeled using the current CFD techniques. Our study shows that CFD techniques can be used to virtually sample field data and test different design parameters and conditions for new samplers.

Acknowledgements

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Protecting Residential Care Facilities Against Pandemic Influenza

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It is widely believed that protecting healthcare facilities against outbreaks of pandemic influenza requires antiviral pharmaceutical resources and vaccines. However, these will not likely be available early in a pandemic. Moreover, outside developed countries, control policies will be mainly based on non-pharmaceutical interventions (NPIs). The containment of pandemic influenza within acute-care hospitals is problematic due to open connections with communities. However, other health care institutions, e.g., those providing care for the disabled, can potentially control community access. We modeled a residential facility using a stochastic SLIR compartmental model to make explicit conditions under which NPIs alone might prevent introduction of a pandemic virus. The model projected that with currently recommended staff/visitor restrictions and social distancing measures, influenza introductions are inevitable in all but mild pandemics, with rapid internal propagation. The model identified staff re-entry as the critical pathway of contagion and provided estimates of reduction in likelihood of introduction required. Using information on latency for candidate pandemic viruses, we were able to develop NPIs that were evaluated for social plausibility by collaborators who operate residential facilities. Different combinations of NPIs, some novel, promise protection over the range of pandemic severities; but combinations effective against pandemics other than mild imply social disruption that increases with severity.

Introduction

The last influenza pandemic was almost 40 years ago. A steadily larger series of human cases of H5N1 avian influenza with a case fatality rate over 50% stands as a harbinger of the devastating potential a novel pandemic virus might pose. Several researchers have explored the effect of various measures for mitigating a pandemic [1, 2, 3]. Mathematical models utilizing historical data on the 1918-19 influenza pandemic in US [4,5] suggested that timely implementation of non-pharmaceutical interventions (NPIs) at the community level may have been somewhat effective in curtailing pandemic effects. However, these studies also concluded that most interventions began too late and were halted too soon. A recent study [3], focused on the application of NPIs for pandemic control in both the social community and acute-care hospitals found that open community access made containment within acute-care hospitals improbable. However, other health care institutions have the potential to restrict community access to a greater degree. These include approximately 16,000 institutions

within the US that provide care for individuals who require assistance with activities of daily living, the disabled (mostly elderly) and the mentally-and developmentally-challenged [6]. We used a Susceptible-Latent-Infected-Recovered (SLIR) type stochastic compartmental model to represent residential-care facilities and to identify specific NPIs and combinations thereof that could actualize community access control. Managers of residential care facilities provided real-world feedback iteratively to our modeling directing development of practical implementations sufficient for protection over the full range of projected pandemic categories.

Materials and Methods

Transmission model. The model (Figure 1) quantified the dynamics of influenza transmission in a residential care facility. It included residents of the facility, and tracked staff and visitors in the facility and community. Residents were classified according to their epidemiological state: Susceptible (S_R), Latent (L_R), Asymptomatic (A_R), Early-Infectious (I^1_R), Late-Infectious (I^2_R), Recovered (R_R), Deceased (D_R), Treated (T_R), and Prophylaxed (P_R). Infection occurred upon contact with symptomatic (infectious) individuals. A fraction of latent individuals received prophylaxis and recovered while the remainder and the untreated progressed to the symptomatic or asymptomatic infectious classes. The asymptomatic infectious were assumed to transmit disease at a lower rate than symptomatic individuals, and eventually recovered. In some scenarios these persons were assumed to acquire temporary protection through antiviral prophylaxis at various levels of efficacy. A portion of individuals who received antiviral prophylaxis post-exposure were assumed to return to the susceptible class. An additional proportion of effectively-prophylaxed individuals (P_R) who became infected were assumed to escape developing clinical disease following exposure and ultimately recover. We recorded the instantaneous location of visitors and staff (e.g., community or facility) and their epidemiological states: Susceptible (S), Latent (L), asymptomatic (A), Infected (I), Recovered (R) and Treated (T). Visitors located in the community or in the facility were indexed by VC and VF, respectively. Similarly, staff located in the community or a facility were indexed by SC and SF, respectively. We indexed individuals of the general community by C. We assumed that facility staff worked 8-hour/5-day schedules and that only one third of residents have visitors who spent on average 2 hours per week in these facilities. The force of infection (risk of infection) inside a facility involved the interaction of residents, staff on-duty, and visitors. Similarly, the force of infection for individuals outside a facility included contributions from contacts among staff off-duty, visitors in the community, and general community members. Reduction in transmission measures such as isolating symptomatic residents and/or the use of protective devices (e.g., face masks) and social distancing were implicitly modeled in the force of infection.

Population and Parameter Assumptions. We simulated a facility of 200 residents and assumed that a third of these individuals

received visitors each week. Staff engaged in direct care were assumed to spend 3 contact hours per resident/per day. The total population (community, staff, and visitors) was based on the current estimate for the US (300M). Conventional NPIs modeled included reducing visiting hours, increasing staff shift lengths and introducing temporary staff working part-time to accommodate absenteeism, isolation of symptomatic residents, utilizing measures designed to reduce transmission within the facility (e.g., masks and gloves), screening visitor and staff entry, and decontaminating materials brought into the facility. In the baseline scenario, none of these interventions were considered implemented. Three levels of preparedness plans were generated including the following: extending staff at-work periods from 8-hour shifts/5-day work periods to 12-hour shifts/4-day work periods, reducing visiting hours from 2-hour periods to complete restriction, and introducing transmission-reduction measures implemented by staff and visitors that were assumed to vary from 0% to 95% in effectiveness. Disease-related parameters assumed were taken from experimental data on human subjects for A(H3N2) viruses (P.Glezen, R. Couch, private communication) and were as follows: Latency period of 2.4 days; progression to infection occurring a mean of 2.5 days post exposure with a standard deviation of 1 day; a recovery period of 5 days. Disease-induced mortality rates were 0.005 (treated with antiviral agents) and 0.01 (untreated); residents were assumed to be treated within 12 hours of exposure with efficacy levels ranging from 30-50%.

Results

We solved the compartmental model (Figure 1) numerically via Poisson simulation using Matlab (The Mathworks, Inc). For each pandemic preparedness scenario, we carried out 50 stochastic realizations from which we computed summary statistics. We simulated the baseline scenario and three plans based solely on NPIs that were configured iteratively to provide protection over a range of pandemic severities as measured by the basic reproduction number (R_0) [2,7,8].

Baseline Scenario. This scenario assumed no control interventions. The results indicated that the epidemic size (Table 1) inside a facility was high even for relatively mild outbreaks ($R_0=1.4$).

Assessing the Role of NPI's. We chose three levels of NPI intervention strategies according to the severity of the pandemic. We found that conventional NPIs sufficed to prevent introduction of the virus only in mild pandemics, and that strict and demanding levels of NPIs were needed to bar more transmissible viruses.

Plan for Category Level 1-2. This plan increased staff shifts from 8 to 12 hours, assumed social distancing practices to be implemented by staff and visitors that directly reduced residents' risk of infection by 50%, isolated symptomatic residents, and reduced visiting hours from 2 to 1 hour. These modest restrictions were effective in reducing epidemic size. For a pandemic with R_0 of 1.4, this scenario reduced the epidemic size (median) from 157 to 8 cases. The probability of

an outbreak (given an introduction) was reduced by 50% for moderate pandemics ($R_0 = 1.8$). However, a virus introduction could not be prevented regardless of the severity of the pandemic.

Figure 1. Figure 1a describes the compartmental model of residents of an ADL-challenged facility. Figure 1b depicts the dynamics of staff and visitors (inside and outside a facility). Residents interact directly with visitors and staff inside the facility (populations indexed by VF and SF).

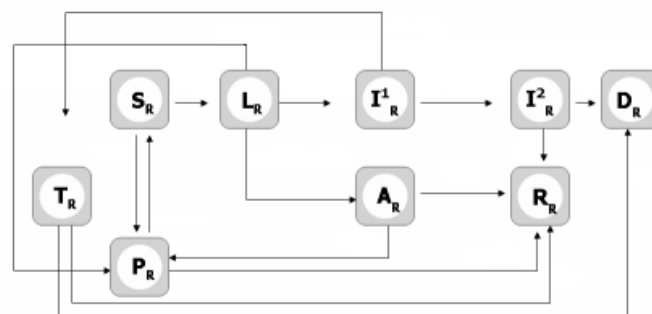


Figure 1a

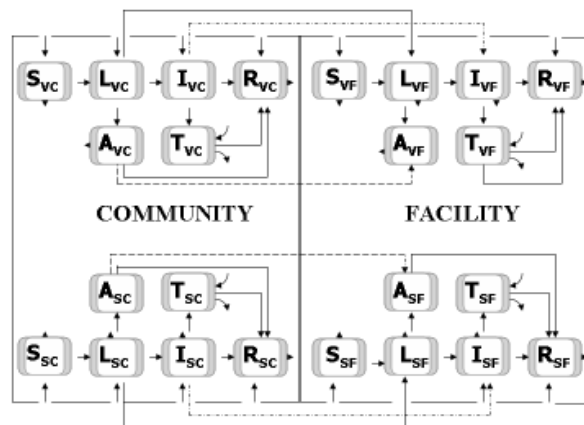


Figure 1b

Plan. Category Level 3-4. Modeling Category 1-2 pandemics allowed us to identify employee re-entry as the principal conduit of virus introduction. Multi-day shifts with on-site residence reduced this risk directly, but was considered socially unworkable for periods longer than a few days. Using expert estimates of time from infection to symptomatic illness (Personal communication, P. Glezen and R. Couch), we devised scheduling schemes that dramatically reduced the likelihood of introduction of the pandemic virus. For an employment schedule comprised of four full days on-site and four days at home, a 2.3-day self-sequestration period involved the employee entering sequestration at home on the evening of the second day off-work. The employee entered sequestration if he/she and all members of the household were asymptomatic and afebrile at the time scheduled for entry

Options for the Control of Influenza VI

and reported to the facility at the end of the sequestration period (the morning of the fifth day) only if all in the household remained asymptomatic and afebrile. This 4 day-on/4 day-off/2.3 day self-sequestration schedule lowered the likelihood of introduction of the virus by about 20-fold compared with daily 12-hour shifts; and was considered socially acceptable by collaborators who operate residential care facilities. In addition, this plan further assigned complete visitor restrictions (e.g., communication with residents only electronically or from behind impermeable barriers). These scenarios turned out to be effective in preventing introduction of the pandemic virus into the facility for all severity levels except the highest ($R_0=2.8$). *Plan: Category Level 5.* For severe pandemics we evaluated a plan that included all interventions used in the Plan for Category 3-4 pandemics, increased self-sequestration periods to 3.3 days (while maintaining a 4-day-on/4-day-off staff shift scheduling), and increased social distancing measures to the level of 95% reduction in residents' risk of infection from returning employees and visitors. This plan required virtually complete facility isolation and visitor restriction, and included high levels of viral mitigation imposed on all goods and high-priority services entering the facility. In simulation, this level of NPIs completely eliminated the possibility of pandemic introduction.

Table 1. Event A denotes the introduction of disease in a single facility, B|A is the event of an outbreak occurs given an introduction, $p(A)$ and $p(B|A)$ denote the respective probabilities of these events. The final epidemic size is given by $Episize_i$ (Final Epidemic Size), where i denotes the maximum and median. Epidemic threshold is established at 5% (10 cases) clinical attack rate.

R_0	$p(A)$	$p(B A)$	$Episize_{max}$	$Episize_{median}$	R_0	$p(A)$	$p(B A)$	$Episize_{max}$	$Episize_{median}$
Baseline					Category 1-2				
1.4	1	1	181	157	1.4	0.86	0.44	91	8
1.6	1	1	188	173	1.6	0.82	0.44	114	7
1.8	1	1	188	181	1.8	0.90	0.54	112	15
2.0	1	1	193	185	2.0	0.98	0.68	154	58
2.2	1	1	196	188	2.2	0.96	0.70	165	94
2.4	1	1	195	189	2.4	0.94	0.84	177	115
2.6	1	1	198	191	2.6	0.98	0.90	171	141
2.8	1	1	197	192	2.8	0.98	0.98	182	156
Category 3-4					Category 5				
1.4	0.18	0.04	15	0	1.4	0	0	0	0
1.6	0.22	0.02	15	0	1.6	0	0	0	0
1.8	0.14	0.06	59	0	1.8	0.04	0	1	0
2.0	0.26	0.08	73	0	2.0	0	0	0	0
2.2	0.34	0.12	96	0	2.2	0.06	0	1	0
2.4	0.24	0.12	122	0	2.4	0	0	0	0
2.6	0.28	0.12	137	0	2.6	0.02	0	1	0
2.8	0.54	0.34	138	5	2.8	0.02	0	1	0

Discussion

Our results indicated that in the absence of interventions, the introduction of influenza into residential care facilities was inevitable. We found that introductions occurred very rapidly and resulted in outbreaks with overwhelming attack rates, much higher than those observed in the general community. These results suggested that current recommendations for managing absenteeism due to pandemic illness by increasing the total

number of employees might act to increase the likelihood of introduction of the pandemic virus. The Plan for Category 1-2 pandemics indicated that even levels of NPIs that produce only minor social disruption could significantly reduce the impact of mild pandemics on institutions that can control public access. This Plan yielded a two-fold reduction in the likelihood of pandemic introduction and dramatically reduced clinical attack rates. However, implementing a plan that succeeded in Category 3-4 pandemics involved employee commitments to multi-day continuous presence at the facility, to behaviors designed to reduce the likelihood of becoming infected during periods away from a facility, and to self-sequestration within the home for the latter portion of multi-day off-work periods. Simulations based on this plan predicted 90% reductions in the likelihood of an outbreak and in the number of cases within a facility for all but the most severe pandemic evaluated ($R_0=2.8$). Managers and the staff of real-world residential care facilities were instrumental in developing the NPI measures proposed in this plan. This study demonstrated that plans for protection of a health care institution against pandemic influenza based solely on non-pharmaceutical measures may be practicable and attainable. While surviving a pandemic may be possible, it will not be easy or simple. For severe pandemics, social disruption will be inevitable and must increase with severity. The activities of daily life will be altered for all and commitments from every person associated with such facilities are required to withstand what may be mankind's most severe test.

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Modeling of Bio-Aerosol Transport and Dispersion in a Ventilation Room

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The transport and dispersion of aerosol imbedded with influenza virus in a positively or negatively pressurized ventilation room is investigated by computational fluid dynamics (CFD) methods. Lagrangian stochastic (LS) walk model is used with several advantages of direct tracking of fluid particle trajectories, independence of the reference frame, and feasibility in conjunction with complicated flow environment. Influenza viruses are believed spread through the air via turbulent suspension and transport and infect others who walk in. The virus-containing bio-aerosols originate from the respiratory activities such as coughing and sneezing. The resulting germ-laden droplets may evaporate rapidly after they are released. Numerical simulation shows that it is justified to model these aerosols as passive scalars which neutrally follow the turbulent air flow, as a first approximation. Given the location of source (standing, sitting, or sleeping position), the semi-analytic LS model can predict the transport and dispersion of aerosols, and calculate the probability density function (PDF) or influence area of aerosols in the ventilation room. This model can also provide valuable information for ventilation control strategies with respiratory protection, such as enhanced air exchange, air filtration rate, and improved airflow patterns to reduce indoor airborne infection risk. Finally, the modeling results of this study can be extended to design and analyze virtual sampling tools for bio-aerosol particles.

Introduction

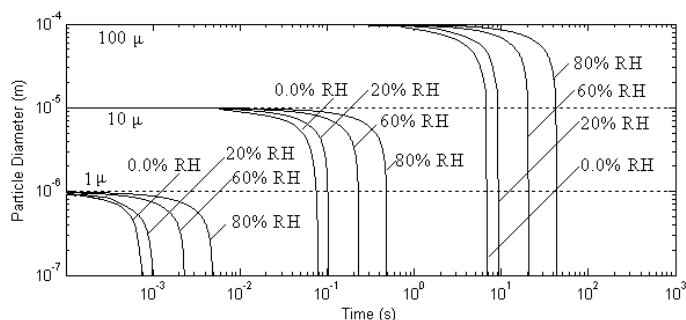
The airborne transmission of disease is of great concern to the public health community because of the pandemic potential of newly-emerging diseases like avian influenza. Bio-aerosols have been referred as an important mode of transmission of influenza by many researchers (Tellier, 2006). Modeling of the dispersion of respiratory virus-laden aerosols in a ventilation room is an important approach to examine the safety and reliability of engineering control and implementation of infectious diseases. Numerical simulation can provide detailed three-dimensional information of infectious aerosols transmission at a given time which is hard to directly obtain from experimental observation or sampling. The spatial probability of distribution of aerosol production by simulation is effective for the risk evaluation of other airborne infections. A more general, yet practical, modeling of bio-aerosols in work places is required. The methodology for this study is to use computational fluid dynamics (CFD) methods to obtain instantaneous and mean flow field, and use Lagrangian stochastic (LS) walk model to analyze aerosol motion in air whose velocity is computed. One advantage of LS model is in obtaining the spatial probability

of distribution of aerosol fairly easily. The approach is different from the Wells-Riley model that has been widely used to evaluate the risk factors for airborne infections (Chen et al. 2006; Fennelly et al., 2004; Nardell et al., 1991). The Wells-Riley model suffers from the negligence of effects of fluid flow (turbulence) on the spatial and temporal distribution of aerosols. Recently, a discrete-time Markov Chain model was used by Nicas and Sun (2006) to analyze infection risk, however, it did not consider effects of turbulence. While several works have been reported to simulate air flow and particle motion in ventilation environment by CFD (Sun et al., 2007; Kao and Yang, 2006; Li et al., 2004; Lu et al. 1996), most simulations have focused on airflow pattern, and neither of them directly analyzed turbulent stochastic of aerosol particles. To better understand aerosol transmission, the stochastic property of aerosols in turbulence should be considered. The objective of this study is to explore possible distribution of risk factors due to turbulence for influenza virus transport and dispersion in positively or negatively ventilated rooms. We restrict the simulation to neutrally buoyant particles in which case marked particles are released into a turbulent flow, and are dispersed by turbulence in the room. Instead of direct solving the particle equation either in an Eulerian or Lagrangian frame, we use LS model (a single-particle Markov-chain model) to simulate individual particle trajectories by assuming that its velocity can be represented by a Markov sequence. Given a prescribed flow field such as turbulent mean velocity, velocity variance and its gradient, the problem is posed to describe statistically the evolution of the aerosol field from a known initial state. A direct application of this study is that the numerical results can be extended to design and analyze virtual sampling tools for bio-aerosol particles. Other possible application is to provide recommendations for personal protective equipment by government health protection agency.

Numerical Modeling

In health-care environment or other work places, aerosol particles with an aerodynamic diameter ($<10 \mu\text{m}$) remain suspended in air for a duration sufficient to permit dispersion through room air (Tellier, 2006). We simulated the temperature and size variation under evaporation for three sizes of aerosol particles using the standard single-droplet equation model (Crowe et al., 1998). Figure 1 shows that large size particles rapidly settle down close to point of emission (90%), only about 4~10% of aerosol particles disperse in room air ($d < 10 \mu\text{m}$), and the time scale for process of evaporation of droplet from 5~10 μm to 2 μm is less than 0.1 s. Considering 1.0 m/s air velocity in the room, the maximum distance for particles with large size to settle down is about 0.1 m for 99% particles involved. The numerical model only investigates the transport and dispersion of aerosol particles within the size of 2 μm after coughing. Therefore, it is reasonable to assume that the dispersion and transport of aerosol particles can be regarded as passive scalars (e.g., carbon dioxide). Thus, the dispersion of passive scalar (flu virus aerosols) by turbulent convection can be considered as an archetypal continuous stochastic process.

Figure 1. Evaporation of aerosol droplets with different sizes as a function of relative humidity (Droplet initial temperature is set as the same as 310.15°K and ambient air temperature of 293.15°K) and assuming a typical velocity profile of a coughing jet (Water droplets) in a room with uniform background flow field.



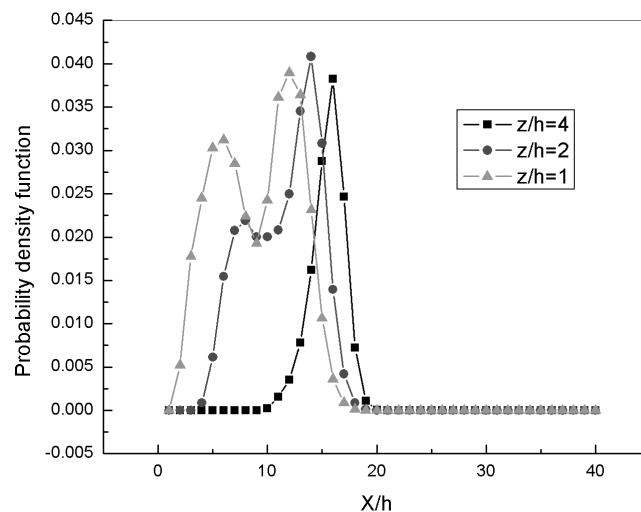
A Reynolds Averaged Navier Stokes (RANS) model was applied to find the turbulent mean flow field that is required by LS model. The continuous phase (air) solution is independent from the aerosol particle trajectories assuming that the particles do not affect the continuous phase. The turbulent diffusion and advection was computed in the vertical and horizontal directions by using a Lagrangian framework. The vertical displacement of aerosol is calculated from $dz=w \cdot dt$ (1) where w is the Lagrangian vertical velocity (fluctuation) and dt is differential time increment. Incremental change in vertical velocity were computed using the Langevin equation, an algorithm that is weighted by a deterministic forcing (which is a function of previous velocity of the particle) and a random forcing term, $dw=a(z,t,w)dt+b(z,t,w) d\xi$ (2). A similar process is used for horizontal directions. The coefficients $a(z,t,w)$ and $b(z,t,w)$ are non-linear functions of w , and are defined to account for inhomogeneous turbulence. The term $d\xi$ defines a Gaussian random forcing with a mean of zero and variance of dt . The terms $a(z,t,w)$ and $b(z,t,w)$ are derived from the Fokker-Planck equation for w (Durbin, 1983), and they are functions of Reynolds stresses, the standard deviation of velocity and , and the Lagrangian integral time scale . The variance of a random number is given by

$$\sigma_r^2 = \frac{1}{N-1} \left\{ \sum_{i=1}^N r_i^2 - \frac{1}{N} \left(\sum_{i=1}^N r_i \right)^2 \right\}$$

(3) In order to present the effects of entrainment and deposition of aerosol particles when in contact with the wall, a constant absorbing factor is used and the entrainment rate is taken proportional to \sqrt{k} , where k is the kinetic turbulent energy of particle. In indoor environment the height of the coughing source was selected as the length scale h . The length, width, and height of a room were 5m, 5m, and 2.5m, respectively. 10,000 particles were released from the coughing source at the same time, and the maximum flight time is 1000 s for statistical averaging. A general requirement is that the maximum flight time should be large enough for statistics.

The time step used in the LS model was $0.17\tau_L$ (0.1~1.0 s). The mean air velocity inside the room and its standard deviation were used as input parameter in the LS model. A numerical code has been developed based on the LS model. This code can directly output the concentration of aerosol and the probability density of its distribution. Figure 2 shows the probability density function (PDF) of aerosol distribution at different levels in a ventilation room. The risk of infection should be proportional to location of peak seen in each curve. There are three issues to be emphasized: First, the accuracy of PDF by LS model is determined by the mean air field and its fluctuation in the ventilation room. Different ventilation layout will result in different PDF profile. Numerical experiments showed that the transport of 70~80% aerosols is limited to 25 h in the horizontal direction if the ratio of room length to room height is larger than 10. Second, the PDF curve is obtained for a limited statistical period (1000 second). Third, different wall condition may result in slightly different PDF profile. Most notable is that we can examine the relationship between the aerosol concentration and the height of coughing source. The lower the height ($z/h=1$), the closer of the peak of PDF to the source. Under the same air flow field, the influence area of the case ($z/h=1$) is five times larger than the case of $z/h=4$. This seems reasonable because aerosols are expanding in vertical direction as the migration of aerosols along the horizontal direction proceeds. The maximum concentration will decrease due to the expansion in vertical direction. In a further work we plan to compare the calculated PDF with that obtained from samplers (Parsons et al., 2007, in this Options VI Proceedings).

Figure 2. Probability density function of aerosols along the horizontal direction at different height (1.0, 2.0, And 4.0, H is the height of the coughing source) by the Lagrangian Stochastic model by using a given mean air velocity in a ventilating room.



Conclusions

A Lagrangian stochastic flight model was applied to examine the risk of infection in a typical ventilation room. This model

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is shown to be feasible for analyzing complicated indoor environment in which turbulence exhibit significant effect on the transport of aerosol particles. The numerical results showed that aerosol particles larger than 10 micron size rapidly deposit in a region of 0.1 to 0.25 m from the coughing source. The results were consistent with experimental data from other researchers. The deposition time scale for aerosol particles with large size (diameter $d > 10 \mu\text{m}$) is less than 0.1 s beyond that such particles evaporate and rapidly reduce in size. The calculated PDF profiles of aerosol particles in a typical ventilation room showed that peak of concentration moved away from the coughing source as aerosols propagate with air (the carrier phase). At the same ventilation condition aerosol particles significantly accumulated at the level of $z/h=1$ from the floor due to turbulent diffusion and indoor environment. This provides a direct estimation of influence area from a single source of influenza virus. To expand the application of Lagrangian stochastic walk model an accurate estimation of fluid flow field including turbulence is prerequisite in our future work.

Acknowledgements

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Validation of the Moving Epidemic Method for Detecting Influenza Epidemics in Europe

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Introduction

Influenza is the most common respiratory infection in human beings. The disease can appear sporadically in the population, but usually there is an annual epidemic wave. Epidemics of influenza occur seasonally, typically during the winter months with great variations in its epidemiological characteristics. Annual patterns depend on the circulating virus strain, susceptibility of the population and on other variables less known but closely related with the infectivity, pathogenecity and virulence. Early warning and monitoring of annual epidemics is one of main objectives in influenza surveillance and a challenge for Public Health Services. Nowadays, sample systems (sentinel networks) exist at regional or national levels, optimising the surveillance by the integration of epidemiological and virological data with sensitivity, accuracy and timeliness. Based in the epidemiological surveillance (notification) of cases of influenza from patients attending primary health care services, a method for detecting seasonal epidemics has been used since 2003 with reliable results in Castilla y León (Spain)^[1]. At the European level, a harmonised methodology for comparison purposes is desired. A modified approach of this model, called the "Moving Epidemic Method (MEM)" is being further developed and evaluated by a Working Group for its systematic use in the European Influenza Surveillance Scheme (www.EISS.org). The aim of this work is to assess whether this method is able to detect the beginning of the historical influenza epidemic using consultation rates for influenza-like-illness (ILI).

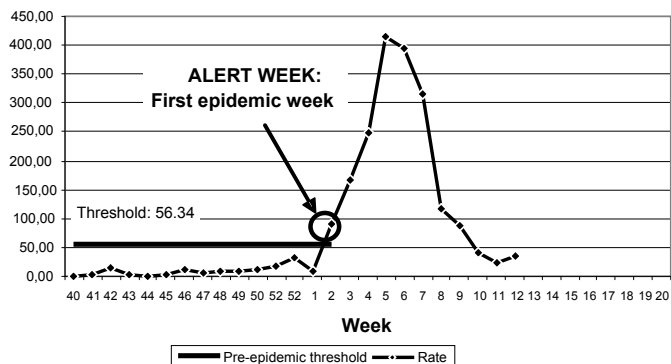
Methods

The Moving Epidemic Method (MEM). A simplified version of the method is shown in the following two steps. The original Method also deals with other issues not discussed in this paper. STEP 1: Determination of the length and timing of the epidemic: The first aim is to determine the length of all epidemics, and to determine the timing of the epidemics. To achieve this, MEM considers, conceptually, that the epidemic period for each season is the minimum number of weeks with the maximum cumulated rate (the set of weeks that maximizes the sum of weekly rates). In practice, the optimal length is chosen among

all possible epidemic lengths by comparing these cumulated rates. Once the length of the epidemic is calculated, the best timing of the epidemic is the set of weeks that maximizes the sum of rates. STEP 2: Calculation of pre-epidemic threshold: Once all epidemics are located, the pre-epidemic period is defined as the set of weeks prior to the epidemic. For each season, the max rate of the pre-epidemic period is chosen and the geometric mean of all these values is calculated. The pre-epidemic threshold is defined as the upper 95%CL of the geometric mean. For surveillance purposes, during an influenza season, the method would detect the beginning of the influenza epidemic when a weekly incidence rate exceeds the pre-epidemic threshold.

Validation analysis. To validate the method, we used a cross-validation process applied to all historical data from 12 European countries registering weekly ILI consultation rates over the last 5 or 10 seasons (some countries have registered 10 seasons, others only 5 seasons). The cross-validation process is the simplest "Leave-one-out", which means that in every iteration one item is used as the validation data, and the rest as the training data. So for every season (the validation season), a threshold is calculated using as the training data the rest of historical seasons (all except the one used to validate). The threshold is calculated with 4 or 9 seasons, depending on the country, and then is applied to the validation season (the excluded season) to ascertain whether the modelled timing of the start week was confirmed. To validate the threshold, we used the positive predictive value (PPV). The PPV is the percentage (in respect of 5 or 10 seasons), in which the start week (alert signal) was confirmed (true positive alerts). An alert signal is confirmed if the weekly rates did not drop below the threshold until the epidemic peak was reached in that particular season. (Figure 1).

Figure 1. Example of a pre-epidemic period and the alert week.



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Results

Table 1 shows the summary of the cross-validation estimates for each country. The cross-validation PPVs have been estimated for each country individually and the results are summarized below. The pre-epidemic thresholds estimated with all data available for each country varies from 22.6 in Slovenia to 230.8 in Italy; The lengths of the epidemics are around 10 weeks with a minimum of 5.7 in Portugal and maximum of 11.4 in Scotland; The average PPV of the alert signal, the indicator of the best fitting method, for these twelve countries, was 79% (minimum 60%, maximum 100%). Half of the countries had a PPV of 80% or higher (6/12).

Table 1. PPV of the alert signal for the included countries.

Country/Region	Weeks	Seasons	Pre-epidemic threshold	Mean length
Belgium	30	5	170.9	10.7
England	33	10	35.4	9.1
Netherlands	33	10	50.8	10.7
Scotland	32	10	69.4	11.4
Spain	31	10	71.4	10.2
Portugal	33	5	29.4	5.7
Switzerland	30	5	66.9	10.2
Italy	28	5	230.8	11.3
Ireland	32	5	24.4	10.6
Slovenia	33	5	22.6	7.7
Denmark	32	5	149.6	10.4
Poland	33	5	110.7	8.3
Wales	34	10	12.1	9.6

Discussion

The estimated threshold is conservative. The model aims to minimize false positive results ("false alert"). Considering this, the results show that half of the included countries had false alerts in 20% or less of the included historical seasons. On the other hand, false negatives results are extremely rare and indicate an unappreciable epidemic period. It has been noticed that most false alerts appear in two circumstances: 1) When a low rate slightly exceeds the threshold: This means that the rate is around the threshold, overtakes it slightly and then goes down again; 2) When the threshold is widely exceeded early in the surveillance period (probably due to erroneous data). These false alerts can be explained by consultations for viruses other than influenza (increased acute respiratory infections or respiratory syncytial virus cases) or by unstable ILI rates without a clear influenza epidemic pattern. In these circumstances, alert signals should be interpreted with caution and complementary information (other epidemiological and virological data) should be taken into account. The method is very sensitive to unstable rates and confounders. It has been developed to model influenza epidemics and should only be used within reliable data in the surveillance framework from which it has been built.

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Optimal Isolation and Quarantine Strategy for Containing Pandemic Outbreak

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Isolation and quarantine strategies in the containment of epidemics are investigated in a mathematical model that demonstrates the importance of quick measure in identifying the infected and the subsequent quarantine of his/her neighbors. In this model, the time delay in the identification of the infected (denoted as τ) and the subsequent isolation of the infected and the quarantine period for his/her neighbors (denoted as τ_0) are two key parameters in the containment of the epidemics. In general, a long quarantine period and short time delay in the identification of the infected are desirable for the containment of the outbreak, but the associated social cost of isolation and quarantine may be too big to be realistic. Our model provides a theoretical framework for the estimation of the cost by properly balancing the risk and the social cost. We also identify a critical parameter π for the SIR model (Susceptible-Infected-Recovered) so that the isolation and quarantine strategy will fail to contain the outbreak when $\pi < 0$.

Introduction

The serious threat posed by the avian influenza virus (H5N1), which may be transmissible among human [1,2], calls for effective strategies to control the pandemic [3-5]. The lesson from the SARS outbreak suggests that the short time delay in the identification and the isolation of the infected, coupled with a suitable period of quarantine is an effective way to contain the spreading of the contagious virus at the initial stage, especially before an effective vaccine is available [6]. Although this strategy of "isolation and quarantine" is a common measure, a theoretical discussion on its effectiveness in the balancing of the risk of pandemic and the associated social cost remained unexplored. In this paper, we formulate a simple variant of the SIR model where we identify two important parameters that determine the effectiveness of the measure. They are the time taken to identify and isolate those infected and the imposed quarantine period [6-9]. One expects that a very conservative and strict quarantine strategy is likely to contain the spreading. However, this inevitably raises many complex issues involving ethical, legal and socioeconomic concerns [10, 11], which must be properly addressed to avoid an unacceptable cost associated with excessive isolation and quarantine. Here we introduce a general model to describe the mathematical consequence of slow identification of the infected (large time delay for

isolation) and the short quarantine period, thereby deriving a general guideline for the calculation of the optimal time delay (certainly not instantaneous) and the proper quarantine period (not too long but sufficient to contain the epidemics).

Materials and Methods

For simplicity, we use a random network [12] to model the social network in a small-sized population, with $N=500$ individuals and the average number of contacts (average degree=number of nearest neighbors: $\langle k \rangle=10$). For the transmission by daily contact, we assume an infection rate $\xi=0.05$ per day. For the infected individual, we assume that the incubation period is one day and the infectious period is T_R days. We further assume that the Poisson distribution describes well the infectious period T_R . As the typical infectious period ranges from two to seven days, with average around four days [3], we set the

parameter a in the Poisson distribution $p(T) = \frac{e^{-a} a^T}{T!}$ and $\langle T \rangle = \sum_{T=0}^{\infty} T p(T) = a = 4$. The number of days T_I used for

the identification and isolation of the infected individual also follows a similar Poisson distribution. Our SIR model then reads

$$\dot{S} = -rIS/N + \beta_R I \quad (1)$$

$$\dot{I} = rIS/N - \beta_R I - \beta_I (1 - \beta_R) I$$

$$S + I = N$$

Here $b_R = \sum_{T_R=0}^{\infty} \frac{p(T_R)}{T_R}$, $b_I = \sum_{T_I=0}^{\infty} \frac{p(T_I)}{T_I}$ and $r = \xi < k$

Note that $\pi = r - \beta_R - \beta_I(1 - \beta_R)$. This parameter π measures the efficiency of the identification and the isolation of the infected and is critical in the early stage of the outbreak (when I is small). In general, the isolation is efficient and the spreading of the virus can be controlled when $\pi < 0$. On the other hand, when $\pi > 0$, the strategy is unlikely to contain the spread, though this measure still contribute in the mitigation of the scale of the outbreak. Our simulations were performed according to the flowchart shown in Fig 1. After a finite time delay τ , an infected individual is identified and will be isolated and his/her nearest neighbors will be quarantined and released after τ_0 days. We simulate the outbreak patterns to evaluate the effectiveness of different quarantine criteria. (Fig 2b, c and d show the average data over 500 simulations for each set of input parameters $\langle T_I \rangle$ and T_Q .)

Results

In general, we find that the smaller the time $\langle T_I \rangle$ involved in identifying the infected, the more efficient is the isolation, and the smaller is the scale of the outbreak. Indeed, the parameter π becomes less than zero for a sufficiently short time delay $\langle T_I \rangle$ and the outbreak is contained. In Fig 2a, we see that the outbreak can be contained in the first fifty days if $\langle T_I \rangle$ is less than three days. However, when $\langle T_I \rangle > 4$, we have $\pi > 0$ and

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the outbreak cannot be contained. While π is an important parameter, we see in Fig 2b and Fig.2d that a long quarantine period can also decrease the scale of the outbreak. However, a long quarantine period will be too costly. This then poses an optimization problem where we seek a optimal quarantine period that minimize the cost. The social cost is a complex function of various factors that involves not only the loss in monetary terms for the isolation and quarantine strategy, but also complex issues such as the loss of individual freedom of movement, interruption of important personal and public schedules, etc. Although we are not modeling the detailed cost structure in this strategy of isolation and quarantine, we can still make a simple assumption that the social cost in the strategy is linearly proportional to the cost involving the infected (such as the cost for hospitalization per person) and the cost involving the quarantined (such as income lost and work delay per person). If we assume that the ratio of the cost per infected individual over the cost per quarantined individual is μ , then the social cost is proportional to, where I is the number of infected individual and Q is the number of individuals quarantined. Since both I and Q can be computed in our model, we can estimate the social cost for a given μ , together with the two parameters in our strategy: T_I and T_Q . The first step in using our model is to compute the three-dimensional plot of social cost versus T_I and T_Q for a given μ . This is done numerically in Fig.2b. From this plot, we can extract the optimal quarantine period which is shown in Fig.2c. Furthermore, we can obtain some insights to this optimal quarantine period using Fig.2d, which plots Q/I versus T_Q . As Fig.2d shows a family of straight lines, we can say that there exists a linear relation between Q/I and T_Q and we can then write

$$\frac{Q(T_Q)}{I(T_Q)} = \lambda(T_I)T_Q \quad (2)$$

With this linear relation, we can write the total cost as

$$\text{Cost} \equiv \mu I(T_Q) + Q(T_Q) = (\mu + \lambda(T_I)T_Q)I(T_Q) \quad (3)$$

To determine the optimal , we take derivative of this cost with respect to the parameter and obtain

$$\frac{d\text{Cost}}{dT_Q} = \frac{d(\mu I + Q)}{dT_Q} = (\mu + \lambda(T_I)T_Q) \frac{dI(T_Q)}{dT_Q} + \lambda(T_I)I(T_Q) \quad (4)$$

As Fig.2b shows that the number of infected decreases with T_Q

that is, $\frac{dI(T_Q)}{dT_Q} < 0$, we see that the first term in Eq.(4) is

negative and the second term positive, implying the existence

of a T_Q that makes $\frac{d\text{Cost}}{dT_Q} = 0$. This shows that there must

be an optimal T_Q for effective quarantine. With this proof, we are assured that the optimal period for quarantine

exists and our numerical method can find it for a given μ and time delay T_I . Usually, the ratio μ can be obtained based on known costs, while the time delay parameter T_I can be obtained based on data of outbreaks in the past.

Discussion

Based on a variant of the SIR model, we develop a model to address the effect of time delay in the isolation of the infected and the subsequent quarantine measure and derive a general criteria based on the critical value of a parameter π , so that simple quarantine measure is efficient and the outbreak can be contained when π is less than zero. A more important result of this work is the demonstration of a procedure to evaluate this traditional strategy in the control of the outbreak of infectious diseases using numerical simulation. We know that if the time delay in finding the infected individual is small, we can contain the outbreak at its initial stage. However, we cannot set it to zero since instantaneous identification is technically impossible. We therefore incorporate this finite time delay (T_I) in our model and consider the effectiveness of various periods of quarantine in the containment of the outbreak. Conceptually, the outbreak can be controlled with a sufficiently long quarantine period. However, the associated social cost may be too high to be practical [6, 7, 9-11]. In order to balance the cost issue with the containment of the outbreak, we show how to calculate the optimal quarantine period that minimizes social cost. We conclude that an efficient quarantine strategy is achievable and can contribute to contain a pandemic outbreak at its source. This is especially relevant now that avian flu poses danger to the general public.

Acknowledgements

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Figure 1. Flowchart of the numerical simulation. Virus spreads with the infection rate, r . The infected individuals recover at the rate β_R . After recovery, their status is susceptible. The infected individual is identified and isolated at a rate β_I . After isolation, the neighbors of this infected individual are quarantined for T_Q days.

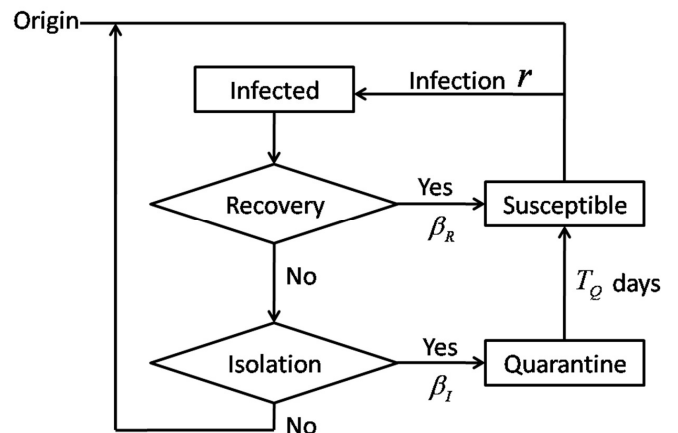
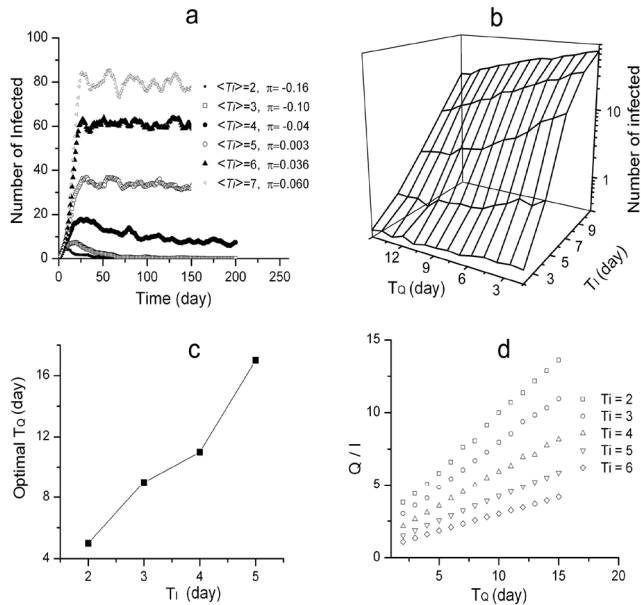


Figure 2. Simulation of the outbreak in a population of 500 individuals. **(a)** The number of people infected as a function of time (for different average time $\langle T_i \rangle$ required to identify and then isolate the infected: $\langle T_i \rangle = 2, \dots, 7$). When $\pi < 0$, the outbreak is contained effectively with the quarantine strategy. **(b)** The average number of infected in the first 200 days with different T_i and T_Q . **(c)** The optimal for controlling the outbreak with minimum cost ($\mu=2$) **(d)** The ratio of the average number of quarantined and infected in the first 200 days with different T_i and T_Q .



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Modeling the Population Benefits of Pediatric Influenza Vaccination Strategies

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Introduction

Although the next influenza pandemic is the focus of scientific and political concerns, it must not be forgotten that the burden of seasonal influenza is high, with an estimated 36,000 influenza-related deaths and 200,000 hospitalizations occurring during the 1990s in the US [1]. Seasonal influenza vaccination is recommended for at-risk groups including persons over 50 years old and children aged 6 to 59 months. Direct protection, however, has its limits: while the influenza vaccination coverage rate in the US increased continuously from 1980 to 2001, reaching 65% in the elderly in 2006 [2], the vast majority (90%) of influenza related deaths still occur in the older segment of the population [3]. Current influenza vaccination strategies are effective but the benefits to the population as a whole are not as high as might be expected and other options should be explored. It has been shown that morbidity and mortality can be further reduced by targeting children while continuing to vaccinate the elderly themselves [4,5,6]. While the highest rates of influenza-related complications and the greatest number of deaths are in those aged >65 years old (yo), attack rates are much higher in children, particularly those attending school. The role of children as major vectors in the spread of influenza has long been recognized and recent research has shown that increased vaccination of children may reduce overall transmission and benefit the population as a whole [7,8]. To test the assumption that vaccinating children reduces population-wide illness, Weycker et al [9] used a transmission model adapted from Elveback [10], and Halloran [11]. They simulated the vaccination of four age groups of children (including from 6 months -18 y.o.) to evaluate the population level benefits during seasonal influenza in terms of deaths, illness, hospitalizations, outpatient visits and over the counter (OTC) medications avoided in a population model that approximated to US society. Some vaccination scenarios, mostly involving vaccinating children, showed significant reductions in influenza cases, deaths and related complications. In the present study, we further refined the transmission model developed by Halloran et al. by dividing the population into narrower age groups which more accurately reflect those considered in ACIP recommendations and the age structure of the US educational system. These adaptations allow the model to more accurately assess the impact of current vaccine recommendation, and hence better forecast the impact of

wider school-based recommendations. We have also updated the externally derived data, including, for example, the CDC data for influenza incidence and vaccination coverage rates. We explored the role of children in influenza transmission by age group to help determine the optimal strategy for childhood influenza vaccination in the US.

Methods

The population-wide benefits of various pediatric seasonal influenza vaccination strategies were modeled and their relative benefits in a typical US community evaluated using a refined stochastic simulation model first developed by Elveback et al., and then refined by Halloran et al. [10] and Weycker et al.[9]. This model simulates disease transmission during the daily interaction of 2,000 hypothetical individuals, representative of a typical US population in terms of age distribution, household composition, and daily interactions based on 2004 census data. We refined the age structure to include nine age groups: 0-5 and 6-23 months old (mo), and 2-4, 5-10, 11-14, 15-18, 19-49, 50-64, and >65 yo. Within the simulation, preschool-age children are assumed to attend a small playgroup or large daycare center; school-age children attend elementary, middle, or high school and some adults are assigned to several workplaces depending on age and household composition. A number of individuals are randomly classed as "high-risk" based on the estimated proportion of individuals at high-risk of influenza-related complications in their age group, e.g. 10% of children, 17% in adults aged 19 to 64, and 100% of >65 yo. Individuals are vaccinated before the influenza season with vaccine efficacy against infection set at 70% in individuals ≤49 yo and 50% in those ≥50 yo. Efficacy against infectiousness is set at 80% for all age groups. During a simulation, the course of an outbreak begins with the random selection of $n_0=12$ initially infected individuals. These individuals can then transmit the infection to susceptible individuals among their daily contacts, and these newly infected individuals can then infect others, and so on. On any given day, each susceptible person in the model has a given probability of contracting influenza from infected contacts. For each daily interaction, a binomial model evaluates each individual's risk of infection depending on the individual's susceptibility (i.e. vaccination status, vaccine effectiveness, age and type of social interactions) and the infectiousness of infected contacts (i.e. vaccination status, stage of infection and type of social interactions). Thus, vaccination reduces disease transmission both directly, by reducing susceptibility among non-infected individuals and indirectly by reducing the infectiousness of those infected. Infected individuals can develop clinical illness or be asymptomatic. Symptomatic individuals may withdraw from the community (i.e. stay at home) and therefore no longer interact with individuals outside their households. Asymptomatic individuals continue their social activities but their infectivity is halved. Daily iterations are repeated until there are no longer any infectious individuals and the epidemic ends. The endpoints of the model are the cumulative number of influenza cases and the number

of hospitalizations and deaths which are calculated by applying a ratio of each outcome to the cumulative number of influenza

Figure 1. Age distribution of vaccinees, influenza cases and deaths in the baseline scenario.

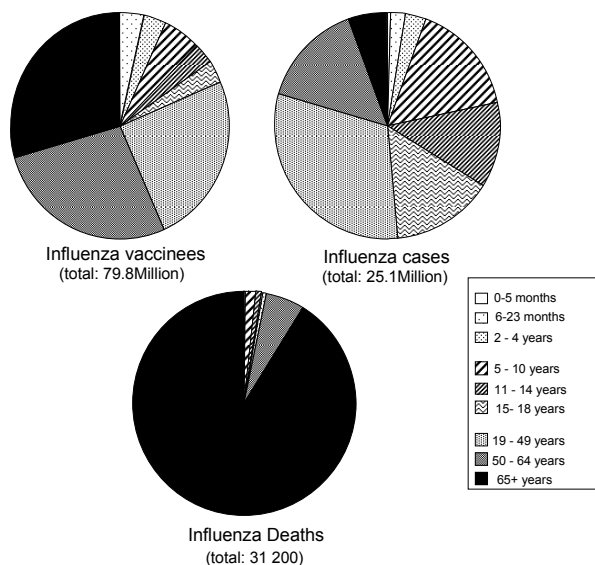
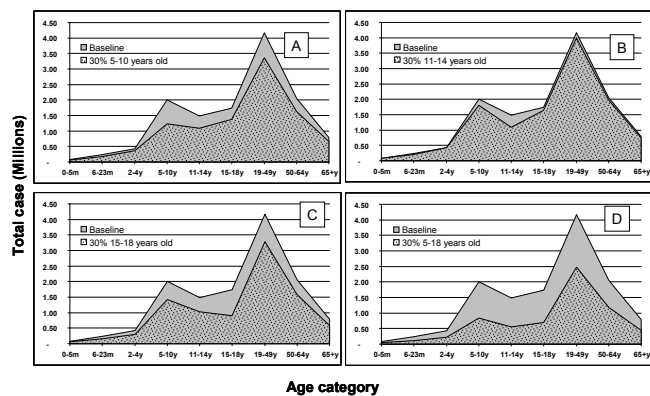


Figure 2. Indirect benefits of childhood vaccination strategies. Total number of influenza cases in baseline and different childhood vaccination strategies (Extrapolated to the whole US population).



cases. Probabilities of hospitalization were derived from a health insurance database covering 3.2 million members and probabilities of deaths were derived from national statistics. The baseline scenario used as a reference in this model corresponds to the actual US 2006 vaccination coverage data by age group and risk level, based on CDC estimates[12]: no vaccination for children below 6 months, 48.4% coverage for 6-24 mo children, 24.5% coverage within low risk children aged 2 to 4 (41.5% within high risk), 14.8% for low risk children aged 5 to 18 (36.6% for high risk), 14.8% for low risk adults aged 19-49 (26% within high risk), 32% within low risk adults aged 50-64 (45.5% within high risk), 64.6% for elderly over 65 years. Then several school aged children vaccination scenarios were tested, as well as universal or increased vaccination rates among elderly.

Results

In the baseline scenario, most cases occur in children but 91% of deaths occur in people >65 years and 97% in people >50 years despite the high vaccination coverage rates in these populations (Figure 1). In 2006, the coverage rate of the 6-23 mo was around 50%. Our results show that increasing the coverage to this level among 5-10 yo, 11-14 yo or 15-18 yo would reduce the global disease burden in the community and produce a similar or better impact than increasing coverage among at-risk populations and elderly to 60% or even 90% (results not shown). Even an increase to 30% in these three age groups would reduce impact of influenza in all age groups by 24.2%, 7.6% and 28.8% respectively (Figure 2 A-C) Influenza vaccination in all children is the most efficacious intervention and amplifies indirect effects (figure 2 D) as previously demonstrated by Weycker [9]. While the “universal vaccination” scenario (high coverage in all age groups) shows the best overall results, all paediatric vaccination scenarios with coverage rates >50% show a significant impact on the overall number of cases and deaths (results not shown).

Conclusions

Our results confirm that children are a major vector of influenza transmission in this hypothetical model. Childhood influenza vaccination can reduce the incidence and mortality as a result of both, direct benefits to the vaccinee, and indirect benefits to others due to the reduced disease transmission. In our model, this kind of fencing strategy has a major impact on mortality among the elderly. Consequently, extending paediatric recommendations in addition to current influenza vaccination strategies should provide increased protection for at-risk populations by providing a barrier to transmission. Adding a cost perspective to this current research would be an interesting extension and allow a more complete evaluation of vaccination strategies in school aged children.

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Proceedings Topic #12

Virus Host Interaction/Pathogenesis

Poster Presentations

Interference-Dominance Properties of Cold-Adapted Influenza Viruses

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Live attenuated cold-adapted (*ca*) influenza vaccines are an effective means for the control of influenza, most likely due to their ability to induced both systemic and mucosal, humoral and cellular immune responses. Recent evidence has demonstrated that cold-adapted influenza vaccines have the additional benefit of providing protection shortly after administration prior to the development of the adaptive immune response. This activity is probably due to the phenomenon of viral interference. The following paper addresses concerns that *ca* influenza virus vaccines may somehow interact with wild-type (*wt*) viruses including avian strains. In the present study we investigated the interaction of temperature-sensitive *ca* reassortant vaccine viruses prepared using Russian cold-adapted master donor strain (MDS) A/Leningrad/134/17/57 (H2N2) over wild-type viruses and apathogenic avian virus.

Introduction

Development of intranasally administered live cold-adapted recombinant vaccines are areas of intensive research for the past four decades. The safety, immunogenicity and efficacy have been demonstrated for live cold-adapted reassortant vaccines developed by this method against influenza in different age groups (young, adults and the elderly) [1-4]. These vaccines have been in widespread use in Russia for several years. Live virus vaccines have advantages over inactivated virus vaccines because of induction a long-lasting and broader immune response, provide protection against antigenic drift and antigenically mismatch influenza strains [4-6]. Moreover because of induction of mucosal immunity in the upper respiratory tract, live virus vaccines can limit viral spread and provide herd immunity [7]. It have been shown, that in addition to its ability to induce broad spectrum of immunity, the cold-adapted reassortant vaccine influenza A viruses can interfere directly with the replication of wild-type viruses in mixed infections of either MDCK cells, or emryonated eggs [8,9]. In the our studies we investigated the interaction of temperature-sensitive *ca* reassortant vaccine viruses prepared using Russian cold-adapted master donor strain (MDS) A/Leningrad/134/17/57 (H2N2) over wild-type viruses and apathogenic avian virus.

Materials and Methods

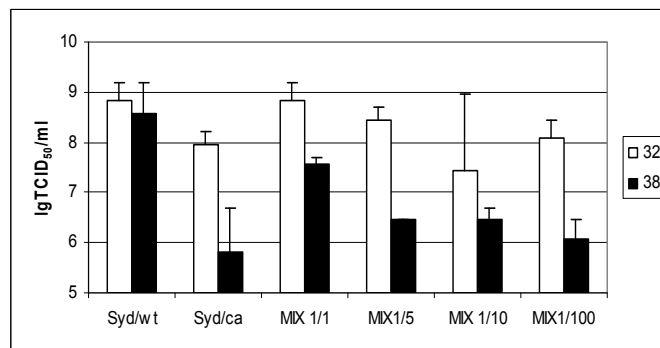
Viruses. Several live cold-adapted vaccine influenza strains prepared by genetic reassortment with *ca* Master Donor Strain A/Leningrad/134/17/57 (H2N2) (Len/17) and their wild-type parents were used in the study: A(H1N1) wild-type and cold-adapted 6/2 reassortant A/New Caledonia/20/99 (NC/*wt* and

NC/*ca*); A(H3N2)-wild-type and cold-adapted 6/2 reassortant A/Sydney/5/97 (Syd/*wt* and Syd/*ca*);A(H5N2): wild-type and cold-adapted 7/1 reassortant A/duck/Potsdam/1402-6/86 (dk/Pot/*wt* and Len17/H5) [10]. Viruses were propagated in allantoic cavity of 10-day-old embryonated hens' eggs at 34°C for 48 hours (human viruses) and at 37°C for 26 hours (avian virus). Fifty percent egg infectious dose (EID₅₀) titers were determined by serial titration of viruses in eggs and calculated by method of Reed and Muench [11]. **Cells.** MDCK cells were maintained in DMEM supplemented with 5% fetal calf serum, antibiotics and non-essential amino acids and used for virus titration. Approximately 1x10⁶ cells were infected with virus at various multiplicities of infection (M.O.I.) (0.001-10) for 1 hour, washed and cultured in 24-well tissue culture plates for 24 hours in DMEM medium contains 1% BSA, antibiotics and 5µg/ml TPCK-treated trypsin. Culture supernatants were collected and virus titers were determined and expressed as Log₁₀TCID₅₀/ml. **Animal experiments.** Eight-week-old male CBA mice were used in the study. Each group of mice was lightly anesthetized with ether and immunized i.n. with 50 µl containing approximately 10⁷ EID₅₀ of each virus separately or simultaneously. Control mice received 50 µl PBS i.n. Three days post-infection mice were sacrificed to obtain tissue samples. Lungs were titrated for virus infectivity estimated as Log₁₀EID₅₀. Weight loss was observed for 14 days after the infection.

Results and Discussion

Homologous interference of cold-adapted reassortant viruses and wild-type viruses in MDCK cell cultures. Cultures of MDCK cells were infected with either epidemic influenza virus A/Sydney/5/97 (H3N2) (Syd/*wt*) or with cold-adapted 6/2 reassortant vaccine virus A/17/Sydney/97/76 (H3N2) (Syd/*ca*) to give the same multiplicity of infection. The other cultures were infected by mixing these two strains to give *wt/ca* virus ratios: 1/1, 1/5, 1/10, 1/100. The growth of wild-type and cold-adapted viruses after separate and mixed inoculation with low MOI (MOI=0.001) are shown in Figure 1.

Figure 1. Replication of wild-type and cold-adapted A/Sydney/5/97 (H3N2) influenza viruses in MDCK cells after single and double inoculation at MOI=0.001 (average data of three experiments).



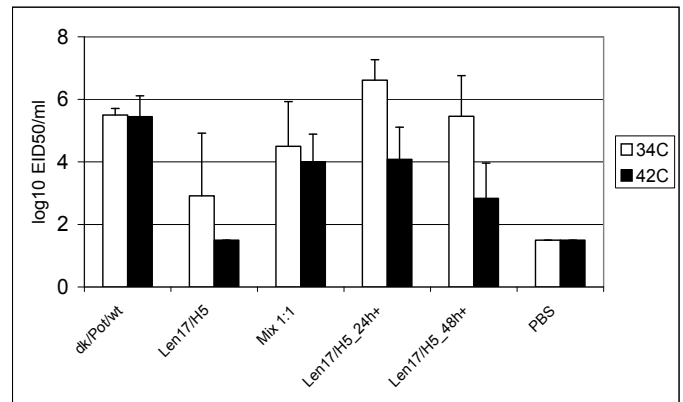
The yields after separate and mixed infections have been tested for infectivity at 32°C (permissive) and 38°C (restrictive for *ca*

virus) to estimate the influence of addition of *ca* virus on the growth of wild-type virus. *Syd/wt* virus grew at the same level at 32°C and 38°C, while the difference in levels of reproduction of *Syd/ca* at 32°C and 38°C was as much as 10^4 TCID₅₀. For the double infected cultures, the yields replicate in 100 times lower level at 38°C compare to replication at 32°C. If the proportion of *ca* virus in the inocula increased we observed more dramatic reduction in the replication capacity at 38°C.

Homologous interference of cold-adapted reassortant vaccine viruses over wild-type viruses in mice. To study whether or not interference observed *in vitro* will happen *in vivo*, a series of experiments were done in mice infected with wild-type virus, a cold-adapted reassortant vaccine strain or doubly infected with both viruses. Groups of male CBA mice were inoculated intranasally under light ether anesthesia with approximately 10^7 EID₅₀ of each strain or were inoculated with a mix of both viruses in a ratio of 1/1. Both H3N2 and H1N1 wild-type viruses replicated well in the lungs of mice, an average of 10^6 EID₅₀/ml; the differences in replication at 34°C and 39°C did not exceed $10^{1.5}$ EID₅₀/ml. Replication of *ca* viruses in the lower respiratory tract of mice was very limited ($10^{1.5}$ EID₅₀/ml). In mice that received a mix of both strains, the replication in the lungs was over 100 times less at 39°C in comparison to replication in the group that received the wild-type strain. The latter was observed when both H1N1 and H3N2 of *wt* and *ca* viruses were tested (data not shown). Next, we studied the replication of wild-type virus after sequential co-infection of cold-adapted virus. Groups of mice were inoculated with cold-adapted variant and 24 and 48 hours later were infected with homologous wild-type virus. The lung homogenates were collected from animals on day three after wild-type virus inoculation. It should be noted that if the time between inoculations (*ca* and *wt* viruses) was increased, we have observed a dramatic decrease in titers at 39°C. In mice that received *ca* vaccine virus and after 48 hours received wild-type virus, the titers in the lungs at 39°C were lower than 10^2 EID₅₀/ml, and the difference in replication at 34°C and 39°C was more than 10^4 EID₅₀/ml. Thus, it is possible that in the case of co-infection of mice with two viruses, some reassortment could take place resulting in production of mutants with temperature sensitive phenotypes or *ca* interference with *wt* virus (data not shown). We next evaluated the phenomenon of homologous interference during co-infection of cold-adapted reassortant avian vaccine influenza virus and wild-type avian influenza virus in mice. In Fig. 2, the levels of replication of avian *wt* and *ca* H5N2 viruses in the lower respiratory tract of mice after separate and mixed inoculation is shown. Avian influenza virus A/duck/Potsdam/1402-6/86 (H5N2) replicated well at 34°C as well as at 42°C and reached approximately $10^{5.5}$ EID₅₀/ml. Thermosensitive cold-adapted vaccine strain Len17/H5 demonstrated limited replication at 34°C (10^3 EID₅₀/ml); at 42°C the replication of this virus was undetectable. When these two viruses were mixed and inoculated into mice, replication in the lungs was $10^{1.5}$ - $10^{2.0}$ EID₅₀/ml at 34°C and 42°C and were lower compared to replication of wild-type virus. When these viruses were given sequentially and cold-adapted vaccine virus was administered 24 hours before

wild-type virus, replication in the lungs exhibited the same trend as simultaneous co-infection. However, when *ca* virus was given 48 hours before *wt* virus, titers in the lungs at 42°C was more than $10^{2.5}$ EID₅₀/ml, 500 times lower than in the group that received *wt* virus alone.

Figure 2. Replication of avian H5N2 wild-type and cold-adapted vaccine influenza viruses in the mouse lungs after separate and mixed inoculation. Mice were inoculated with 10^7 EID₅₀ of each virus intranasally with light ether anesthesia. Lung homogenates were prepared on day 3 after inoculation, in case of sequential administration on day 3 after inoculation of wild-type virus. Samples from three mice were titrated for determination infectivity on the 10 days old chicken embryonated eggs at the optimal 34°C and restrictive 42°C temperature for avian influenza virus. Histogram represents average values of virus titers in the lungs ±SD.



Summary

We observed interference-dominance effects of cold-adapted vaccine influenza viruses over wild-type viruses in MDCK cells. In the case of mixed infection, the replication of wild-type virus was reduced as much as 1000 times compared to the yield from a single infection at restrictive temperature. Interference was more obvious if the proportion of *ca* virus in the inocula was increased. In mice, replication of *ca* viruses in the lungs was diminished. Simultaneous inoculation of cold-adapted and wild-type viruses does not lead to increased pathogenicity over single infection of *wt* viruses. In the case of epidemic A(H1N1) and A(H3N2) influenza viruses as well as in the case of avian A(H5N2) influenza viruses, we observed homologous interference during co-infection of wild-type viruses and cold-adapted vaccine viruses. The reduction of *wt* virus titers in the lungs of mice infected by a mix of viruses have been seen at both permissive and non-permissive temperatures to various degrees, and was more dramatic if the time between inoculations exceeded 24 hours. The molecular mechanisms of interference are not completely understood. There is a possibility of the production of reassortants with a mutant *ts*-phenotype during co-infection of human cold-adapted and avian viruses that needs to be investigated. It is important to remember that a key characteristic of live cold-adapted influenza vaccines is that they prevail over wild-type viruses.

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Studies on Proteolytic Activation of Human Influenza Viruses in Stable Cell Lines Expressing Serine Proteases TMPRSS2 and HAT

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Recently, we demonstrated that the serine proteases TMPRSS2 and HAT from human airway epithelium cleave the hemagglutinin protein (HA) of human influenza A viruses *in vitro*. For further studies on HA cleavage by TMPRSS2 and HAT we generated stable cell lines, MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2, which express either protease under control of doxycycline-dependent transcriptional activation, supporting proteolytic activation and multicycle replication of influenza viruses in the absence of exogenous trypsin. By using these cells we found that HA cleavage and viral spread in HAT-expressing cells were completely inhibited in the presence of aprotinin and soybean trypsin inhibitor. By contrast, viral activation in TMPRSS2-expressing MDCK cells was hardly blocked by aprotinin and not affected by soybean trypsin inhibitor, but strongly inhibited in the presence of ovomucoid trypsin inhibitor and Pefabloc SC. In summary, we conclude that cell lines stably expressing TMPRSS2 and HAT can be used for studies on inhibition and mechanisms of HA cleavage by these proteases.

Introduction

The influenza A virus hemagglutinin (HA) is synthesized as a precursor protein that has to be cleaved by a host cell protease into the subunits HA1 and HA2. Cleavage of HA is a prerequisite for fusion of viral and endosomal membranes at low pH, and therefore, is an important determinant of pathogenicity [4]. Highly pathogenic avian influenza A viruses of subtypes H5 and H7 possess a HA with multibasic cleavage site (consensus sequence R-X-R/K-R) that is cleaved by ubiquitous endoproteases furin or PC6, thus allowing a systemic spread of viral infection [4]. In contrast, the cleavage of HA of mammalian and low pathogenic avian influenza A viruses with a single arginine at the HA cleavage site is restricted to the respiratory tract in mammals and the respiratory and intestinal tracts in birds. HA with monobasic cleavage site can be activated by trypsin *in vitro* [5, 6], but specific proteases that cleave HA *in vivo* have not been identified so far. Recently, we cloned the serine proteases TMPRSS2 (transmembrane protease, serine S1 family member 2) and HAT (human airway trypsin-like protease) from differentiated cultures of human tracheobronchial epithelial cells and demonstrated that both proteases cleave HA and support multicycle replication of human influenza A viruses *in vitro*. Therefore, these proteases

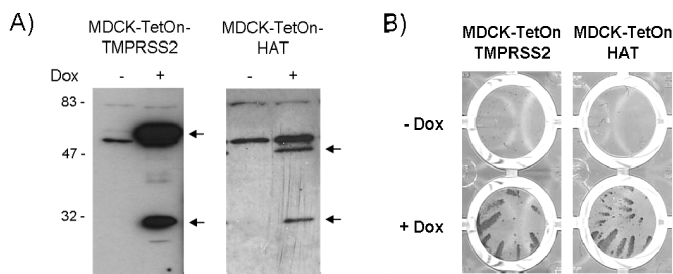
are plausible candidates for proteolytic activation of influenza viruses in the human airway epithelium *in vivo* [2]. Here we describe the generation of MDCK cells stably expressing TMPRSS2 or HAT, which represent a convenient experimental model for studies on virus activation by either protease.

Results and Discussion

Initially, we tried to generate stable cell lines, which constitutively express TMPRSS2 or HAT. However, none of the attempts was successful, perhaps due to a toxic effect of permanently over-expressed proteases in the cells. To solve this problem, we used the tetracycline-inducible gene expression system described by Gossen and Bujard [3]. MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2 cells were prepared by stable co-transfection of MDCK cells with a plasmid encoding for the transactivator protein rtTA and plasmids pTREpur-TMPRSS2-flag and pTREpur-HAT-flag, respectively, that contain the coding sequences of either protease under control of a rtTA-dependent promoter. Either protease was flag tagged at the C-terminus to facilitate protein detection. The constitutively expressed transactivator protein rtTA becomes an active transactivator of transcription in the presence of doxycycline (Dox). Under these conditions, rtTA binds to the Tet responsive element (TRE) in the promoter of the pTREpur-protease plasmids and thus, activates transcription and expression of TMPRSS2 or HAT. To analyze inducible protease expression in MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2, cells were maintained in medium with or without doxycycline for 24 h, and then cell lysates were analyzed by SDS-PAGE and immunoblotting. As expected, expression of either protease was observed in the presence of doxycycline (Figure 1A) in contrast to no expression in the control cells (Dox-). TMPRSS2 and HAT were detected as full-length proteins of 70 kDa and 46 kDa, respectively, and as truncated isoforms, presumably the cleaved catalytic domains (Figure 1A). Expression of different forms of these enzymes was described previously [1, 7]. To investigate whether induced expression of proteases in MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2 cells will ensure functional proteolytic activation of influenza viruses, cells were infected with human influenza virus A/Memphis/14/96 (H1N1) at a low multiplicity of infection with or without addition of doxycycline. At 24 h p.i. cells were fixed and immunostained for viral nucleoprotein (NP). Typical comet-like foci of infected cells were observed in the presence of doxycycline, indicating that progeny virions are efficiently activated. This effect is in contrast to the infection in the absence of doxycycline where only single initially infected cells were observed due to a lack of protease expression (Figure 1A). These results demonstrate that MDCK-TetOn-TMPRSS2 and MDCK-TetOn-HAT cells are suitable for studies on HA cleavage by the corresponding protease.

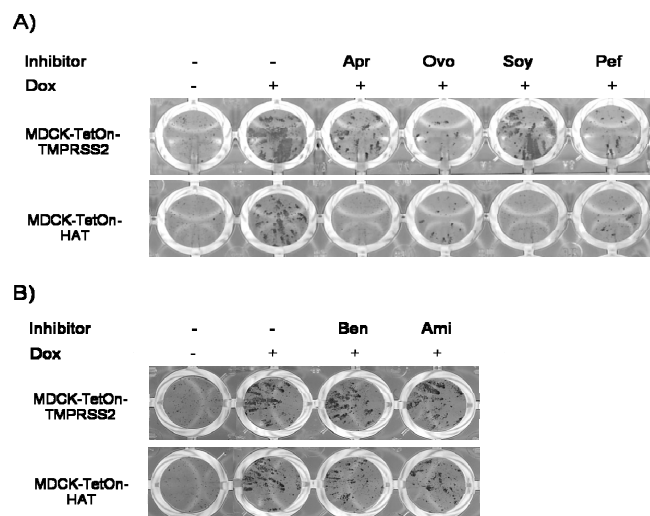
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Figure 1. (A) Doxycycline-inducible expression of hAT-flag and TMPRSS2-flag in MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2, respectively. Cells were incubated in medium with (+) or without (-) doxycycline for 24 h. Cell lysates were analyzed by SDS-PAGE and immunoblotting using antisera against the flag epitope. Labels on the left show molecular masses in kDa. (B) Virus infection in MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2 cells in the absence and presence of doxycycline. Cells were infected with A/Memphis/14/96 (H1N1) at a MOI 0.01 and incubated in the absence (-Dox) or presence (+Dox) of doxycycline for 24 h. Cells were immunostained for viral NP.



The identity of influenza virus-activating protease(s) in human airway epithelium is not defined, and little is known about specific properties of such proteases. Previous studies demonstrated that influenza infection in cultures of human airway epithelium and in human volunteers can be efficiently inhibited by the protease inhibitor aprotinin [8, 9]. Therefore, we wanted to examine the sensitivity of TMPRSS2 and HAT to aprotinin as well as to several other protease inhibitors. For this

Figure 2. Virus infection in MDCK-TetOn-TMPRSS2 and MDCK-TetOn-HAT cells in the presence of protease inhibitors. Cells grown in medium with (Dox+) or without (Dox-) addition of doxycycline were infected with influenza virus A/Memphis/14/96 (H1N1) at a MOI 0.01, incubated for 24 h, and immunostained for viral NP. (A) Infection in the presence of aprotinin (Apr), ovomucoid trypsin inhibitor (Ovo), soybean trypsin inhibitor (Soy) and pefabloc SC (Pef) (each 50 μ M). (B) Infection in the presence of benzamidine (Ben) and ϵ -aminocaproic acid (Ami) (each 50 μ M).



purpose, we tested the ability of various inhibitors to prevent formation of viral foci in infected cultures of MDCK-TetOn-HAT and MDCK-TetOn-TMPRSS2 cells. Aprotinin and soybean trypsin inhibitor completely blocked multicycle viral replication in MDCK-TetOn-HAT (Figure 1A) indicating that HAT is highly sensitive to these inhibitors. In marked contrast, these two inhibitors had only a marginal if any effect on viral spread in cells expressing TMPRSS2. Ovomuroid trypsin inhibitor and Pefabloc SC inhibited viral activation and spread in both cell lines (Figure 1A), whereas benzamidine and ϵ -aminocaproic acid had no effect (Figure 1B). These data suggest that particularly HAT due to its high sensitivity to aprotinin is an appropriate candidate for proteolytic activation of influenza viruses in the human airway epithelium. In summary, we generated stable cell lines with inducible expression of functional human serine proteases TMPRSS2 and HAT, that permit efficient spread of influenza virus infection in the absence of exogenous trypsin. This experimental system can be useful for further studies on detailed mechanisms of HA cleavage by either protease, including inhibition and cellular compartmentalization of HA cleavage, in order to determine the role of HAT and TMPRSS2 in influenza virus infection in vivo.

Acknowledgements

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Convergent Evolution of Interferon Resistance in Influenza A Viruses

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Contemporary strains of highly pathogenic avian influenza (HPAI) H5N1¹ and H9N2 strains replicate in the face of induced hypercytokinemia that include the antiviral type I interferons (IFN). Although resistance to IFN is controlled by the NS1 gene,² the mutational basis for differences in this property is unknown. Using reverse genetics we show that recombinant viruses that differed solely due to mouse-adaptive mutations in NS1 demonstrated increased resistance to IFN. Some NS1 mutations reduced viral replication indicating that viral defenses come at a cost to replication. IFN sensitivity mapped to NS1 binding sites for translation initiation factors and RNA, consistent with roles in protein synthesis. Furthermore experimental evolution of influenza virus in mice recapitulated natural evolution in birds where HPAI H5N1 strains had independently acquired 23A, 103L and 106I mutations. Convergent NS1 evolution indicates that the molecular basis for IFN antagonism is conserved among influenza viruses in both mammalian and avian hosts, having implications not only to antiviral therapy and infection control but also to predicting evolutionary events. Because type I IFN's (IFN α/β) are antiviral cytokines induced early during infection, influenza viruses express NS1 proteins that are IFN antagonists required for effective replication in the presence of IFN (reviewed³⁻⁵). The first influenza virus shown to possess enhanced resistance to IFN was HPAI A/Hong Kong/156/97 (HK97) (H5N1) that is more IFN resistant and more virulent due to unknown mutations in its NS1 gene². Based on the assumption that NS1 mutations selected on adaptation to increased virulence would increase resistance to IFN and provide a genetic mapping of IFN sensitivity we characterized NS1 mutations selected on experimental evolution of human influenza virus to high virulence in the mouse lung. Previous mouse adaptation by 20 serial lung to lung passages of the clinical human H3N2 influenza virus isolate, A/Hong Kong/1/68 (HK-wt), resulted in the selection of highly virulent mouse-adapted (MA) variant clones possessing specific NS1 gene mutations such as HKMA20 and HKMA20c with V23A or F103L mutations respectively⁶. Whereas HK-wt is avirulent for mice (median lethal dose (LD₅₀) >10^{7.7} plaque forming units (pfu), HKMA20 and HKMA20c have enhanced abilities to replicate in mouse lung causing fatal infections at dosages (LD₅₀ = 10^{3.0} and 10^{2.5} pfu respectively) that are comparable to pathogenic Asian H5N1⁷ and 1918 pandemic⁸ viruses (LD₅₀ ≤ 10^{2.9} and 10^{3.5} pfu respectively) and thus are expected to possess similar genetic properties. To further identify adaptive NS1 mutations, more

mouse-adapted clones were characterized including those from independent mouse-passaged series of A/HK/1/68 sister clones where three more distinct NS1 sequence variants were obtained: clones MA41 (M106V mutation), MA51 (M106I), and the MA53 double mutant (M106I+L98S) each of which induced significantly higher levels of IFN β associated with greater lung infection and pathology (data not shown). The effect of IFN on the yield of virus from infected cells during a single step growth cycle was monitored by infection of primary mouse embryo fibroblasts following pretreatment with or without saturating doses of mouse IFN α/β (200 U/ml). IFN sensitivity was assessed as the relative reduction (fractional yield) with and without IFN pretreatment. The HK-wt parent strain was inhibited to 1/15th of the untreated yield (ie 15 fold inhibition) by IFN whereas all mouse-adapted variants with NS1 mutations were more IFN resistant being inhibited from 2 to 4 fold indicating that they had acquired increased resistance due to mutation selected on mouse adaptation ($p < 0.05$, t test; not shown). To confirm that the observed NS1 mutations were controlling IFN resistance, viruses with recombinant genomes were generated that differed solely due to the presence of each of the 5 different mutant NS1 genes in isolation on the A/WSN/33 genetic backbone using the 12 plasmid system of Kawaoka⁹. It was possible to obtain wild type recombinant genome, rHK-wt, as well as variants possessing NS1 segments from MA20c, MA41, MA51 and MA53 mutants; however the MA20 NS1 gene was not rescuable. In addition, recombinants possessing the F103L and M106V genome segments were significantly attenuated in growth in both mouse fibroblasts and epithelium (data not shown). Assaying interferon resistance for recombinant WSN viruses in mouse epithelium (M1 kidney cells treated with 200 or 20 U/ml recombinant mouse IFN- β Figure 1a and b or Figure 1c and d) showed that all rescued NS1 mutants tested (M106V was omitted due to insufficient titers) demonstrated increased resistance to IFN, relative to the rWSN x NS/HK-wt, to levels that were comparable to those seen for the parental HK-wt and mouse-adapted strains shown earlier ($p < 0.01$ by t test, Figure 1). The M106I mutation was similar in IFN resistance when coupled with the L98S mutation in vitro however the mouse adapted virus with both mutations had previously demonstrated enhanced replication and IFN induction in mice. We also showed that WSN recombinants that expressed genome segment 8 from the pathogenic avian H9N2 strain, A/Chicken/Beijing/1/95 (CK/BJ) that possessed the NS1 23A, 103L and 106I mutations in combination was more resistant to IFN than any of the individual HK-NS1 mutants. This observation is consistent with a role for these mutations in IFN resistance in this naturally evolved NS1 gene. The mouse adaptive NS1 gene mutations map to RNA¹⁰ (V23A) and translation initiation factor binding sites for poly-A binding protein I¹¹ (PABP1) (V23A) and eukaryotic initiation factor 4G1¹² (eIF4GI) (L98S, F103L, M106I, and M106V) (Figure 2). While all 5 of these mutations were associated with IFN resistance in mouse-adapted variants, only the F103L and M106I mutations were confirmed to control IFN resistance in isolation.

Figure 1. Adaptive NS1 mutations increased resistance to IFN. The infectious yield of viruses from mouse epithelium, with (grey bars) and without (black), pretreatment with 200 (n=5) or 20 U/ml (n=3) of recombinant mouse IFN β , (panels a, b and c, d respectively). The fractional yield is calculated as (yield untreated)/(yield IFN treated).

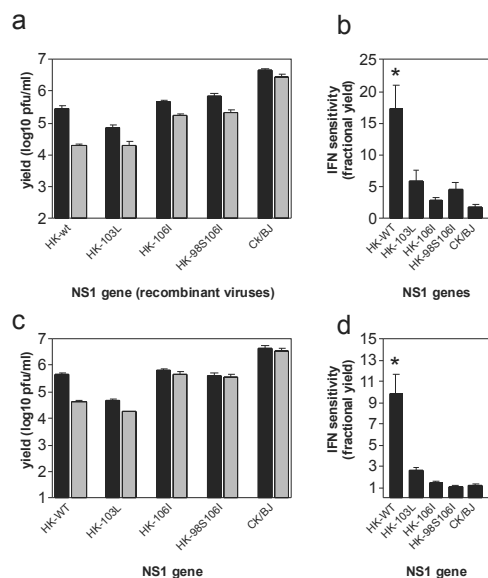
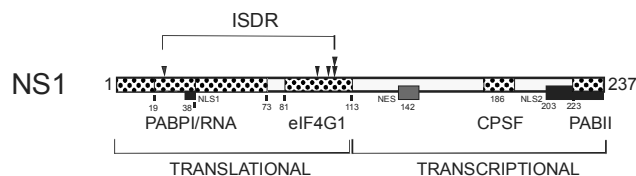


Figure 2. Adaptive NS1 mutations define an interferon sensitivity determining region (ISDR). NS1 mutations are indicated with arrows on a genetic map of NS1 interaction sites. Protein interaction sites are indicated for poly-A binding protein 1 (PABP1), eukaryotic initiation factor (eIF4G1), cleavage and polyadenylation specificity factor (CPSF), poly-A binding protein nuclear 1 (PABNP1).



Both virulent mouse-adapted and avian strains possess IFN resistance mutations in common. Specifically the 1997 H5N1 NS1 gene possesses 3 residues in common with those identified in this study (23A, 103L and 106I). Thus most of the 5 mouse-adaptive NS1 mutations were independently selected in avian viruses in avian hosts (most avian strains possess 23A) indicating that these mutations are general determinants of NS1 biology that are common among viruses with similar properties of high virulence but derived in different host species. We propose that the 3 identical NS1 mutations are also operating to resist IFN in these virulent avian viruses.

Methods

Rapid directed evolution. Mouse adaptation employed clones of the human influenza clinical isolate, A/HK/1/68 (H3N2) (HK-clone#) (not shown), that were serially passaged for 21 cycles of high dose infection in mouse lung before plaque isolation on MDCK cells as described previously (HK-(clone#)(MA-isolate#))⁶.

NS1 gene sequences were determined by direct sequencing of RT-PCR amplified cDNA as previously described⁶. Virus stocks were prepared in MDCK and chicken allantoic cavity and infectivity measured by MDCK cell plaque assay; titres are given in MDCK plaque forming units.

Reverse genetics. The A/HK/1/68 genome plus segment 8 of each mutant genome and A/Chicken/Beijing/1/96 (CK/BJ) were inserted into the pHH21 plasmid directly from cDNA using ligation independent cloning as described earlier¹³ and were rescued into HK⁶ or WSN virus backbones using MDCK and 293T cell co-culture as described by Kawaoka⁹. Virus populations were produced by 2 passages in MDCK cells. NS1 gene sequences were confirmed for rescued viruses.

Monitoring mouse Lung infection. Mice were infected intranasally and sacrificed 1 or 2 days post-infection for virus titration or IFN assay or immunofluorescent staining, respectively. Virus and IFN were titrated for extracts obtained by sonicating lungs in 4 parts (w/w) PBS on ice. Immunofluorescent and histopathological staining was performed on frozen sections as described previously¹⁴.

Interferon Sensitivity Assay. The effect of interferon treatment on reducing viral replication was determined by plaque assay of virus yields from Balb-c mouse embryo fibroblasts obtained by trypsinizing 13 day embryos (Charles River, Quebec) or mouse epithelium (M1 cells obtained from ATCC) that had been pretreated with defined dosage of IFN (20 or 200 U/ml) for 24 before infection for 0.5 h at an moi of 2, washing 2 with PBS, and incubation at 37°C for 24 hr in the presence of medium supplemented with trypsin. Mouse L929 cell IFN α/β was obtained from NIAID (Gu02-901-511) and recombinant mouse IFN β was obtained from Sigma-Aldrich Canada (#19032). Infectious yield was measured by MDCK plaque assay.

Interferon Assay. Mouse IFN β was titrated relative to control IFN by commercial ELISA as describe by the manufacturer PBL Biomedical Laboratories.

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Inefficient Transmission of H5N1 Influenza Viruses in a Ferret Contact Model

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The ability to infect and transmit efficiently among humans is essential for a novel influenza A virus to cause pandemic. To evaluate the pandemic potential of H5N1 influenza virus, a ferret contact model that comprised of one inoculated and two contact ferrets was used to study the transmissibility of four human H5N1 viruses isolated during 2003–2006. The effects of viral pathogenicity and receptor binding specificity (affinity to synthetic sialosaccharides with α 2,3- or α 2,6-linkages) on transmissibility were assessed. A/Vietnam/1203/04 and A/Vietnam/JP36-2/05 viruses, which possess “avian-like” α 2,3-linked sialic acid (SA) receptor specificity, caused neurological symptoms and death in ferrets inoculated with 10^3 TCID₅₀. A/HongKong/213/03 and A/Turkey/65-596/06 viruses, which show binding affinity for “human-like” α 2,6-linked SA receptors in addition to their affinity for α 2,3-linked SA receptors, caused mild clinical symptoms and were not lethal to ferrets. No transmission of A/Vietnam/1203/04 or A/Turkey/65-596/06 viruses was detected. One contact ferret developed neutralizing antibodies to A/HongKong/213/03 in the absence of clinical signs or detectable virus shedding. In two groups, one of two naïve contact ferrets had detectable virus after 6 to 8 days housing together with the A/Vietnam/JP36-2/05 virus inoculated ferrets. Infected contact ferrets showed severe clinical signs, although little or no virus was detected in nasal washes. This limited virus shedding explained the absence of secondary transmission from the infected contact ferret to the other naïve ferret that was housed together. Our results suggest that despite their receptor binding affinity, circulating H5N1 viruses retain molecular determinants that restrict their spread among mammalian species.

Introduction

As H5N1 influenza viruses expand their geographic and host range, it is of great concern if the viruses have gradually acquired the characteristics that allow efficient transmission between humans. To date, transmission to humans has been inefficient, occurring through close contact with infected poultry or consumption of its undercooked meat or blood (22), and reported probable human-to-human transmission has been limited (3,22,23). On the other hand, incidents of direct

transmission of H5N1 viruses from infected domestic or wild aquatic birds to mammalian hosts (human, felids, stone martens) continue to be reported (4,25,28). These incidences of direct avian to mammalian transmission events may allow the H5N1 viruses to gradually become adapted to and spread among mammalian species, including humans. Receptor distribution in the human airway is proposed to restrict efficient inter-human transmission of H5N1 influenza virus (19). Human influenza viruses specifically recognize α 2,6-linked SA receptors, which are dominant on epithelial cells in the upper respiratory tract (19). In contrast, avian influenza viruses specifically recognize α 2,3-linked SA receptors, which are located in the lower respiratory tract (19,24) and are not easily reached by the large droplets (diameter >10 μ m) produced by coughing or sneezing (1). Amino acid changes in the receptor binding site of the HA glycoprotein may alter the virus' receptor binding specificity, as observed with initial isolates from the human pandemics of 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) (13,21). Because some of the circulating H5N1 avian viruses have demonstrated un-characteristic affinity for α 2,6-linked SA receptors (20,26), it is important to evaluate their transmissibility in a suitable animal model. Ferrets have been shown to be an appropriate animal model (11) for study of the pathogenicity (6,12) and transmissibility (8,9) of influenza viruses. On the basis of H5N1 virus cell tropism in their lower respiratory tract, ferrets have also been proposed to be a good small animal model of human H5N1 pneumonia (24). We used a ferret contact model to study the transmissibility of four representative H5N1 viruses isolated from humans during 2003–2006. These isolates, selected from two different clades based on the phylogenetic analysis of HA gene, differ in their pathogenicity and in their affinity for avian-like (α 2,3-linked SA) and human-like (α 2,6-linked SA) receptors.

Materials and Methods

Viruses and cells. The A/Hong Kong/213/03 (HK/03), A/Vietnam/1203/04 (VN/04), A/Vietnam/JP36-2/05 (VN/05), and A/Turkey/65596/06 (TK/06) human H5N1 influenza virus isolates were obtained through the WHO Global Influenza Surveillance Network. Experiments were conducted in a USDA-approved biosafety level (BSL) 3+ containment facility.

Infectivity. The 50% tissue culture infectious dose (TCID₅₀) and plaque forming units (PFU) were determined in MDCK cells, and the 50% egg infectious dose (EID₅₀) was determined in 10-day-old embryonated chicken eggs. TCID₅₀ and EID₅₀ values were calculated by the Reed-Muench method (18).

Binding of virus to sialic acid-containing substrates. Virus binding affinity to fetuin (possessing both α 2,3- and α 2,6-linked SA) was tested in a direct solid-phase assay with the immobilized virus and horseradish peroxidase–conjugated fetuin (5). Virus binding affinity to sialylglycopolymers obtained by conjugation of a 1-*N*-glycyl derivative of 3'-sialyllactose (p3'SL) or 6'-sialyllactose (p6'SL) was tested by competitive assay based on inhibition of binding to peroxidase-labeled fetuin (14). The dissociation constants (K_{diss}) of virus–receptor analog complexes were calculated as the means of four individual experiments for each virus.

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Transmissibility in the ferret contact model. Transmissibility was tested in 4- to 6-month-old female ferrets of similar body weight obtained through the ferret breeding program at St. Jude Children's Research Hospital or from Marshall Farms (North Rose, NY). All studies were conducted under applicable laws and guidelines and after approval from the St. Jude Children's Research Hospital Animal Care and Use Committee. Each experimental group comprised one inoculated ferret and two naïve contact ferrets. Ferrets were inoculated intranasally with 10^3 TCID₅₀ of virus under isoflurane anesthesia. At 2 days post-inoculation (d.p.i.), two naïve contact ferrets were placed in the same cage with inoculated ferret. To monitor virus shedding, nasal washes and rectal swabs were collected from all ferrets every other day for 14 days and were titrated in MDCK cells. Seroconversion was monitored by determining the neutralizing antibody titers of the pre-exposure and post-exposure ferret sera by the method described previously (7). The susceptibility of ferrets that survived after a 21-day observation period was also confirmed by re-challenging with 10^3 TCID₅₀ homologous virus.

Collection and virus titration of organs. To determine the tropism and replication efficiency of the H5N1 viruses in ferret organs, virus titers (TCID₅₀/gram tissue) in organs were determined from two ferrets inoculated with 10^3 TCID₅₀ of each H5N1 virus on 5 d.p.i.

Histology analysis and immunohistochemistry. Formalin-fixed and paraffin-embedded tissue sections were stained with hematoxylin and eosin for histological evaluation or with pooled monoclonal antibodies against influenza A virus nucleoprotein (NP) protein for detection of influenza viral antigen.

Results

Selection and characterization of H5N1 viruses. Four H5N1 human isolates were selected from clades 1 (HK/03, VN/04, and VN/05 viruses) and 2 (TK/06 virus) based on the phylogenetic analysis of the HA genes. Three of these isolates contained amino acid changes in the conserved HA receptor-binding residues (Table 1). We measured the receptor binding affinity of the four H5N1 viruses to high-molecular-weight sialic substrates (Table 1). All four isolates exhibited affinity for p3'SL (synthetic sialosaccharides with α 2,3-linkage). The HK/03 and TK/06 viruses showed a greater affinity for p6'SL (synthetic sialosaccharides with α 2,6-linkage) than is typical of avian H5N1 influenza viruses, and both of these viruses had an N substitution at HA receptor binding residue 223. The growth and infectivity of the H5N1 viruses were comparable in MDCK cells and in embryonated chicken eggs (Table 1).

Transmission in ferrets. The transmissibility of the H5N1 viruses was assessed with the ferret contact model. No significant difference was observed between the four viruses' nasal wash titers on days 2, 4, and 6 after inoculation with 10^3 TCID₅₀ (Figure 1). Nasal wash titers usually peaked at 4 d.p.i. (range, 2-6 d.p.i.), and peak titers were 4.0-6.5 log₁₀ TCID₅₀. Between 2 and 6 d.p.i., virus (1.7-2.5 log₁₀ TCID₅₀) was also detected in the rectal swabs of ferrets inoculated with VN/04 and VN/05 (Figure 1).

Table 1. Affinity of the H5N1 influenza viruses to sialyl substrates and virus growth.

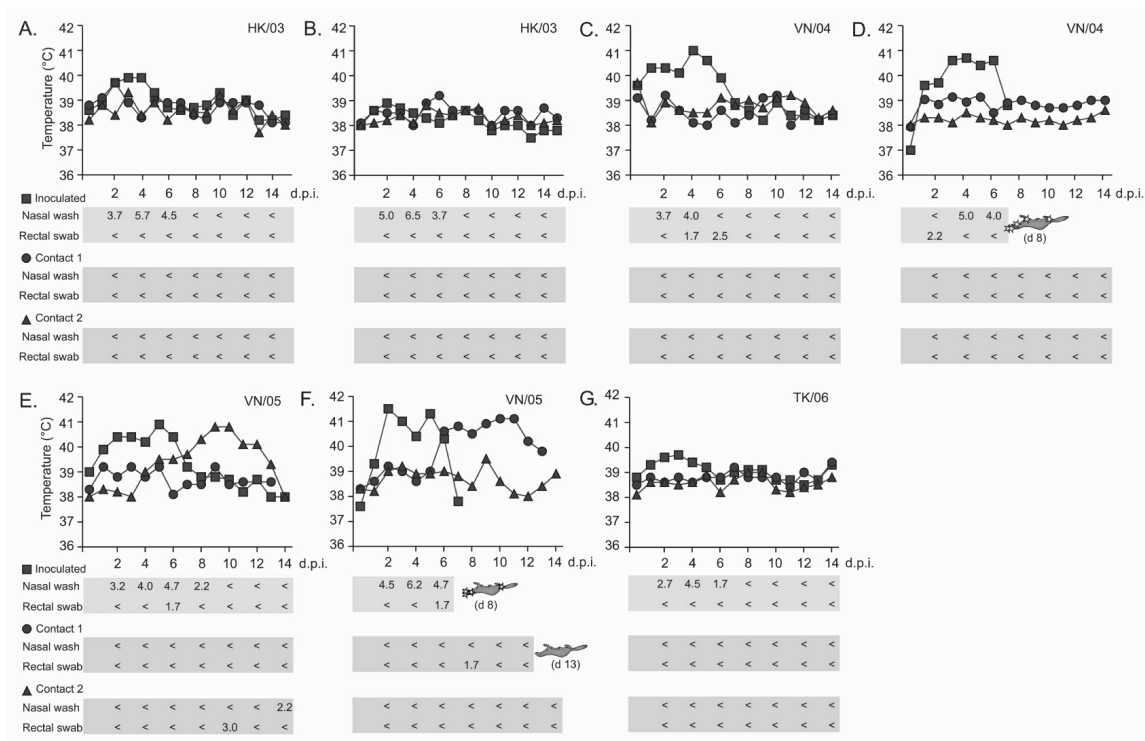
Virus ^a	Changes in HA receptor binding site ^b	Affinity for sialyl substrate ^c			Virus growth ^d		
		Fetuin	p6'SL	p3'SL	log ₁₀ TCID ₅₀ /mL	log ₁₀ PFU/mL	log ₁₀ EID ₅₀ /mL
HK/03, E2/E1	S ¹³³ , N ²²³	0.18 ± 0.03	5.78 ± 0.82	1.05 ± 0.21	8.5 ± 0.0	8.1 ± 0.03	8.5 ± 0.4
VN/04, E2/E1	S ¹³³ , S ²²³	0.13 ± 0.03	> 100 ^e	1.21 ± 0.05	9.0 ± 0.0	9.1 ± 0.01	9.5 ± 0.3
VN/05, C1/E1	A ¹³³ , S ²²³	0.15 ± 0.08	> 100 ^e	0.12 ± 0.02 ^e	8.4 ± 0.2	8.4 ± 0.1	9.3 ± 0.4
TK/06, E2/E1	S ¹³³ , N ²²³	0.15 ± 0.06	10.34 ± 1.40	0.44 ± 0.05	8.5 ± 0.0	8.3 ± 0.3	8.8 ± 0.4

- a Virus passage history: C, passaged in MDCK cells; E, passaged in embryonated chicken eggs
- b Changes in conserved residues. H5 numbering.
- c $K_{\text{diss}} = \text{mean} \pm \text{SE} \times t_{\alpha, n-1}$ (μM sialic acid), where t_{α} is Student's coefficient with probability $\alpha = 0.95$, from four independent experiments. Fetuin possesses both α 2,3- and α 2,6-linked SA, while p3'SL and p6'SL are synthetic sialosaccharides with α 2,3- and α 2,6-linkages, respectively.
- d Titers expressed in log₁₀ scale. All data are the mean \pm SD from three independent experiments.
- e $p < 0.05$, t-test

Transmission of virus from inoculated donors to naïve contact ferrets was confirmed by virus isolation from nasal washes or rectal swabs and/or by seroconversion. No transmission of the VN/04 or TK/06 viruses was detected. One of four contact ferrets exposed to HK/03 had developed neutralizing antibody (titer = 1:80) when tested after 21 days of contact with the inoculated ferret but did not shed detectable virus or show signs of illness (Figure 1). In two independent ferret groups, VN/05 virus was isolated from one of the two naïve contact ferrets of each group, after 6 and 8 days of contact with the inoculated ferret, respectively (8 and 10 d.p.i. of the inoculated ferret) (Figure 1). One contact ferret in each group remained seronegative after 21 days of contact (Figure 1), demonstrating inefficiency of VN/05 transmission as well as the absence of secondary transmission from the infected contact ferret to the other naïve contact. Although virus titers were high in the nasal washes of inoculated ferrets, little or no virus was detected in the nasal washes of the infected contact ferrets (Figure 1). Despite limited virus detection, both infected contact ferrets showed elevated temperature (maximum elevation at 2.8°C and 2.4°C to the baseline temperature, respectively) (Figure 1), lethargy, weight loss (24.2% and 11.4% to the original body weight, respectively), and neurological signs (uncontrolled movement or hind limb paralysis). One infected contact ferret died after 11 days of contact due to severe clinical symptoms.

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Figure 1. Temperature changes and virus Titers (\log_{10} TCID₅₀/mL) of human H5N1 influenza viruses in a ferret contact model. Shown are results for one donor (inoculated) and two recipient (contact) ferrets in two independent tests of each virus. (A,B) HK/03 virus; (C,D) VN/04 virus; (E,F) VN/05 virus; (G) TK/06 virus. Overturned ferret figures indicated time of death, and the yellow stairs indicate sites of virus isolation [virus was detected in the nasal turbinate, olfactory bulb, brain, trachea, lungs, and large intestine of VN/04 inoculated ferret (D); virus was detected in the nasal turbinate, olfactory bulb, brain, and large intestine (F)].



Pathogenicity of the H5N1 viruses in ferrets. The four human H5N1 isolates differed in their pathogenicity in inoculated ferrets. VN/04 and VN/05 were lethal and caused neurological signs, temperature elevation (maximum elevation, 2.4-3.7°C), and weight loss (11%-29%) similar to the disease signs previously reported (6,12). In contrast, ferrets inoculated with the HK/03 and TK/06 viruses showed only slight temperature elevation (0.9-1.4°C) and weight loss (2.4%-9.7%). All four human H5N1 isolates were detected in multiple organs; however, only the VN/04 and VN/05 viruses were detected in the brain. In contrast, the H3N2 human influenza virus was detected only in the nasal turbinate. Of the four H5N1 viruses, VN/04 replicated most efficiently in all ferret organs, except in the trachea. Differences in pathogenicity were also apparent on histopathologic examination. Histological changes in the brain, lung, and liver were detected from VN/04 and VN/05 infected ferrets (inoculated or contact ferrets) but, changes were only detected in the lungs of HK/03 and TK/06 infected ferrets.

Discussion

We studied the transmissibility of four 2003-2006 human H5N1 isolates that differed in their pathogenicity in ferrets and their binding affinity for synthetic sialosaccharides with α 2,3- and α 2,6-linkages. The inefficient transmission of both the low-pathogenic HK/03 and the high-pathogenic VN/05 viruses

suggests that there is no direct correlation between the viral pathogenicity and the transmissibility in this animal model. The inefficient transmission of HK/03 and the non-transmission of TK/06 virus demonstrates that the observed binding affinity for human-like α 2,6-linked SA receptors is not sufficient to allow efficient transmission of H5N1 virus among ferrets. It is likely that further adaptations are required for the surface glycoproteins as well as the internal genes of the avian-derived H5N1 viruses to cooperate efficiently with the mammalian cell machinery. In a previous study using the same experimental design, we found that ferrets inoculated with 10^3 TCID₅₀ of the human Wuhan/95 (H3N2) influenza virus shed virus in nasal washes for 6 days, with a peak virus titer ($6.3 \log_{10}$ TCID₅₀/mL) at 2 d.p.i. (27). Although some of the H5N1 viruses in this study had comparable peak nasal wash titers (4.0 - $6.5 \log_{10}$ TCID₅₀), the peak occurred later, at 4 d.p.i. (range, 2-6 d.p.i.). Whereas human H3N2 virus was transmitted efficiently from the inoculated ferret to both naïve ferrets after 2 to 4 days of contact (27), transmission of the VN/05 H5N1 viruses was not detected until after 6 to 8 days of contact, consistent with the reported incubation time for human H5N1 infection (2,10,17,22). The observation that Wuhan/95 (H3N2) virus (27) reached peak titer earlier than the four H5N1 human isolates suggests that replication efficiency in the upper respiratory tract may explain the greater transmission efficiency of the Wuhan/95 virus. The different replication efficiency

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of H5N1 and human H3N2 influenza viruses in the upper respiratory tract may reflect the preferential targeting of different subsets of human airway epithelial cells by viruses with human-like vs. avian-like receptor binding specificity (15). However, it was also shown recently that in spite of lacking $\alpha 2, 3$ -linked SA receptors, productive H5N1 viral replication can still be detected in the *ex vivo* cultures of human nasopharyngeal, adenoid, and tonsillar tissues (16). We observed the HK/03 and TK/06 viruses did not show significantly greater replication efficiency in the upper respiratory tract (reflected by nasal wash and nasal turbinate titers) or the trachea than did VN/04 and VN/05 viruses, suggesting that additional molecular determinants restrict their replication efficiency in mammalian cells. The two contact ferrets infected with VN/05 virus experienced severe pneumonia, neurological signs as well as systemic virus spread, and one died of infection. However, little or no virus was detected in the nasal washes. Inefficient viral replication in the upper respiratory tract, together with pneumonia and systemic virus spread in these contact ferrets, suggests that infection may have initiated in the lower respiratory tract via small infectious droplet nuclei (1)(19). Further, the detection of virus in the rectal swabs of both VN/05 infected contact ferrets suggests that virus may have infected animals via feces-contaminated food or water. Overall, our results show that circulating H5N1 viruses, including those with increased receptor binding affinity for human-like synthetic sialosaccharides with $\alpha 2, 6$ -linkage, are not transmitted efficiently in the ferret contact model. However, the transmission of the HK/03 and VN/05 viruses indicates that some of the circulating H5N1 viruses may transmit better than others in mammals. The molecular determinants for efficient transmission appear to be complicated and required further detailed studies.

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Structural and Biochemical Properties of Clinical Influenza Viruses Passaged in Human CACO-2 Cells

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CACO-2 cell line derived from human intestinal epithelium was used to isolate virus from influenza patients in Moscow in 2003. Influenza H3N2 viruses were isolated from CACO-2 with higher efficiency than in MDCK cells and chicken eggs. (1) The increased tropism to CACO-2 cells correlated with high adsorption of clinical viruses to human cells. The ratio of sialic receptors of the 2-3 and 2-6 types was found to be similar in CACO-2 and MDCK suggesting that not only sialic acid, but also additional cellular coreceptor could be involved in the adsorption/entry process of clinical viruses to human cells. (2) Quasispecies characterized by deletion of 66 nucleotides (22 amino acids) in stalk region of the NA gene was dominant in naso-pharyngeal washes of all patients whereas during passaging in CACO-2 and MDCK cells of isolates from different patients it was either stably retained as prevalent quasispecies or rapidly replaced for that one containing full length NA. (3) During passaging of virus strains in CACO-2 and MDCK cells concordant strain-specific mutations occurred in HA and NA implying the existence of different patterns in functional cooperation between HA and NA in human and avian influenza viruses. (4) Virus isolates 2003 showed an about 2-fold increase in the number of glycosylation sites of HA and NA when compared to isolates from 1968-1970. (5) The M2 protein of Moscow isolates was sensitive to amantadine. The data suggest that (i) clinical viruses possess specific adsorption/entry mechanism into human cells and (ii) increasing numbers of glycosylation sites in HA and NA and short stalk of NA facilitate influenza virus survival in humans.

Introduction

Influenza viruses have the capacity to cross interspecies barriers and adapt to new hosts [7]. Transmission to man may give rise to new pandemic viruses which then undergo further genetic evolution in the human host by two principle processes: (i) adaptation to host cell factors and (ii) changes in antigenicity that allow escape from the immune response of the host. Monitoring of virus genome changes can throw light on the mechanism(s) of virus virulence and adaptation to a new host. This study is concerned with virus changes appearing after the transfer of viruses from human patients to cell cultures, such as MDCK (canine kidney cells) and embryonated chicken eggs usually used for virus isolation [5]. We describe a system to minimize interspecies-dependent changes in virus isolates that arise following culture. We have used human cells (CACO-2) for

isolation of virus from influenza patients [8].

Results and Discussion

Replication of clinical viruses in CACO-2 and MDCK cells was studied by the method of immune foci. The CACO-2 and MDCK cell cultures were infected by viruses passaged in the CACO-2 (C-virus) and MDCK (M-virus) cells and incubated under agarose to observe the size of viral foci [8]. Thus, C-viruses were found to be 10^2 - 10^4 more infective for the CACO-2 cells than for the MDCK cells, whereas the M-viruses did not display such a difference and had a similar tropism to both cell cultures. Laboratory viruses A/Aichi/68 (H3N2) and A/PR/8/34 (H1N1) effectively replicated in both cell cultures producing large foci of similar size. Then the virus adsorption on receptors of CACO-2 and MDCK cells was studied. The C- and M-viruses were biotinylated and virus adsorption was assessed by biotin-specific staining [7]. The viruses passaged in CACO-2 cells more effectively adsorbed on the CACO-2 than on the MDCK cells, whereas the M-viruses adsorbed similarly on both cells. Thus, the selective adsorption of the C-virus on the CACO-2 cells correlated with its higher infectivity to these cells. Differences in adsorption of influenza viruses are known to be associated with the ratio of 2-3 and 2-6 receptors on the cells [3]. We determined contents of the receptors in the CACO-2 and MDCK cells using the selective binding of the SNA (Calbiochem 431792) and MAA (Sigma L8025) lectins specific for receptors of 2-6 and 2-3 types, respectively. Fractions of the cells positive by the 2-6 and 2-3 receptors were about 60% both in CACO-2 and MDCK cultures. Thus, these two cultures were enriched in both type receptors that allowed us to exclude a trivial explanation of the higher affinity of C-virus for CACO-2 cells than for MDCK by the higher content of the 2-6 receptors. Next, it was suggested that the difference in the adsorption of the C- and M-viruses on the CACO-2 and MDCK cells could be caused by host-dependent modification of the viral proteins HA and NA. To elucidate the role of secondary cell-specific modifications (glycosylation, sulfatation, acylation, etc.) of the HA and NA proteins in the adsorption of the viruses, the C- and M-viruses were cross-grown in MDCK and CACO-2 cells, respectively. After one-cycle cross-replication of the virus its hemagglutination profile, infectivity for the CACO-2 and MDCK cells, and amounts of virus in the cell medium and on the infected cells were evaluated (Table 1). First, the host exchange was not accompanied by changes in the hemagglutination profile of the virus. After cross-replication C-virus retained its "human" profile and agglutinated only human erythrocytes and M-virus did not change its "avian" phenotype and agglutinated both avian and human erythrocytes. Second, in the cross-infected cells the virus ratio on the cell surface and in the extracellular medium was different. The virus content was high on the surface of MDCK cells infected by the C-virus and it was associated with formation of small size foci (Table 1). This observation could be explained by weak neuraminidase elution of the M-virus from the CACO-2 cells at the stage of viral budding and its insufficient transmission onto the neighboring

cells. It is possible that function of viral NA is influenced by host-specific variations in oligosaccharide chains localized in the active site region.

Table 1. Virus progeny in CACO-2 and MDCK cells cross-infected with M- and C-virus variants.

Input virus	Target cells	HA profile ¹⁾ hum chk	Virus amounts (#) ²⁾ cells c.f.		Focus size ²⁾
Moscow/343/03 (C ₁₂)	MDCK	2 ⁷ <2 ³	38	62	M
	CACO-2	2 ⁷ <2 ³	41	59	M
Moscow/343/03 (M ₁₂)	MDCK	2 ⁷ 2 ⁷	2	98	M
	CACO-2	2 ⁷ 2 ⁶	95	5	S
Aichi/2/68	MDCK	2 ⁸ 2 ⁸	5	95	L
	CACO-2	2 ⁸ 2 ⁸	3	97	L

1) CACO-2 and MDCK cells were infected (MOI ~1) with C- and M-variants of the A/Moscow/343/03 virus (passage 12) or laboratory virus A/Aichi/2/68 (H3N2) grown in chicken embryos, and 30 h.p.i. culture fluids (c.f.) the cell pellets (cells) were collected. In these samples, the hemagglutination titers were determined with 1% human (hum) and chicken (chk) erythrocytes and virus titers were evaluated by immune focus titration in CACO-2 cells. Considering the obtained virus titers and initial volumes of samples, the virus amounts (%) were determined.

2) Virus foci developed in target cell monolayers under agarose overlay containing trypsin (in the case of MDCK) and 3 days after infection were immune-stained and analyzed using light microscope (x60). By the amounts of virus-positive cells the foci were identified as large (L), middle (M), and small (S) containing 250 and more, 50-150, and 1-10 cells per focus, respectively.

Human CACO-2 cells displayed an increased susceptibility to clinical viruses and isolates passaged in this culture (homologous viruses), than to heterologous variants passaged in canine cells. Importantly, both the increased adsorption and infectivity of clinical viruses in homologous cells were not associated with the difference in the contents of the main sialyl-containing cellular receptors of the 2-6 type. This parameter was virtually the same in MDCK and CACO-2 cells. It suggests that something other than terminal residues of sialic acid in the cellular receptor could regulate host dependence of virus reception and the whole transmembrane complex with its cell-specific environment could be involved in this process. Such a receptor complex could be formed by oligosaccharide chain and protein backbone of the receptor itself and additional cellular coreceptor(s). This receptor/coreceptor complex of human CACO-2 cells seems to be more adequate than in MDCK cells for replication of clinical influenza viruses. Primary structures of HA (GenBank ac.n. DQ 086157-086161, 089634-089639), NA (DQ 090706-090710, 091199), and M (DQ 098266-098269, 100422-100424) genes of three H3N2 viruses A/Moscow/328/2003, A/Moscow/343/2003, A/Moscow/346/2003 isolated from three influenza patients were studied. Viral genes in clinical washes and early (passages 4-6) and late (p12-15) virus passages in CACO-2 and MDCK cells were compared. First, general analysis of HA genes has shown 12 and 11 potential N-glycosylation sites (motif Asn-X-Thr/Ser) in the HA of strain 328 and 343, 346, respectively. 6 new sites appeared after 1968 located in antigenic areas A and C (amino acids 122-165[6]) in the vicinity of the HA receptor center. The appearance of the latest 11'th and 12'th sites in the HA coincided with the loose ability of modern clinical influenza H3N2 viruses to agglutinate chicken erythrocytes and to infect MDCK cells. Thus, it seems likely that during evolution human

influenza H3N2 viruses increase the number of glycosylation sites to protect HA polypeptide backbone from antibody attacks and to enhance its specificity to human receptors. Moreover, this hyperglycosylative influenza virus evolution hides a threat of the future inefficiency of killed and subvirion vaccines because massive carbohydrate cover in HA and NA will protect their polypeptide backbone from neutralizing antibodies. Second, comparison of polypeptide part of the HA receptor center, which is formed by aa 98, 134-138, 153, 155, 183, 190, 193-195, 224-229 [6], of C and M variants did not reveal any regular changes specifically-linked with passaging in CACO-2 or MDCK cells. The most important positions 193, 226, and 228 were occupied by Ser, Val, and Ser, respectively, showing a human motif possessing affinity to sia2-6gal receptors. Only between C and M variants of A/Moscow/328/03 and A/Moscow/343/03 strains homologous mutation Val226→Ile and heterologous one Lys453→Glu between C and M variants of A/Moscow/328/03 strain (GenBank DQ089635 and DQ089636) were revealed, whereas C and M variants of strain 343 did not have these mutations (ac.n. DQ089637, DQ089638). These observations suggest that ability of M variants to agglutinate chicken erythrocytes appeared after 3-5 passages in MDCK, in contrary to C variants lacking this property [9], was not coupled with mutations in the HA receptor center and could be provided by the other mechanisms specific for each virus strain. The most prominent changes were revealed in primary structure of the NA gene. (1) Ten potential N-glycosylation sites were identified whereas early 1968 viruses had 7 sites. (2) Evolutionary amino acid mutations were found to accumulate in the antigenic areas A, B, and C [10]. Additionally, high variability was revealed in amino acid positions 143-148, 430, 434-438, and 462-469 that allows us to refer to this area as an additional antigenic domain D in human influenza N2. (3) Significant differences between C and M variants were determined in A/Moscow/328/03 virus. The M-virus variant had mutations Thr148→Ile, Asp151→Glu and lost the N-glycosylation site at Asn 146. Moreover, in contrast to all variants of strains 343 and 346, C and M variants of strain 328 had substitution Asn245→Ser removing glycosylation site at this position. (4) All Moscow clinical isolates 328, 343, 346 were found to contain 7 residues of U at 5'-region in the NA gene. Before 2000 all human H3N2 strains contained 5-6 residues of U in this tract. Significance of this poly U tract lengthening in influenza virus biology is not clear. All Moscow clinical viruses isolated in CACO-2 cells were found to contain the deletion of 66 nucleotides (22 amino acids at positions 35-58) in stalk region of NA gene. Sequencing of the NA gene isolated from naso-pharyngeal washes has shown exactly the same 66 nt deletion in all isolates from different patients (ac.n. DQ886602, 886603, 887750-51). Stability of the NA deletion during virus propagation in cultured cells was different among virus isolates. Strains 328 and 356 rapidly lost the NA deletion and restored full NA gene after 2-3 passages in CACO-2 and MDCK cells whereas viruses 343 and 346 stably retained deletion during at least 20 passages in both cell lines. Rapid restoration of the NA length is most probably explained

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by the existence of the full length NA quasi-species in virus population in human respiratory tract. This quasi-species of certain strains, such as 328 and 356, was more replicative in cultured cells and rapidly become dominant in virus population whereas in the other strains, such as 343 and 346, the shortened NA did not give replicative advantages in cultured cells. These observations suggest that (i) population of human influenza H3N2 viruses in one outbreak is heterogeneous, and (ii) shortening of NA gene reflects adaptation of influenza H3N2 virus to replication in human respiratory tract.

Table 2. Patterns of the NA deletion and coupled HA and NA glycosylation sites in human and avian influenza viruses.

Characteristics	Human (Moscow H3N2)		Avian (H5N8, H7N1, etc.)	
HA glycosylation	Asn ₁₆₅ (+)	Asn ₁₆₅ (-)	Asn ₁₅₈ (+)	Asn ₁₅₈ (-)
NA stalk region	L	S	S	L
NA glycosylation	Asn ₂₄₅ (-)	Asn ₂₄₅ (+)	Asn ₂₃₅ (+)	Asn ₂₃₅ (-)
	Asn ₁₄₆ (+)	Asn ₁₄₆ (+)	Asn ₁₄₇ (+)	Asn ₁₄₇ (+)

HA and NA glycosylation sites and stalk deletion in the NA gene were obtained here for human A/Moscow/2003(H3N2) viruses and for avian H5N8, H7N1, H5N1, H7N3 viruses were taken from [1,4]. S and L mean short and long forms of NA, respectively.

It was reported that laboratory influenza A viruses adapted to chicken eggs could have a large deletion (from 33 to 48 nucleotides in length) in the stalk region of the NA [2]. This stalk deletion in the NA was considered as characteristic of a chicken-adapted avian influenza viruses [4]. To maintain efficient replication in MDCK the short-stalked NA of different avian influenza viruses was found to require the HA containing glycosylation site at Asn158. Conversely, the HA lacking Asn158 appeared to complement reciprocally with the long-stalked NA [1,4]. In our study of human clinical viruses H3N2 this NA-HA cooperation differed significantly from avian viruses (summarized in Table 2). First, in contrast to avian strains, the shortened NA of all human strains was coupled with the lack of the HA glycosylation-site at Asn165 (equivalent to Asn158 in H5 and H7). Second, short-stalked NA in strains 343, and 346 was coupled with the additional NA glycosylation-site at Asn245 in close vicinity to the NA active site, while in strain 328 the long-stalked NA did not contain the Asn245 glycosylation-site. These data suggest that human clinical viruses differed from avian viruses in patterns of reciprocal compensatory mutations in HA and NA during adaptation to cells of different origin.

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Effects of Cyclosporin A on the T Cell Immune Response and Pathogenesis in Specific Pathogen Free Chickens in Response to Infection With a Low Pathogenic Avian Influenza H9N2 Subtype Virus

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The pathogenesis and host immune responses of T cell suppressed chickens in response to infection with a low pathogenic avian influenza (LPAI) virus of the H9N2 subtype were investigated. Cyclosporin A (CsA) treatment led to suppression of cell mediated immunity such as CD8⁺ T cells and reduced expression of IFN- γ mRNA. T cell suppression correlated with high viral load in the oropharynx and cloaca of H9N2 LPAI virus infected specific pathogen free (SPF) chickens. Elevated levels of viral RNA in peripheral blood lymphocytes was present only in immunocompromised chickens. Viral protein and associated cellular apoptosis was observed in the kidneys of immunocompromised chickens, particularly in those which died. Our findings suggest that T cells and T cell-mediated responses may be important in viral clearance and the pathogenesis of H9N2 LPAI virus infection in immunocompromised SPF chickens and may help to explain the pathogenesis of human influenza virus infection particularly in immunocompromised persons.

Introduction

As two previous pandemic strains (H2N2 in 1957 and H3N2 in 1968) were also derived from LPAI viruses [1], the LPAI H9N2 virus subtype may be an important candidate for a future human pandemic [2]. Infection of the LPAI H9N2 subtype virus in agricultural poultry settings sometimes results in fatal disease complicated by the presence of secondary pathogens [3] or co-infection with immunosuppressive viral diseases. Similarly, human patients receiving immunosuppressive drug therapy appear to be more susceptible to influenza infection [4]. Recently, peripheral lymphopenia has been described in human patients infected with the H5N1 subtype [5, 6]. Therefore, we hypothesized that immunosuppression could enhance the pathogenicity of LPAI H9N2 subtype virus infection in SPF chickens. Here, the immunopathogenesis of the LPAI H9N2 virus subtype in immunocompromised SPF chickens was investigated.

Materials and Methods

Chickens and virus. Three week-old SPF chickens and A/HS/K5/01 [H9N2](HS/K5) virus were used. All experiments using infectious virus were conducted in a biosafety level 2 (BSL-2) facility.

Cyclosporine A (CsA). 100mg of cyclosporine A ('Cypol', Chongkundang, Korea)/kg body weight was administered by intramuscular injection in the pectoral muscle of a 3 week-old chicken every 3 days throughout the experiment.

Grouping. Sixty four chickens were divided into four groups. Ten days prior to infection, chickens in group 1 and 2 were injected with CsA. At 31 days of age (experiment day 0 day p.i.), chickens in group 2 and 3 were inoculated by intranasal route with 10⁶EID₅₀/100 μ l LPAI H9N2 virus. Group 4 was untreated control.

Virus titration. On day 2, 3, 5, and 7 post infection (p.i.), virus shedding from oropharyngeal and cloacal swabs was evaluated by egg inoculation. On days 5 p.i., chickens in each group were sacrificed and trachea, thymus, lung, spleen, kidney, bursa, cecal tonsil and peripheral blood mononuclear cells were collected for virus detection by quantitative real time RT-PCR [7].

FACs analysis & quantification of cytokine mRNA expression level. On day 1, 2, 3, 5, 7, and 14 p.i., blood was collected into heparin tubes from chickens of each group. For FACs analysis, 10⁶ lymphocytes were incubated with anti-chicken CD3, CD4, CD8, and $\gamma\delta$ T cell monoclonal antibodies (Southern Biotech, USA) at 4°C for 30 min. Positively stained cells were analyzed by FACScan (Becton Dickinson, USA) and WinMDI 2.8 software. mRNA expression levels of IFN- γ , IL-18, IL-1 β , and IL-6 were determined by quantitative real-time RT-PCR, using QuantiTect™ Probe RT-PCR (Qiagen, USA) as previously described [8].

IHC & TUNEL staining. On days 5, and 14 p.i., brain, trachea, lung, thymus, heart, liver, spleen, cecal tonsil, bursa, and pancreas were collected from 3 chickens of each group and fixed in 10% buffered formalin. IHC staining was performed in an automated staining machine (Ventana Medical Systems Inc., USA) using Mab against type A influenza A nucleoprotein (Serotec, UK). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was carried out using the In Situ Cell Death Detection Kit, POD (Roche, Germany).

Results

Clinical signs and virus shedding of chickens after infection. Five out of eight chickens in CsA+H9N2 group (group 2) showed clinical signs such as facial and eye swelling, depression, and weight loss (Table 1). Two out of 8 chickens in this group died and they had significantly higher virus shedding than the group 3 on 5 days p.i. in cloacal swabs ($p < 0.05$). Only one out of eight chickens in H9N2 group (Group 3) showed eye swelling, and they had no mortality.

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Table 1. Clinical signs and mortality in CsA treated 3-week-old SPF chickens after challenge with H9N2 LPAI virus.

Group	Treatment	Clinical signs		Mortality			
		Sick	B.W (g ± S.D) ^A	Dead	MDT(days) ^B	PI ₁₄ ^C	HI log ₂ ± S.D.
1	CsA	0/8	616.8 ± 4.1	0/8	-	0	0
2	CsA + H9N2	5/8	486.8 ± 99.7**	2/8	7	1.225	6.3 ± 0.5
3	H9N2	1/8	557 ± 61.3 [†]	0/7	-	0.125	6.4 ± 0.5
4	Control	0/8	687.4 ± 76.2	0/8	-	0	0

^A Body weight was measured 14 days post infection.

^B Mean death time.

^C Pathogenicity index : the mean score per bird per observation over a 14 day period when each day, birds are scored 0 if normal, 1 if sick, 2 if dead.

** $p < 0.01$ by Student's t-test compared with chickens in CsA group (Group 1).

[†] $p < 0.05$ by Student's t-test compared with uninfected control chickens.

Virus detection from tissues or PBMC on days 5 p.i. Presence and titer of virus within the PBMC was successfully detected by quantitative real-time RT-PCR using Taqman[®] probe. In PBMC, virus was detected only in all chickens in CsA+H9N2 group (Group 2), and virus titer was significantly higher ($p < 0.001$) than that of chickens in H9N2 group (Group 3).

Lymphocytes subpopulation & cytokine mRNA levels in PBMCs. The percentage of CD8+ T cells of chickens in H9N2 group (Group 3) were significantly elevated ($p < 0.05$) on days 5 p.i.,

in comparison with uninfected chickens (Group 4). However, CD8+ T cells of chickens in CsA+H9N2 group (Group 2) were dramatically decreased (Fig. 1, $p < 0.05$) on days 5 p.i., in comparison with chickens in CsA group (Group 1). As shown in Fig. 2, IFN- γ mRNA levels of chickens in CsA+H9N2 group (Group 2) decreased between 1 and 5 day p.i. However, IFN- γ mRNA levels of chickens in H9N2 group (Group 3) significantly increased on days 5 p.i. ($p < 0.05$).

IHC & TUNEL assay. In IHC staining, influenza viral antigens were detected only in tubular epithelial cells and lymphocytes within the kidneys of chickens which died on days 7 p.i. in the CsA+H9N2 group (Group 2). The kidneys of chickens which died on days 7 p.i. in CsA+H9N2 group (Group 2) were markedly stained in brown, indicating cellular apoptosis.

Discussion

In mice, evidence has shown that although influenza virus induces a protective antibody response [9], cytotoxic T lymphocytes (CTL) play a more functional role in recovery from infection and resolution of pneumonia [10]. It is also known that cell-mediated immunity plays a critical role in protection of chickens from early infection of the virus [11]. CD3⁺ lymphocyte numbers in H5N1-infected individuals correlated inversely

Figure 1. Flow cytometric analysis of the effect of CsA treatment and H9N2 infection on the relative proportion of CD3+ (A), CD4+ (B), CD8+ (C) and non-CD8+CD4+ (D) T cells in the peripheral blood of SPF chickens. * $p < 0.005$ by student's t-test.

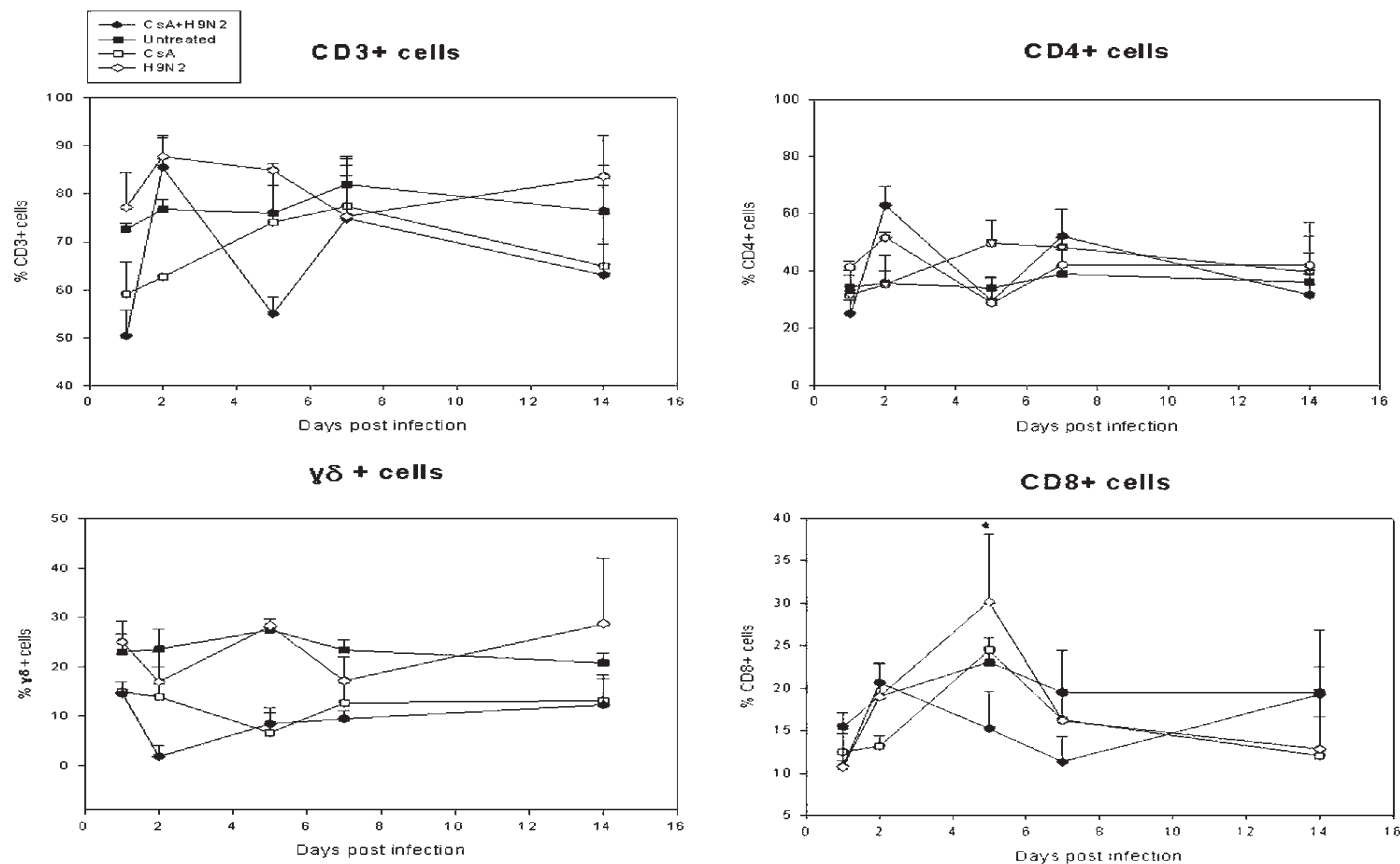
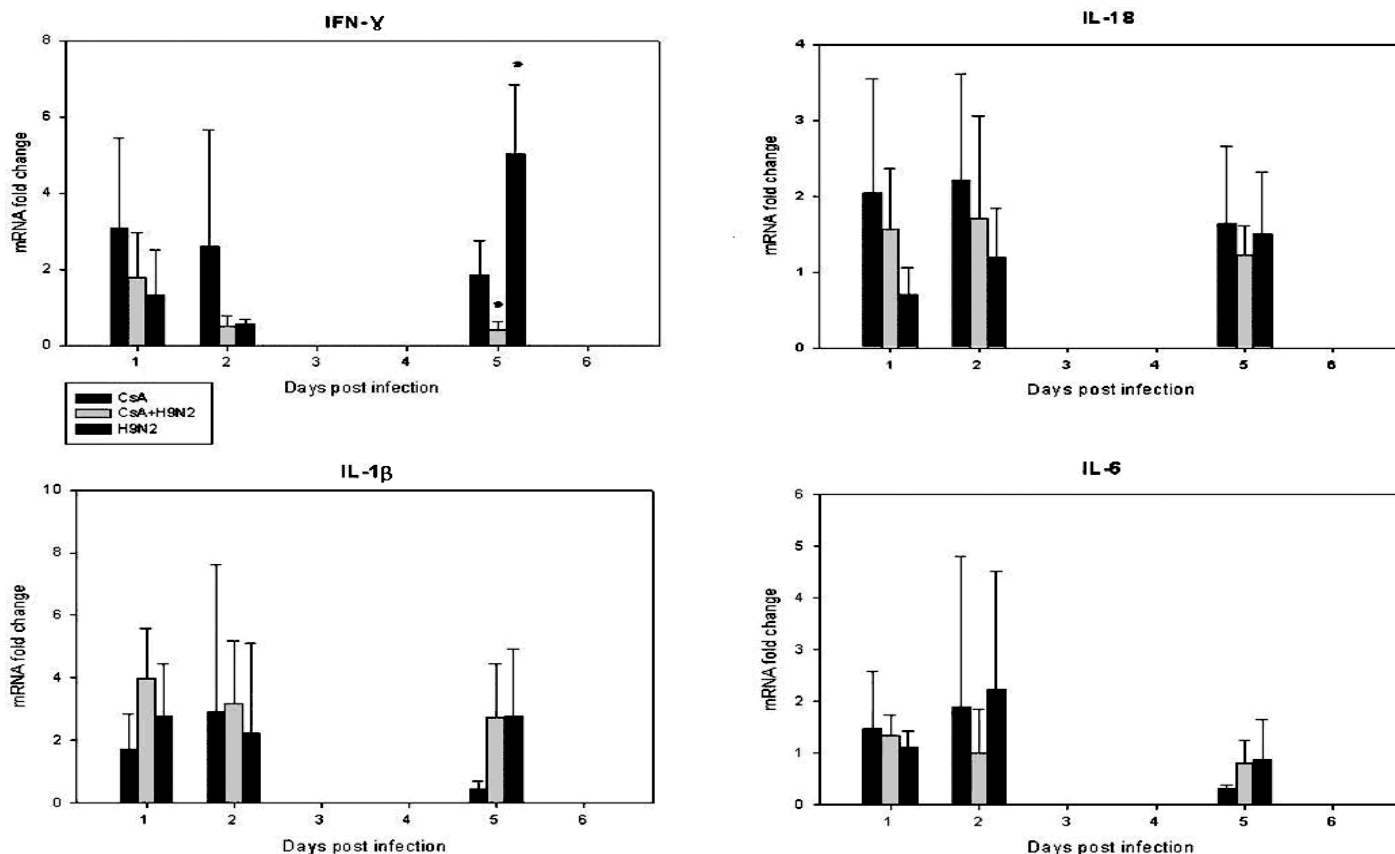


Figure 2. Quantification of cytokine mRNA extracted from peripheral blood mononuclear cells (PBMCs) of chickens after H9N2 LPAI virus experimental infection. The error bars show standard error for triplicate samples from three birds in experiment. * $p < 0.005$ by student's t-test.



with pharyngeal viral RNA load, suggesting an association between lymphopenia and the level of viral replication [5]. Here, we determined CsA induced CD8⁺ T cell suppression, decreased INF- γ mRNA level, higher level of virus shedding from oropharynx and cloaca, and associated mortality in chickens. These results indicate that T cell immunity plays an important role for the pathogenesis of the H9N2 LPAI virus in SPF chickens. Diminished cell mediated immune responses such as CD8⁺ T cells and INF- γ gene expression hinder viral clearance. Apoptosis of kidney cells caused by LPAI virus infection may contribute to the pathogenesis of the virus. These findings reveal pathogenic mechanisms by which immunocompromised chickens succumb to infection with the H9N2 influenza virus subtype.

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Does H9N2 Avian Influenza Virus Acquire High Pathogenicity For Chickens?

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H9N2 avian influenza virus strains have caused outbreaks in domestic poultry in Eurasian countries, giving serious economic losses. Since H9N2 viruses have been isolated not only from domestic birds but from pigs and humans, H9 virus is one of the candidates causing next pandemic in humans. In the present study, we investigated whether virus with H9HA become highly pathogenic avian influenza virus strain by acquisition of a pair of dibasic amino acid residues at the cleavage site on the HA molecule. The mutant H9 viruses with basic amino acid residues by substitution at the cleavage sites of the HA were infectious but remained apathogenic for chickens. Mutant H9 viruses with HA that acquire a pair of dibasic amino acid residues by insertion mutation were not recovered, indicating that the insertion of basic amino acid residues at the cleavage site of the H9HA may lead to disfunction of the HA molecule.

Introduction

Each of the known subtypes of influenza A virus (H1 to H16 and N1 to N9) are circulating in water birds, especially in migratory ducks [1]. Virus isolates from water birds usually do not infect chickens. Low pathogenic avian influenza viruses (LPAIVs) capable to infect chickens emerge through multiple passages in geese, quails, or turkeys. LPAIV may become highly pathogenic for chickens after chicken-to-chicken transmission more than six months [2]. Subtypes of the hemagglutinin (HA) of highly pathogenic avian influenza viruses (HPAIVs) are restricted to H5 and H7 subtypes [2]. The HAs of HPAIV differ from those of LPAIV by virtue of possessing insertion of more than a pair of di-basic amino acid residues at their cleavage sites [3]. This HA is susceptible to ubiquitous proteases such as furin and PC6, which recognize multiple basic amino acids, to cleave the HA and to spread to a variety of organs, leading to systemic infection. By contrast, HAs of LPAIV do not possess a series of basic amino acids at the cleavage site and are cleaved only by trypsin-like proteases which are secreted from the cells in the respiratory or intestinal tracts, so that the virus replication is localized, resulting in mild or asymptomatic infections. In recent years, H9N2 avian influenza virus strains have caused outbreaks in Eurasian countries, giving serious economic losses (Figure 1) [4-7]. Since H9N2 viruses have been isolated not only from domestic birds but from pigs and humans, H9 virus is one of the candidates that cause next pandemic in humans [8]. In the present study, we investigated whether virus with H9HA become HPAIV strain for chicken by artificial mutation to substitute or insert a pair of di-basic amino acid residues at the cleavage site on the HA molecule.

Figure 1. Occurrence of H9N2 LPAI in poultry since 2001.



Materials and Methods

Virus. A/chicken/Yokohama/aq55/01 (H9N2) (Y55) was kindly provided from Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan), who isolated the virus from chicken meat imported from China upon quarantine [9]. The virus was propagated in 10-day-old chicken embryos for 48h at 35°C.

Reverse genetics. Viral RNA was extracted from the allantoic fluid of chicken embryos infected with Y55 by using a commercial kit (TRIzol LS Reagent, Sigma-Aldrich, St.Louis, MO, U.S.A) and reverse-transcribed with the Uni12 primer [10] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A). The viral complementary DNAs were cloned into pCR2.1-TOPO vector (Invitrogen) or pGEM-T Easy Vector (Promega, Mannheim, Germany). After confirmatory sequencing, T-vector clones were digested with *BsmBI* and inserted into pHW2000 vector [11]. MDCK cells and 293T cells were maintained in Minimum essential medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% calf serum and D-MEM (Gibco BRL, Gaithersburg, MD, U.S.A) containing 10% FBS, respectively. Before transfection, confluent 293T and MDCK cells in 75cm² flask were trypsinized, and 10% of each cell line was mixed in 12ml Opti-MEM I (Gibco BRL); 2ml of this suspension was seeded into one well of six-well tissue culture plates (Nunc Inc., Naperville, IL, U.S.A). The cocultured 293T and MDCK cells were used for the transfection. TransIT-293 (Panvera, Madison, WI, U.S.A) was used according to the manufacturer's protocols to transfect the cells. Briefly, 2ul of TransIT-293 per 1ug of DNA was mixed, incubated at room temperature for 45min, and added to the cells. The transfection mixture was replaced with Opti-MEM I after 6h of incubation at 37°C. Thirty hours later, Opti-MEM I containing 1ug/ml trypsin was added. At 48 to 72 h post-transfection, the culture supernatant was collected and propagated in 10-day-old chicken embryos. The recovered viruses were stored and titrated as described below.

Site-directed mutagenesis. The desired mutations were introduced into the HA genes using a QuikChange II site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The mutant viruses

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rescued as described above, and the HA genes were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

Plaque assay. Confluent MDCK cells were incubated 1h at 35°C with 10-fold dilutions of virus. The cells were washed and overlaid with MEM containing 0.7% Bacto-agar (Difco, Sparks, MD, U.S.A) and 0.5ug/ml of trypsin and incubated at 35°C for 2 days.

Measurement of the intravenous virus pathogenicity index (IVPI). IVPI test was performed as described by Capua and Mutinelli [12].

Results and Discussion

We generated mutants of A/chicken/Yokohama/aq55/01 (H9N2) (Y55) with basic amino acid residue substitutions at the cleavage site by site-directed mutagenesis and reverse genetics. The amino acid sequences at the cleavage sites of their HAs are as follows: Y55 (PARSSR/G), mY55A (PARSKR/G), mY55B (PARKKR/G), mY55C (PRRKKR/G), and mY55D (RRRKKR/G) (Table 1).

Table 1. Property of H9N2 mutant viruses.

Viruses	Amino Acid sequence in HA cleavage site	PFU/ml in MDCK cells		IVPI
		Trypsin +	Trypsin -	
WT Y55	PARSSR/G	1.0×10 ⁸	- *	0.0
RG Y55	PARSSR/G	1.2×10 ⁸	-	0.0
mY55A	PARSKR/G**	2.3×10 ⁹	-	0.0
mY55B	PARKKR/G	1.3×10 ⁷	-	0.0
mY55C	PRRKKR/G	8.9×10 ⁷	-	0.0
mY55D	RRRKKR/G	9.0×10 ⁷	-	0.0

*No plaque formation.

There were no differences in growth and infectivity in MDCK cells and chicken embryos between original virus (WT Y55) and mutant strains. Thus, it was revealed that the substitution of basic amino acid residues did not interfere with the replication of the virus. Mutant viruses required trypsin-like protease to replicate effectively in MDCK cells. HI titer of the serum samples obtained from IVPI test showed that mutant viruses infected chickens (data not shown). IVPI of each of the mutant viruses showed 0.0 as did WT Y55, H9 mutant viruses, thus, did not acquire pathogenicity for chickens. These results suggest that mutant viruses with the HA with basic amino acid residues by substitution at the cleavage sites of the H9HA did not cause systemic infection in chickens because their mutated HAs were cleaved only by trypsin-like protease which are secreted from cells in the respiratory and intestinal tract. Mutant H9 viruses with HA that acquire a pair of dibasic amino acid residues by insertion mutation were not recovered (data not shown), indicating that the insertion of basic amino acid residues at the

cleavage site of the H9HA may lead to dysfunction of the HA molecule.

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Polarity of Influenza H5N1 Virus Infection in Respiratory Epithelial Cells and the Impact of Basolateral Release of Cytokines in Disease Pathogenesis

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Introduction

Highly pathogenic avian influenza virus (H5N1) is the first avian influenza virus that documented to cause respiratory disease and death in human. The biological basis for the severe human disease and high fatality rate remains unclear. We have previously demonstrated that when compared to human H1N1 and H3N2 influenza viruses, infection of influenza H5N1 virus led to the hyper-induction of pro-inflammatory cytokines in human primary macrophages and non-polarized respiratory epithelial cells in vitro. We also reported that patients with H5N1 disease have unusually high serum concentration of chemokines, e.g., IP-10. We therefore hypothesized that the differential hyper-induction of pro-inflammatory cytokines may contribute to the unusual severity of human H5N1 disease. In human, pathology of influenza A (H5N1) viruses are primarily respiratory pathogens targeting the respiratory epithelium, resulting in high level of cell death and tissue damage. As respiratory epithelial cells are primary target cells for replication of influenza viruses, a full understanding of how influenza virus interacts with respiratory epithelium is vital to advance our knowledge of its tropism and pathogenesis.

Methods

Primary human respiratory (bronchial and alveolar) epithelial cells were isolated and seeded onto collagen-coated transwell filters and cultured under air-liquid interface until cells were polarized and differentiated. The replication kinetic of influenza virus was assessed by virus yield titration (TCID₅₀) in MDCK cells. The cytokines expression profile induced by influenza H5N1 viruses (A/HK/483/97 and A/Vietnam/3046/04) was compared with human H1N1 virus by quantitative RT-PCR and ELISA.

Results

Polarized and differentiated bronchial and alveolar epithelial cells models are established for the study (Figure 1). We demonstrated that influenza A viruses (H5N1 and H1N1) can infect and productively replicate in the bronchial and alveolar epithelial cells leading to cytopathic effects. We found that the respiratory epithelial cells infected on the apical rather than the basolateral surface showed high levels of viral replication. Progeny influenza virus was released into the apical chamber at titers up to 3 logs higher than those recovered from the

basolateral surface of polarized cell cultures (Figure 2). Influenza H5N1 viruses were more potent inducers of cytokines in human respiratory epithelial cells when compared to the H1N1 virus. We show that these cytokines are released preferentially at the basolateral aspect of the polarized epithelial cells (Figure 3).

Conclusion

Our data suggest that influenza H5N1 viruses enter and are released from the apical domain of respiratory epithelium, while the pro-inflammatory cytokines are released preferentially from the basolateral aspect of the respiratory epithelium. These finding may provide important insights into the mechanism of replication and pathogenesis of influenza H5N1 virus in humans.

Figure 1. Polarized and differentiated bronchial and alveolar epithelial cells cultured at air-liquid interface using transwell inserts.

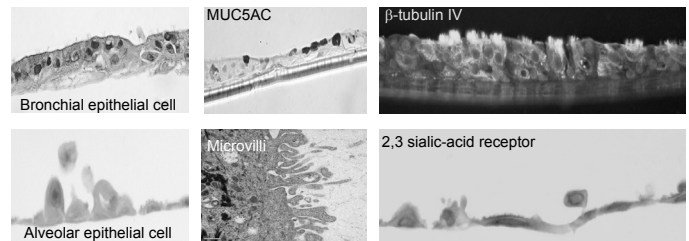


Figure 2. Apical entry and release of influenza H5N1 virus in human respiratory epithelium. The replication kinetic of influenza virus infected through the Apical (▲) and Basolateral (◻) surface of the respiratory epithelium was assessed by virus yield Titration (TCID₅₀/MI) at 48 hours post infection.

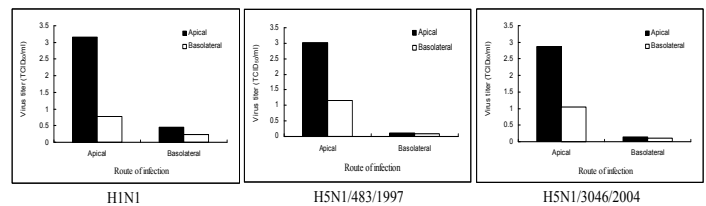
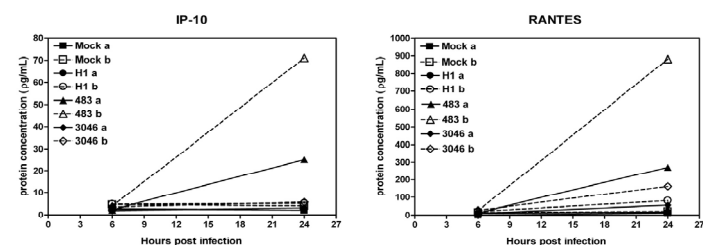


Figure 3. Basolateral release of proinflammatory cytokines induced by influenza (H5N1 and H1N1) viruses in human respiratory epithelium. IP-10 and RANTES were detected by ELISA from the cell supernatant collected from the Apical (A) and Basolateral (B) Surface of transwell inserts post infection.



Pathogenesis of H5N1 Avian Influenza Virus Reassortants in Chickens

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Highly pathogenic H5N1 avian influenza viruses produce severe disease and mortality in chickens. Identification of viral genes important for cell tropism and replication efficiency helps identify virulence factors. To determine which viral gene or genes contribute to the virulence of H5N1 avian influenza viruses in chickens, we used reverse genetics to generate single-gene recombinant viruses and examined their pathogenicity in chickens. Intranasal inoculation of two week-old chickens with the recombinant avian influenza virus rEgret/HK/02 resulted in 100% mortality and high viral titers in tissues. Inoculation of chickens with rCk/Indonesia/03 produced only 50% mortality with significantly less viral replication in tissues. Reassortants combining genes of the mentioned viruses demonstrated that exchanging the hemagglutinin and PB2 genes considerably affected pathogenicity; this was reflected in differences in mortality, viral replication and spread in tissues. The NS gene also had an effect on viral replication and spread in tissues; however, no effect on mortality was observed. Differences in mRNA cytokine levels in lung and spleen were observed among the different groups, indicating a role of the innate immune response on the outcome of infection.

Introduction

Highly pathogenic (HP) H5N1 avian influenza viruses (AIV) produce severe disease and mortality in chickens. The study of the factors that determine influenza virus virulence is an area of research that has important implications for public health and agriculture. The determinants of pathogenicity of influenza virus are not totally defined. Previous studies have pointed towards the importance of different influenza virus genes in determining virulence in various hosts. H5 and H7 influenza viruses containing a motif of multiple basic amino acids adjacent to the cleavage site of the hemagglutinin (HA) glycoprotein display a broader range of tissue replication and that leads to a systemic disease in chickens that is usually fatal (10). However, the cleavage site is not the only factor that affects the virulence of AIV. It is apparent that other AIV genes contribute to the severity of the HP phenotype, including those that encode the polymerase and receptor specificity (3). Another gene, the nonstructural (NS) gene, and one of its products, NS1, enhances AIV pathogenicity by preventing the induction of interferon (IFN)(1, 6), an early step in the innate defense of the host against the virus. To further explore which viral genes contribute to the virulence of H5N1 avian influenza viruses in chickens we used

reverse genetics to generate single-gene recombinant viruses. Two HPAI H5N1 viruses were used in this study to generate these viruses; A/Egret/HK/757.2/02 and A/CK/Indonesia/7/03. These viruses have the same multiple basic amino acids (aa) in the cleavage site of the HA gene, however have different virulence phenotypes. We generated single-gene reassortant viruses containing the HA, NA, PB2 and NS genes of A/Ch/Indonesia/7/03 in the A/Egret/HK/757.2/02 background and studied their pathogenicity in chickens.

Materials and Methods

Generation of infectious reassortant viruses. Two HPAI H5N1 viruses, A/Egret/HK/757.2/02 and A/CK/Indonesia/7/03 were used in this study for the generation of reassortant viruses. The parent viruses induced similar mortality in chickens but different pathology in ducks. Construction of transcription and expression plasmids and generation of reassortant viruses were performed as previously described (5, 7).

In vivo characterization of reassortant viruses. Two-week-old SPF Leghorn chickens were intranasally inoculated to determine the pathogenicity of the recombinant H5N1 viruses. The experimental design has been previously described (8). Briefly, chickens were separated into a control group and 6 virus-inoculated groups of 11 birds each. Virus-inoculated groups were given 10^5 EID₅₀ of the reassortant viruses. Two birds from each group were euthanized at 2 days post-inoculation (dpi) and their tissues collected in formalin for histopathology. Immunohistochemistry was conducted to determine the extent of virus replication in tissues. Portions of the brain, lung, spleen, skeletal muscle, and heart were also collected for virus detection by real-time RT-PCR. The remaining 8 birds were observed for signs of illness over a 14 day period.

Real-time RT-PCR for AIV. Viral RNA from the tissues collected at 2dpi was extracted with Trizol LS (Invitrogen Corp., Carlsbad, CA) according to the manufacturers' instructions. The RNA was analyzed by real-time RT-PCR (RRT-PCR) with AIV matrix lyophilized beads as previously described using a SmartCycler II (Cepheid, Sunnyvale, CA) (2). The results were reported as the average obtained from tissues from two birds per group and is expressed as 40 - cycle threshold (40-C_t) values.

Quantitative RRT-PCR for IFN alpha and IL-4. Cytokine mRNA levels in control and AI infected bird tissues were quantitated as previously described (4). Total RNA was prepared from spleen and lung cells from three birds using the RNeasy mini kit (Qiagen, Valencia, CA). Primers for IL-4 and IFN-alpha were designed using sequences obtained from GenBank. Quantitative RRT-PCR was performed using the One-step RT-PCR kit containing SYBR Green (Bio-Rad Laboratories, Richmond, CA). Amplification and detection of specific products were performed using the Bio-Rad iQ5. A standard curve was generated for the cytokine and 28S rRNA-specific reactions. Each RRT-PCR experiment contained the appropriate controls. Tissues from each bird were individually tested in triplicate.

Results and Discussion

In order to examine the role of influenza virus genes in the pathogenicity of the virus in chickens, we studied the differences in virulence of recombinant H5N1 viruses by determining pathology, distribution of virus antigens and viral load in tissues (Tables 1 and 2). Differences in the severity of avian influenza virus infection were associated with marked differences in viral replication. Cell tropism of the viruses was similar; however the level of viral staining in tissues varied depending on the virus and correlated with the viral load in tissues as measured by real-time RT-PCR. The presence of lesions in the different tissues examined also correlated with the presence of AIV antigen staining. Comparison of gene sequences from the recombinant rEgret and rIndo viruses showed a 98.1% aa similarity and 11 different aa in the HA gene. The rEgret NA was 95.2% similar with rIndo presenting a 20 aa stalk deletion and also 21 aa differences. The PB2 gene was 98.7% similar at the aa level among these viruses with 10 aa differences, and the NS gene was 95.8% similar with 11 aa differences. Inoculation of chickens with the rescued recombinant virus rEgret resulted in 100% mortality and high viral replication in tissues. Inoculation of chickens with the rescued rIndo virus produced only 50% mortality with significantly less viral replication in tissues. Reassortant viruses demonstrated that the HA, NS and PB2 genes affect the pathogenicity of the virus; however the neuraminidase (NA) gene did not appear to have any effect since viral replication of the reassortant virus containing this gene was similar to the rescued rEgret virus, even though this gene differed substantially among the two viruses. Exchange of the HA gene considerably affected virulence which was reflected in decreased mortality and viral replication and spread in tissues demonstrating the importance of this gene in pathogenesis of the virus. The HA genes had identical cleavage sites however there were 10 aa differences, some of them located on the receptor binding site of the glycoprotein, which could explain the differences observed in pathogenicity of the viruses. The NS gene also had an effect on viral pathogenesis; however, had no effect on mortality. Several studies have reported that the NS1 protein is responsible for IFN antagonist activity (1, 11). The virus bearing the NS gene from A/CK/Indonesia/7/03 induced a weaker IFN response than the other viruses. However this did not result in a replication advantage indicating that other factors are involved. Exchange of the PB2 gene, contrary to what was expected, resulted in higher viral replication and spread in tissues compared. The introduction of this gene into the regret backbone resulted in a replicative advantage thereby increasing its virulence. This corroborates the important role for the polymerase genes in influenza virus pathogenesis (9). Differences in mRNA cytokine levels in lung and spleen of infected birds were observed among the viruses studied. IL-4 and IFN alpha levels in the lung were significantly increased for rEgret and rEgret/Indo-NA when compared to controls, which correlates with the pathogenicity of these viruses. However the cytokine response measured for rEg/Indo-PB2 in the lung was lower than expected considering it was also pathogenic. IL-4

and IFN alpha levels in the spleen were significantly decreased in chickens infected with rEgret/Indo NS. Cytokines are an important component of the adaptive immune response and can affect disease manifestation. It should be noted that we only measured IL4 and IFN alpha in 2 different tissues at only one time point. Clearly more work on the correlation between cytokine response and viral replication, cell tropism, and pathogenicity needs to be conducted.

Table 1. Mortality, mean death time and viral antigen distribution in 2-week-old chickens inoculated intranasally with 10^5 EID₅₀ of the reassortant viruses. Tissues collected from two chickens at 2 days after inoculation.

	rEgret	rIndo	rEgret / Indo-HA	rEgret / Indo-NA	rEgret / Indo-NS	rEgret / Indo-PB2
Mortality	100%	50%	75%	100%	100%	100%
Mean death time (days)	3.0	7.0	4.2	3.1	3.2	2.2
Trachea	++	-	+	++	+	++
Lung	++	-	+	++	+	+++
Heart	++	-	-	++	+	+++
Brain	++	-	-	++	-	+++
Pancreas	-	-	-	-	-	++
Adrenal	++	-	-	-	-	+
Intestine	+	-	-	-	-	+++
Liver	+++	-	-	++	+	+++
Kidney	+	-	+	++	+	++
Spleen	++	-	-	++	++	+++
Bursa	+	-	-	-	+	+++
Thymus	+	-	+	+	+	+++
Muscle	+	-	-	-	-	++
Gizzard	+	-	-	++	-	++
Proventriculus	+	-	-	++	-	+
Gonads	-	-	-	++	+	+++
Cecal tonsils	+	-	-	++	-	+++
Esophagus	++	-	-	-	+	++

- = no antigen staining; + = infrequent; ++ = common; +++ = widespread

Table 2. AIV real-time RT-PCR results from tissues collected from 2 chickens two days after inoculation with the reassortant viruses. Results reported as 40-cycle threshold values (40-CT).

	Brain	Lung	Spleen	Heart	Muscle	Average all tissues
rIndo	4.5	4.1	4.0	4.9	4.5	4.4
rEgret	13.9	14.5	14.6	14.5	14.5	14.4
rEgret /IndoHA	6.8	6.7	5.3	6.3	3.2	5.4
rEgret /IndoNA	14.7	14.5	15	14.3	15.5	14.8
rEgret /Indo-NS	10.1	10.4	10.2	10.4	9.6	10.1
rEgret /Indo-PB2	15.3	16.7	16.5	15.5	14.8	15.8

Acknowledgements

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Identification of the Molecular Determinants Required for Duck H9N2 Influenza Virus Replication in Chickens

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H9N2 influenza viruses have been frequently isolated from chickens in Asia since 1994. The natural reservoir of the viruses is wild waterfowl such as migratory ducks. It is believed that duck influenza viruses are the origin of H9N2 viruses that cause epidemics in chickens. However, we found that H9N2 viruses isolated from ducks (A/duck/Hokkaido/9/99) hardly replicated in chickens, whereas another H9N2 virus isolated from chickens (A/chicken/aq-Y-55/01) efficiently replicated in the respiratory tissues of chickens. To identify the determinants restricting the replication of H9N2 viruses in chickens, we established a reverse genetics system for these duck and chicken H9N2 viruses (RG-Dk/Hok and RG-Ck/Y55, respectively) and reassortant viruses in various combinations between RG-Dk/Hok and RG-Ck/Y55, and compared their abilities to replicate in chickens. We found that HA was the most critical component for the replication of H9N2 virus in chickens. No difference in hemagglutinating activities for duck or chicken red blood cells between these viruses was found. We then examined the susceptibility of RG-Dk/Hok and RG-Ck/Y55 to proteases in the mucosal secretions of chickens. RG-Ck/Y55 replicated in MDCK cells with media supplemented with chicken tracheal or intestinal wash instead of trypsin, but RG-Dk/Hok did not, suggesting that RG-Dk/Hok HA had lower susceptibility to the proteases of chickens. However, mutant Dk/Hok whose cleavage site was replaced with that of Ck/Y55 grew neither in cultured cells with the mucosal secretion nor in respiratory tissues of chickens. A molecular modeling study revealed three different amino acids near the cleavage sites of Dk/Hok and Ck/Y55 HAs, all of which were highly conserved in duck and chicken H9N2 viruses, respectively. Accordingly, the charge distributions on the HA cleavage site regions of Dk/Hok and Ck/Y55 were mainly negative and positive, respectively. Taken together, these results suggest that the inability of duck H9N2 virus to replicate in chickens depends on its lower HA cleavability with chicken proteases, though it is defined not only by the cleavage site sequence itself but also by another structural restriction that may affect the interaction of HA with the HA-cleaving proteases of the chicken.

Introduction

H9N2 influenza viruses have been frequently isolated from chickens in Asia since 1994^{1,2,3}. A natural host of the viruses is wild aquatic birds such as migratory ducks. It is believed that

duck influenza viruses are the origin of H9N2 influenza viruses that cause epidemics in chickens. However, we found that H9N2 viruses isolated from wild ducks (A/duck/Hokkaido/9/99) hardly replicated in chickens, while another H9N2 virus isolated from chickens (A/chicken/aq-Y55/01) efficiently replicated in the respiratory tissues of chickens⁴. To identify the molecular determinants that restrict replication of H9N2 viruses in chickens, we established a reverse genetics system for these duck and chicken H9N2 viruses (RG-Dk/Hok and RG-Ck/Y55, respectively) and reassortant viruses in various combinations between RG-Dk/Hok and RG-Ck/Y55.

Methods

The eight genes of Dk/Hok and Ck/Y55 were cloned as described previously⁵. Viruses were rescued by using the eight-plasmid system⁵. Five-week-old chickens were infected intranasally with 0.1 ml of allantoic fluid containing 10^{7.0} 50 % egg infectious dose (EID₅₀) of these viruses. The tracheal swabs of chickens were collected at 3 days postinfection and virus titers in the swabs were determined by EID₅₀.

Results and Discussion

We found that reassortant viruses possessing HA gene from Ck/Y55 and the other genes from Dk/Hok replicated efficiently in chickens (Table 1), suggesting that HA is the most critical component for the replication of H9N2 virus in chickens. To examine the receptor binding preference of RG-Dk/Hok and RG-Ck/Y55, hemagglutination tests for these viruses by using duck or chicken red blood cells were performed. We found no difference in hemagglutinating activities for both cell types between these viruses, suggesting that receptor binding capacity of HA is not the primary factor for the replication in chickens. We then examined the susceptibility of RG-Dk/Hok and RG-Ck/Y55 to proteases in the mucosal secretions of trachea and intestine of chickens. We compared the replication of RG-Dk/Hok and RG-Ck/Y55 in MDCK cells, using the media supplemented with chicken tracheal or intestinal wash instead of trypsin that is widely used to cleave HA in cell culture. RG-Ck/Y55 replicated in MDCK cells with media containing chicken tracheal or intestinal secretions, but RG-Dk/Hok did not (Figure 1). These results suggest that HA of RG-Dk/Hok have lower susceptibility to the proteases in the mucosal surface of chickens. It is unlikely that this difference is due to the sensitivity of RG-Dk/Hok to anti-HA inhibitors in the secretions since one-step entry of the viruses was not blocked even in the presence of the secretions. Dk/Hok and Ck/Y55 have different amino acid sequence in the cleavage site of HA (ASDR and RSSR, respectively), both of which are highly conserved among duck and chicken isolates, respectively. Therefore, we produced RG-Dk/Hok and RG-Ck/Y55 mutant viruses whose cleavage site sequences were modified to swap between RG-Dk/Hok and RG-Ck/Y55, and examined their ability to grow in vitro and vivo. The mutant RG-Dk/Hok having the cleavage site of RG-Ck/Y55 did not replicate in vitro and vivo, while the mutant RG-Ck/Y55 having that of RG-Dk/Hok replicated. These results suggest

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that the difference of the primary structure of HA cleavage site may not be crucial for limited replication of Dk/Hok in chickens. Three-dimensional (3D) molecular models of Dk/Hok and Ck/Y-55 HAs, constructed by homology modeling (Insight II), revealed the difference in three amino acids near cleavage site (455N, 480Q, 483Q in Dk/Hok; 455K, 480R, 483K in Ck/Y-55), all of which are highly conserved among duck and chicken H9N2 viruses. Accordingly, the electrostatic potentials on the surface around HA cleavage sites showed that the charge distribution of Dk/Hok is mainly negative, while that of Ck/Y55 is mainly positive. Taken together, these results propose that inability of duck H9N2 virus to replicate in chicken tissues depends on not only the difference of cleavage site itself but also another structural restriction that may affect the interaction with the HA-cleaving proteases in chicken.

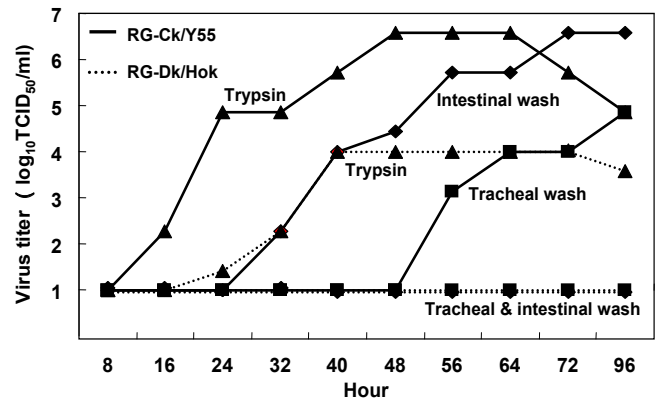
Acknowledgements

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Table 1. Virus recovery from chickens experimentally inoculated with H9N2 viruses.

Virus	Gene segments								Virus titer			
	PB2	PB1	PA	HA	NP	NA	M	NS	$(\log_{10} \text{EID}_{50}/\text{ml})$			
RG-Ck/Y55	■	■	■	■	■	■	■	■	4.7	4.7	3.2	4.5
RG-Dk/Hok	□	□	□	□	□	□	□	□	-	-	-	-
RG-55PB2	■	□	□	□	□	□	□	□	-	-	-	-
RG-55PB1	□	■	□	□	□	□	□	□	-	-	-	-
RG-55PA	□	□	■	□	□	□	□	□	-	-	-	-
RG-55HA	□	□	□	■	□	□	□	□	5.5	1.7	3.2	5.0
RG-55NP	□	□	□	□	■	□	□	□	-	-	-	-
RG-55NA	□	□	□	□	□	■	□	□	-	-	-	-
RG-55M	□	□	□	□	□	□	■	□	-	-	1.5	1.2
RG-55NS	□	□	□	□	□	□	□	■	-	-	-	-

Figure 1. Replication of H9N2 viruses in MDCK cells with media containing trypsin or chicken tracheal or intestinal wash. The progeny viruses released were collected at the indicated time points and titrated in MDCK cells by performing TCID₅₀ assay.



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Two-Phase Control of Apoptosis in Influenza Virus-Infected Cells By Up-Regulation of Akt and P53 Signaling

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PI3k-Akt and p53 pathways play anti- and pro-apoptotic roles in cell death, respectively. Whether these pathways are recruited in influenza virus infection was studied here. Phosphorylation of Akt (Akt-pho) was found to occur early after infection (5-9 h.p.i). Nuclear accumulation, phosphorylation of p53 (p53-pho), and expression of its natural target p21/waf showed low constitutive expression levels at this period, whereas all three parameters were markedly elevated at the late apoptotic stage (17-20 h.p.i.). However, p53 activation did not play a leading role in death of cells infected with recombinant NS1-deficient virus where accelerated apoptosis characterized by the lack of p53 and Akt activation developed. Apoptosis in influenza-infected cells was Akt-dependent and was accelerated by Ly294002, a specific inhibitor of PI3k-Akt signaling, and down-regulated by the viral protein NS1 which was shown here to activate host Akt. Ly294002 was also found to elevate virus production concomitantly with the acceleration of apoptosis. The obtained data suggest that influenza virus (i) initiates NS1-dependent anti-apoptotic PI3k-Akt signaling at early and middle phases of infection to protect cells from fast apoptotic death, (ii) provokes both p53-dependent and alternative p53-independent apoptotic cell death at the late stage of infection, and (iii) uses proapoptotic factors of the host cell in the middle stage of infection to stimulate its own production.

Introduction

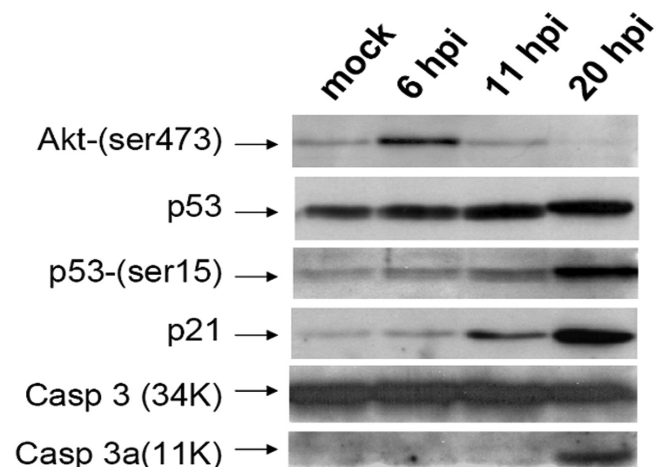
Influenza virus is a cytolytic virus that induces cell death in most cell types by apoptosis and, only in certain cells, by necrosis by caspase-dependent mechanisms [1-3]. Two types of virus-host interactions should be considered in the development of apoptosis/necrosis. In infected cells apoptosis is induced only by endocrine or intrinsic mechanisms, whereas infected cells may trigger paracrine or extrinsic apoptosis in non-infected neighboring cells through released signaling molecules. In either case apoptosis is clearly regulated by virus and host factors. Viral factors play different roles in apoptosis. Viral NA, PB1-f2, M1, M2 were shown to have proapoptotic potential. The non-structural viral protein NS1 was found to down-regulate apoptosis in infected cells [3]. Virus-specific RNAs serve as unique activators of host protein kinase R (PKR), TLR3/TLR7, RIG-1, MDA-5, and IPS-1 signaling that trigger NF-kB/IFN cascades recruited into host pro- and anti-apoptotic pathways [4-7]. Host cell factors, such as Fas/FasL, Bcl-2, TGF- β , mitogen-activated Raf/MEK/ERK and AP-1/JNK, NF-kB, ASK-1,

TLRs 3, 7 and 8, tumor suppressor p53, chemokine CCL5-CCR5, and nitric oxide are up-regulated in influenza-infected cells [7]. These apoptotic mechanisms may trigger pro- and anti-viral programs [8]. However, it is still unclear whether influenza virus initiates anti-apoptosis pathways to maintain its own replication or whether the host triggers pro-apoptotic ones as a programmed anti-stress response to eradicate virus infection. Here we investigated anti- and pro-apoptotic cell signaling pathways that could be recruited in influenza virus-infected cells.

Results and Discussion

Up-regulation of Akt in influenza virus-infected cells. Akt and p53 signaling was monitored in influenza-infected MDCK and CV-1 cells. These cell lines are sensitive to influenza virus infection and maintain high virus replication. The results are shown in Fig. 1. The phosphorylated form of Akt was clearly detected in infected CV1 cells, and there was a transient up-regulation of Akt-pho at the early-middle phase of infection (5-9 h.p.i). Akt-pho up-regulation was clearly seen in cells infected with all viruses studied. Notably, Akt and Akt-pho were controlled in parallel and only elevation of Akt-pho and a constant constitutive level of Akt were observed indicating posttranslational activation of Akt. The time course of Akt activation was found to overlap with logarithmic phase of virus growth. This suggests that Akt protects cells from apoptotic death at the early stage of infection.

Figure 1. Patterns of Akt, p53, p21/waf and caspase 3 in cells infected with influenza virus. CV-1 cells were infected with Aichi/68 (H3N2) viruses at MOI 5 and incubated for different times. At these intervals the same amounts of cells were analyzed with PAGE-WB using antibodies specific for Akt, Akt-(ser473), p53, p53-(ser15), p21/waf, caspase 3 and its activated form 3a. Positive bands were visualized with secondary anti-species antibodies-HRP by ECL.



Up-regulation of p53 in influenza virus-infected cells. Next, we have studied the dynamics of p53 phosphorylation in virus-infected cells. To estimate the activation of p53, p53 phosphorylated on serine 15 and p21/waf, a natural reporter

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of p53 transcriptional activity, were tested by WB (Fig 1). The background level of p53 was not markedly increased during the course of infection, whereas p53-pho was up-regulated at the late stage of infection around 17-20 h.p.i. At this time, the cells had gone into apoptosis as indicated by activation of caspase 3 and appearance of its cleaved 11 kD subunit (caspase 3a) and DNA degradation. p21/waf was also up-regulated at the late stage in parallel with p53 phosphorylation supporting the concept that p53 was activated. These data show that activation of transactivator p53 occurred at the late apoptosis stage in influenza virus-infected cells.

Apoptosis development and dynamics of Akt and p53 in cells infected with delNS1 virus. Protein NS1 was shown to down-regulate apoptosis in infected cells [3]. This implied that NS1 may interact with anti-apoptotic Akt or pro-apoptotic p53 signaling. To study this suggestion we have used recombinant A/PR/8/34 virus lacking the NS1 gene (delNS1 virus). Cells infected with delNS1 showed earlier and more intensive apoptosis than wild type (WT) virus. Notably, the patterns of Akt-pho and p53-pho were found to be significantly different in WT- and delNS1-infected cells. (i) Transient phosphorylation of Akt was achieved on the middle stage in CV-1 cells infected with WT virus, whereas only background levels of Akt-pho were seen in delNS1-infected cells. These data suggest that NS1 plays a role in stimulation of anti-apoptotic Akt signaling that leads to a delay of apoptosis. (ii) Only minor levels of p53 phosphorylation and the lack of concomitant up-regulation of p21/waf were observed in delNS1 virus-infected cells, contrary to WT-infected cells where both mediators were up-regulated. The lack of p53 activation and the development of rapid apoptosis indicate that there is an alternative pathway(s) of p53-independent apoptosis in delNS1 infected cells.

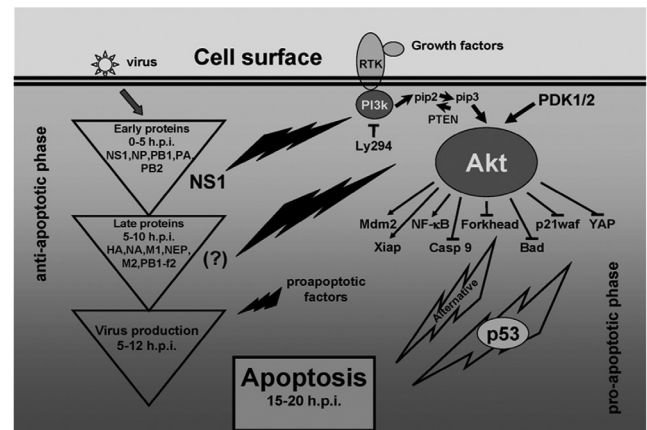
Up-regulation of Akt in cells transfected with a DNA vector expressing NS1. The above results suggested that influenza NS1 protein could be responsible for the Akt stimulation in infected cells. This concept was tested in NS1 transfection experiments. CV1 and 293T cells were transfected either with empty pCAGGS or pCAGGS/NS1 plasmid DNAs, and intracellular levels of Akt-pho were evaluated by WB technique. Accumulation of Akt-pho appeared to be significantly higher in cells transfected with the pCAGGS/NS1 vector than with the empty one. Interestingly, the Akt-stimulating activity of NS1, calculated as a Akt-pho/NS1 ratio was about 10 times higher in pCAGGS/NS1-transfected cells than in virus-infected cells. Thus, the Akt-stimulating potential of NS1 seems to be redundant in infected cells. These data indicate that the NS1 protein is an activator of host Akt.

Interplay of apoptosis and PI3k-Akt signaling in influenza virus-infected cells. PI3k-Akt signaling is known to initiate an antiapoptotic program and its stimulation may increase cell resistance to virus stress [9]. On the other hand, suppression of the PI3k-Akt pathway might promote earlier apoptosis and interfere with virus replication. These suggestions were tested in an experiment where PI3k-Akt signaling was suppressed by Ly294002, a cell permeable inhibitor. First, phosphorylation of Akt was effectively suppressed by Ly-294002 in influenza-

infected cells. Second, apoptosis markers, such as cell damage, reactivity with cyto-death antibody (clone M30), caspase-dependent cleavage of viral NP, caspase 3 activation, and chromatin DNA laddering, developed earlier in Ly-treated cells than in placebo cells. Apoptosis markers appeared in Ly-treated CV-1 cells as early as 9-12 h.p.i., whereas in non-treated cells these markers were detectable at 14-16 h.p.i. Ly294002 did not induce apoptotic signs in mock-infected cells indicating the provoking role of virus in apoptosis enhancement when the host Akt pathway was inhibited. These data support the concept that up-regulation of PI3k-Akt signaling counteracts early apoptosis in influenza-infected cells.

Interference of PI3k-Akt signaling with virus reproduction. We finally addressed the question whether the PI3k-Akt signaling pathway interferes with virus reproduction. To answer this question we evaluated virus replication in infected cells when PI3k-Akt signaling was inhibited by Ly294002. In different experiments with Ly-treated cells the virus yield was 3-8 times higher as compared to non-treated control cells. The apoptosis-coupled increase of virus yield was found both in CV-1 and in MDCK cells infected with Aichi/68 and WSN/33 viruses. The observation that inhibition of PI3k-Akt signaling with Ly294002 accelerated apoptosis and concomitantly increased virus production suggests a stimulatory role of host apoptotic factors in virus replication.

Figure 2. Two phases of apoptosis in influenza virus-infected cells. Serine/threonine kinase Akt is activated by phosphatidylinositol-3-kinase (PI3k)-dependent signaling that is artificially blocked by Ly294002. PI3k receiving a signal from extracellular factors via receptor tyrosine kinase (RTK) generates pip3, a second messenger essential for the translocation of Akt to plasma membrane and its activation by PDK 1/2 through the phosphorylation at Thr309 and Ser473. At the early phase of infection (0-5 h.p.i.) viral proteins NS1, NP, and polymerase proteins PB1, PB2, PA are synthesized when the Akt signaling is initiated through the action of host PI3k and the viral NS1. At the later stage other viral proteins may be additionally involved in the Akt pathway. This Akt signaling can promote a numerous anti-apoptotic pathways. During next middle stage (5-10 h.p.i.) a synthesis of late proteins: HA, NA, M1, NEP, M2 and PB1-f2 is amplified and logarithmic phase of virus growth develops. At the end of this stage pro-apoptotic signaling begins to overbalance the anti-apoptotic one and some apoptotic factors, such as caspase 3, may stimulate virus production. At the late stage (15-20 h.p.i.) the apoptosis develops either through p53-dependent or alternative p53-independent pathways and virus production ceases. Time-periods are shown for permissive for influenza viruses MDCK cells. Symbols (↑) and (T) show up- and down-regulations, respectively.



Results and Discussion

The role of apoptosis in virus replication deserves specific consideration. Apoptosis is believed to be a host defense mechanism that eliminates infected cells. However, there is also increasing evidence that apoptosis may promote virus replication. Proapoptotic host factors, such as caspase 3 and activation of Fas/TRAIL/NF- κ B signaling, were reported to facilitate influenza virus replication [4,10]. Also, host caspases promote permissive replication of the Aleutian mink disease parvovirus by cleaving the non-structural protein NS1 [11]; and the non-structural protein NS5A of HCV [12,13]. In each case caspase cleavage modulated nucleo-cytoplasmic shuttling of virus [10-12]. Here we observed a stimulatory role of apoptotic in influenza virus replication. Mild acceleration of apoptosis by Ly294002 increased virus production. What factor(s) was responsible for influenza virus stimulation in our study remains to be seen. Importantly, Ly294002 only partially shifted apoptosis to the middle phase (10-12 h.p.i.) without total abrogation of the anti-apoptotic program and to permit fast apoptosis at the early phase (4-5 h.p.i.) and to suppress virus production. It is clear that at this middle phase virus production has already been completed. Hence, Ly 294002 is still able to reduce Akt and accelerate apoptosis, but it is not potent enough to effectively stop virus replication. Obtained data support the concept that apoptosis process has two different functions in virus infection. On one side, apoptosis promotes clearance of infected cells and initiates immune response mechanisms. These host programs develop at the final stage of virus replication through p53-dependent and alternative p53-independent apoptosis/necrosis. On the other side, during evolution influenza virus has adapted to apoptosis and exploits this program for its own benefit. To accomplish this the virus (i) has developed instruments to restrain intrinsic apoptosis at an early stage after cell invasion, such as activation of the antiapoptotic Akt pathway by NS1, and (ii) uses mediators of the apoptotic process, such as activated caspase 3, to stimulate its own replication.

Acknowledgements

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Cytokine Induction Profiles Of Avian And Human Influenza Viruses – A Preliminary Report

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The proinflammatory cytokine dysregulation is thought to contribute to the severity of human H5N1 infection. This study compared the profiles of cytokine/chemokine gene expression induced by avian and human influenza viruses. **Methods and Materials:** Quantitative real-time RT-PCR was used to compare the profiles of cytokine/chemokine and chemokine receptor gene expression in human lung epithelial cells (NCI-H292) following infection with influenza A/HK/483/97(H5N1/97), A/Thai/KAN1/04(H5N1/04), A/Canada/504/04 (H7N3/04), and H1N1 (a clinical isolate collected from a hospitalized patient in 2002 designed CU02V13003). **Results:** The quantitative real-time RT-PCR results showed that during early post-infection, there was greater up-regulation of mRNA for TNF-alpha, RANTES, IP-10, CXCL-9, and IL-10 in H5N1/97, H5N1/04, and H7N3/04 infected cells than those infected with H1N1. During late post-infection, the mRNA levels for TNF-alpha, RANTES, IP-10, CXCL-9 remained high in H5N1/97, H5N1/04, H7N3/04 infected cells. However, in the late phase, the IL-10 mRNA level dropped in H5N1/97 and H7N3/04 infected cells, but not in H5N1/04 infected cells. Furthermore, during the late phase, IFN-gamma, CCL-3, IL-6, IL-8 and BCA mRNA levels were also up-regulated in H5N1/97, and H5N1/04 infected cells. The levels of CCR2 and CXCR5 receptor gene expression were elevated in H5N1/04 in late post-infection. However, there was a decrease in CCR2, CXCR4 and CXCR5 gene expression in H5N1/97 and H7N3/04 in late post-infection. In contrast, the H1N1 infected cells showed a decrease in CXCR4 mRNA level in the early phase, and a decrease in CCR2 mRNA level in the late phase after infection. **Conclusions:** There are differences in cytokine/chemokine response of NCI-H292 cells to human and avian influenza virus infection *in vitro*. The H5N1/97 and H5N1/04 viruses are more potent inducers of proinflammatory cytokine/chemokine, particularly TNF-alpha, RANTES and IP-10.

Background

In Hong Kong in 1997, the "H5N1" highly pathogenic avian influenza (HPAI) A virus was first recognized to be capable of infecting humans. (17, 4). Since late 2003, HPAI H5N1 viruses have spread across Asia to the Middle East, into Europe and Africa. As of July 2007, approximately 315 laboratory-confirmed human cases of H5N1 virus infection have been reported to the World Health Organization (7,16). Over 50% of human infections have been fatal. The proinflammatory cytokine dysregulation detected in severe human H5N1

disease is thought to contribute to the severe outcome (3). Dysregulation of cytokine production may result in unwanted inflammation and cell damage (5). Elucidating the differences in cytokine production in response to infections with high and low pathogenic influenza viruses is crucial to identify novel treatment options. In this study, the profiles of cytokine/chemokine and their receptor gene expression induced by H5N1/97, H5N1/04, H7N3/04 influenza viruses of avian origin with those of contemporary human H1N1 viruses were compared.

Methods and Materials

The profiles of cytokine/chemokine as well as their receptor genes expression induced by viruses with different pathogenicity: A/HK/483/97 (H5N1/97), A/Thai/KAN1/04 (H5N1/04), A/Canada/504/04 (H7N3/04) were compared with that of human H1N1 virus in human lung epithelial cells *in vitro*. Human lung epithelial cell line – NCI-H292 cells were seeded at 1×10^7 cells in T75 culture flasks and were infected at a multiplicity of infection of one. After one hour of virus adsorption, the virus inoculum was removed, and the cells were washed with PBS and incubated in RPMI 1640 medium (GIBCO, Grand Island, NY). Samples of culture supernatant were collected for virus titration analysis. RNA was extracted from cells for analysis of cytokine/chemokine and their receptor gene expression. In order to investigate the cytokine/chemokine responses during both the early and late phases after viral infection, the samples were collected at the following time points: 3, 6, 18, 24 and 30 hr post-infection. Quantitative real-time RT-PCR was used to compare the profiles of 30 cytokines/chemokines as well as their receptor gene expression induced by the different viruses. Dnase-treated total RNA was isolated by the TRIzol-total RNA extraction kit (Invitrogen, Carlsbad, CA). cDNA was synthesized from mRNA with poly(dT) primers and Superscript III reverse transcriptase and quantified by real-time PCR analysis using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City; CA). Comparative CT methods were used to analyze the results using beta-actin as the endogenous control.

Results

It was found that the human and avian viruses replicated to similar titres after infection of NCI-H292 cells at a multiplicity of infection (m.o.i.) of one. Quantitative real-time RT-PCR results showed that during the early phase of infection (i.e. 3 and 6 hr post-infection), there were induction of several pro-inflammatory cytokines/chemokines. Up-regulation of mRNA for TNF-alpha, regulated on activation, normal T cell expressed and secreted (RANTES), IP-10, CXCL-9, IL-10 was greater after infection with the H5N1/97, H5N1/04, H7N3/04 viruses than those infected with the H1N1 virus. During the late phase after infection (i.e. 18, 24 and 30 hr post-infection), it was also found that the mRNA levels for TNF-alpha, RANTES, IP-10, CXCL-9 remained high in H5N1/97, H5N1/04, and H7N3/04 infection as compared with that of H1N1 infection. However, in the late

phase, the IL-10 mRNA level dropped in H5N1/97 and H7N3/04 infection, but not in H5N1/04 infection (Figure 2).

Figure 1. Changes of TNF-alpha, RANTES, IP-10, IL-10, CXCL-9, IFN-gamma, BCA, CCL-3, IL-6 and IL-8 gene expression level at various time points post-infection (i.e. early phase and late phase) with H1N1, H5N1(97), H5N1(04) and H7N3 viruses.

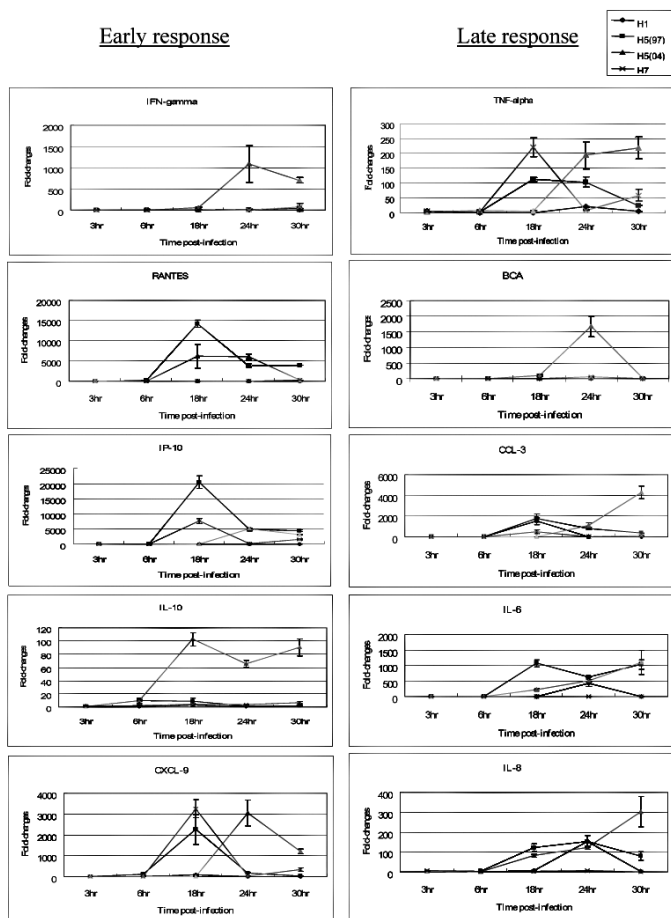
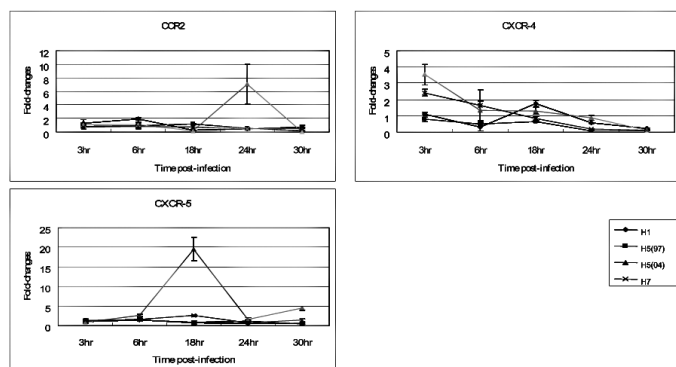


Figure 2. Changes of CCR-2, CXCR-4 and CXCR-5 receptor gene expression levels at various time points post-infection with H1N1, H5N1(97), H5N1(04) and H7N3 viruses.



Furthermore, during the late phase after infection, IFN-gamma, CCL-3, IL-6, IL-8 and BCA were also up-regulated in H5N1/97, and H5N1/04 infected cells. The optimal time for the up-regulation of these clusters of mRNA in H5N1/97 infection occurred at 18 hr post-infection and that for H5N1/04 infection occurred at 24 hr post-infection (Figure 1).

Among the chemokine receptor gene expression measured in our experiments, CCR2 and CXCR5 genes was elevated for H5N1/04 in the late phase after infection. However, there was a decrease in CCR2, CXCR4 and CXCR5 gene expression for H5N1/97 and H7N3/04 in the late phase after infection. For the H1N1 infection, it showed a decrease in CXCR4 mRNA level in the early phase and a decrease in CCR2 mRNA level in the late phase. Therefore, besides the cytokine/chemokine expression up-regulation, the elevated level of CCR2 and CXCR5 receptor gene expression may also be important for the high pathogenicity of H5N1/04 (Fig. 2). Discussion: Uncontrolled cytokine production is thought to be responsible in part for the increased mortality observed in avian flu infected patients (11, 15, 17). Lung epithelial cells are the key targets of influenza virus infection (14). The results of this study indicated that the human and avian viruses replicated to similar titres after infection of NCI-H292 cells at a multiplicity of infection of one. That means, the differential hyper-induction of cytokines was not explained by differences in the replication kinetics between the virus subtypes. During the early phase of infection, up-regulation of mRNA for TNF-alpha in avian flu infection was observed. Although TNF-alpha was first noted for its role in killing tumor cells (2), it has pleiotropic functions that include inflammatory response and host resistance to pathogens (1). TNF-alpha may activate nuclear factor-kB (NF-kB) by inducing phosphorylation and degradation of IκB, and leads to the translocation of NF-kB to the nucleus where it binds to promoters. It has been reported that NF-kB regulates many kinds of genes and plays a crucial role in inflammatory diseases (6,9). It has been reported that NF-kB binds to the RANTES promoter (8,13). Thus, RANTES expression may be induced by TNF-alpha in avian flu infection. RANTES attracts monocytes, eosinophils, basophils and T cells, and selectively CD4+ T cells. Its production from the bronchial epithelial cells contributes to the infiltration of the inflammatory cells in airway during viral infection (10). Another elevated chemokine - IP-10, is a macrophage chemo-attractant and mediates inflammatory response by further recruitment of circulating leukocytes into the inflamed tissue (3). In H5N1/04 infection, the level of CCR2 was elevated. CCR2 recruits alveolar monocytes and neutrophils during the acute inflammatory process, and enhanced monocyte accumulation and extensive neutrophil influx is associated with loss of pulmonary endothelial/epithelial barrier function. Thus, whether the elevated level of CCR2 in H5N1/04 infection might results in abnormal neutrophil and monocyte responses causing pulmonary inflammation and loss of vascular integrity (12).

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Conclusions

Our findings suggest that there are differences in cytokine/chemokine as well as their receptor expression responses of human NCI-H292 cells to human and avian influenza viruses. The responses of NCI-H292 cells to avian influenza viruses were found to be much stronger than those to human virus. The H5N1/97 and H5N1/04 subtypes of influenza A virus were more potent inducers of proinflammatory cytokine/chemokine, particularly TNF-alpha and IP-10 in human lung epithelial cells. This hyper-induction of cytokines/chemokines as well as chemokine receptors may be a key to the high pathogenicity of avian viruses.

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PB1-F2 and the Change in Virulence of Influenza A(H1N1) Viruses

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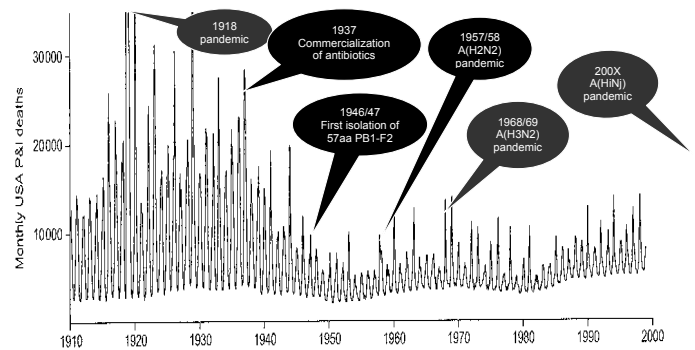
Introduction

Can actions of mankind influence the nature of mutations fixed in the evolution of influenza viruses? What is PB1-F2 and what does it do? The eleventh influenza protein, PB1-F2, was discovered serendipitously during a search of the influenza genome for open reading frames¹. This protein (translated from a +1 frame in the PB1 gene) is produced rapidly after influenza infection, localizes to the mitochondrial membrane where it is believed to form random pores, disrupts this organelle, instigates apoptosis of infected cells, and is rapidly degraded. Expression does not occur in all cell types. Monocytic cell lines and fresh-isolates of monocytes are infected, but epithelial cell lines are spared¹. PB1-F2 may also operate "in trans", affecting uninfected cells through an extracellular presence¹. PB1-F2 acts to delay viral clearance². It has been experimentally determined that viruses expressing an intact PB1-F2 are more virulent than the same viruses in which this polypeptide is nonfunctional³. Apoptosis of cells responsible for innate immunity (tissue macrophages and monocytes) could be responsible not only for the delayed clearance observed, but also for increased susceptibility to secondary bacterial infection...the principal complication of severe influenza infection⁴. All three pandemic viruses of the 20th century emerged with novel genes for intact PB1-F2. *PB1-F2 sequence data*. PB1-F2 sequences thought to represent functional proteins have been identified with sequence lengths of 79 to 101 amino acids (aas) in influenza A viruses that infect a large number of species⁵. Only sequences longer than 86 aas were predicted to contain an amphipathic helix thought to be capable of disrupting mitochondrial function⁶. However, a very recent paper suggests that an effective Mitochondrial Targeting Sequence exists in sequences as short as length 79 aas⁷. Currently, about 90% of all human influenza A sequences and about 96% of avian influenza sequences in GenBank are thought to have a full-length, functional PB1-F2 sequence⁵. Two reviews of PB1-F2 sequences noted that influenza A(H1N1) sequences isolated before 1947 are full length, whereas those isolated in and after 1956 were truncated to length 57 aas^{5,6}. Polypeptides expressed from PB1-F2 sequences of length 57 aas do not localize to mitochondria. *Objectives of the present study*. Since A(H1N1) human viruses were supplanted by A(H2N2) viruses in 1957/58, these observations were suggestive that fixation of the truncated version (57aa) of PB1-F2, may have resulted in a dramatic decrease in virulence of A(H1N1) viruses, precipitating the emergence of a new pandemic virus. We reviewed the ISD database at Los Alamos National Laboratory to verify that A(H1N1) viruses with the truncated PB1-F2 sequence were not seen before 1956⁸.

Results

The ISD database contains 374 PB1 sequences from A(H1N1) viruses. Eighteen sequences were reported for the years 1918-1946. Four PB1 sequences were from viruses isolated in 1947. Three contained full-length PB1-F2 sequences. One of these, A/USA/L3/1947 H1N1, was of length 57 aa. Only six A(H1N1) sequences in the database were from isolates from 1947-57. By year, these were [1947 (1), 1950 (1), 1954(1), 1956(2), 1957(1)]. A very recent review has noted that a truncated PB1-F2 sequence was found in 1950⁷. No A(H1N1) PB1 sequences appear in the database until 1977. Of the 351 sequences in the database isolated in/after 1977, 347 are of length 57 aas.

Figure 1. Monthly deaths due to pneumonia and influenza for the US, 1910-2000. (Permission to reproduce this figure pending, Earn, Dushoff & Levin. TRENDS in Ecology & Evolution Vol.17 No.7 July 2002, 334-40, Figure 3a)



Conclusion and Discussion

It appears that a mutation inactivating PB1-F2 became fixed in A(H1N1) viruses in 1947. Why would a mutation reducing virulence become fixed in a dominant A-type influenza virus after 30 years of successful circulation? After the two catastrophic seasons of emergence of the 1918 virus, the pattern of pneumonia & influenza (P&I) mortality rates for the US from 1920/21 to 1936/37 consisted of 5 similarly large peaks spaced 3-4 years apart with an ascending staircase of peaks in the intervening seasons (Figure 1). Over the next decade, however, P&I mortality dropped by two-thirds. After, 1947, P&I mortality rates were about constant until the A(H2N2) pandemic of 1957/8. In 1937, the first year of commercialization of antibiotics, over 90% of all-cause excess mortality was attributable to P&I. Rapidly incorporated, antibiotic usage dramatically reduced mortality associated with secondary bacterial pneumonia. Complicating pneumonia delays viral clearance; a benefit to the virus. The PB1 gene is known to be under negative selection pressure (highly conserved). Analysis of the PB1-F2 gene selection pressure produces ambiguous outcomes (slight preponderance of evidence for positive selection), but consensus is that PB1 evolution dominates PB1-F2 evolution. If there were a modest evolutionary cost associated with preserving a full-length PB1-F2 gene in an alternate frame, the erosion of benefit from the function of

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the encoded polypeptide may have opened the pathway to mutation with resulting reduced virulence. We suggest that the truncation mutation in A(H1N1) viruses became fixed in response to the widespread adoption of newly available antibiotics and the resultant amelioration of secondary bacterial pneumonia. An action of mankind altered the setting in which the virulence benefit of the full-length PB1-F2 sequence was no longer sufficient to offset the constraint on evolution of the essential PB1 gene. If this mutation had been fixed in 1956, we might be concerned that similar wide-spread actions of mankind (e.g., completely universal vaccination against the currently circulating virus) might correspondingly reduce the fitness of the currently circulating virus sufficiently to abruptly precipitate a new pandemic. However, the ten-year hiatus suggests that viruses successor to the currently dominant viruses may not be immediately "in the wings". This experience also suggests that humankind will ultimately require a strategy more comprehensive than vaccination against the currently circulating strains for managing the influenza virus.

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Biphasic Effect of rTGF- β 1 on Inducible Nitric Oxide Synthase (iNOS) During Experimental Influenza Infection in Mice

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Influenza virus infection activates the interferon (IFN) inducible gene, nitric oxide synthase 2 (iNOS). This process is tightly regulated by a complex network of cytokines. Among these, transforming growth factor- β 1 (TGF- β 1) is known to suppress IFN expression. NO has been shown to contribute to the pathogenesis of influenza virus via modulating the apoptosis. Methods: Eight-week-old BALB/c mice were intranasally instilled with 4.1×10^3 PFU of A/Udorn/317/72 (H3N2) virus in 50 μ l of allantoic fluid or mock infected with 50 μ l of allantoic fluid. rTGF- β 1 was administered to mice by intravenous injection of 0.5 μ g/ Kg body weight of mouse. The mice were euthanized on days 3, 5 and 7 post infection (p.i.) for analysis of the parameters. Cytokine IFN- γ in BALF, iNOS expression in the lung homogenate. Results: We observed a significant increase of INF- γ on 3rd and 5th day and decreased to basal level on 7th day p.i. Simultaneous administration of rTGF- β 1 with virus instillation inhibited release of INF- γ level on 3rd day and 5th day. The iNOS expression was detected on 3rd day p.i. and maximum level was observed on 5th day p.i. in virus instilled group. However simultaneous administration of rTGF- β 1 with virus significantly reduced the level of iNOS on 3rd, 5th and 7th day p.i. Conclusions: rTGF- β 1 acts as an immunomodulatory cytokine and inhibits cytokine mediated apoptosis by modulating inflammatory cytokine INF- γ , which in turn down regulated the interferon inducible genes iNOS.

Introduction

Influenza virus (IV) infection causes inflammation in the upper respiratory tract and the lungs. The virus targets respiratory tract epithelial cells. Histological studies of the upper respiratory tract and/or lungs typically reveal necrosis of epithelial cells and infiltration with neutrophils [1]. Early cytokines such as interferons, TNF- α and interleukin-1 produced at the site of infection mediate both symptom formation and inflammation [2]. Virus infection activates the interferon (IFN) inducible gene, nitric oxide synthase 2 (iNOS) [3]. The production of nitric oxide (NO) via the iNOS is regulated by a complex network of cytokines. TGF- β negatively regulates the inflammatory response by regulating lymphocyte influx to the airway, modulating apoptosis and suppressing the iNOS expression [4, 5]. It is therefore important to elucidate the role of TGF- β in IFN- γ induced expression of iNOS during influenza virus infection.

Materials and Methods

Mice. BALB/c mice weighing 20 to 25 g were obtained from National Institute of Nutrition, (Hyderabad, India). These animals were maintained in a controlled room (25 \pm 5°C with automatic 12-h cycles of lighting) and had free access to water.

Virus. The influenza A virus (A/Udorn/317/72/H3N2) was obtained from The Centers for Disease Control, USA. Influenza A virus was grown in embryonated eggs. For infection of mice, viral stocks were diluted to a concentration of 4.1×10^3 PFU in 50 μ l of allantoic fluid or mock infected mice received 50 μ l of normal allantoic fluid.

Experimental Groups. Mice were distributed in four groups, (I) *Control group.* mice were mock infected by instillation of 50 μ l of allantoic fluid. (II) *Virus group:* mice were instilled with influenza A virus. (III) *rTGF- β 1 treated virus group:* infected mice were treated with 5.0 μ g/Kg rTGF- β 1, given intravenously in a volume of 200 μ L. (IV) *rTGF- β 1 treated control group:* mock infected mice were administered intravenously 5.0 μ g/Kg body weight of mouse per 200 μ l rTGF- β 1. **Cytokine assay.** The concentration of IFN- γ in the supernatant of BALF was determined by using ELISA kits (BD Biosciences) according to the manufacturer's instructions.

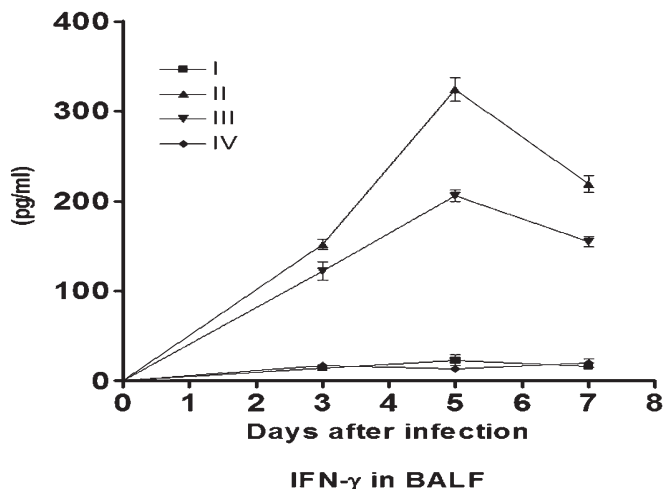
Inducible NO synthase mRNA expression in the lung. Total cytoplasmic RNA was extracted from mouse lungs with the Trizol total RNA isolation kit (GIBCO BRL, Life Technologies, Gaithersburg, MD). Total RNA (1 μ g) from each sample was reverse transcribed with oligo(dT) as a primer. Primers specific for mouse inducible NO synthase (iNOS; GenBank accession no. M84373; sense primer 5'-CTT TGT GCG GAG TGT CAG TGG-3' and antisense primer 5'-TTC TTC CTG ATA GAG GTG GTCC-3') and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. M32599; sense primer 5'-GTC TTC ACC ACC ATG GAG AAG GCT-3' and antisense primer 5'-TGT AGC CCA GGA TGC CCT TTA GTG-3') were used. PCR amplification was carried out in a thermal cycler for 25 cycles for GAPDH and 30 cycles for mouse iNOS. The number of cycles was determined by titration as the acceptable number of cycles that could amplify visible products on Gel Star-stained agarose gels (FMC Bioproducts, Rockland, ME) during the exponential phase of the PCR. The cycling parameters routinely used were denaturation at 94°C for 30s, annealing at 56°C for GAPDH or 60°C for iNOS for 45s, and extension at 72°C for 45s. Gene transcript levels for all samples were amplified for iNOS and GAPDH simultaneously, and each PCR analysis was completed in triplicate. The expected amplified fragments were 223 bp and 192 bp for mouse GAPDH and iNOS, respectively. GAPDH was used to control for variation in the efficiency of RNA extraction, reverse transcription, and PCR. Quantification of the amplified product was obtained by densitometric scanning of the stained gels with ImageQuant (version 1.1, Molecular Dynamics, Cambridge, MA). The mean values of the three determinations were used for final analysis, and the normalized iNOS mRNA levels were derived by dividing the iNOS mRNA by the GAPDH mRNA for each tissue sample.

Options for the Control of Influenza VI

Results

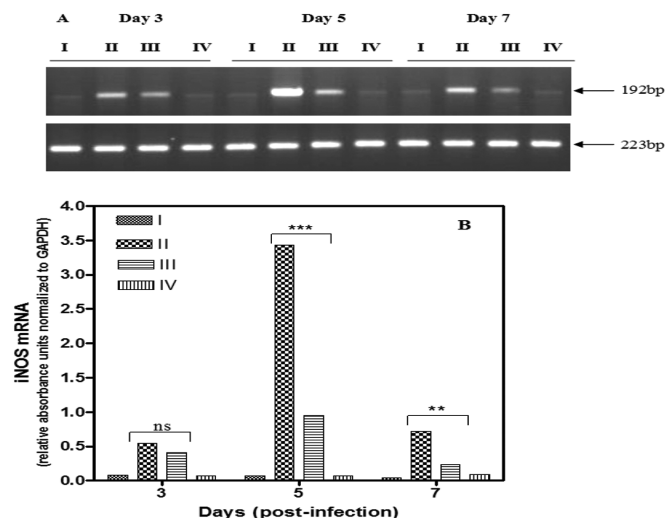
Level of IFN- γ . There was a substantial change observed in IFN- γ concentration. Level of IFN- γ in BALF increased on 3rd, 5th and 7th day p.i. and maximum level was observed on 5th day p.i. (Figure 1A). Treatment with rTGF- β decreased IFN- γ significantly $p < 0.05$ on 3rd day and $p < 0.001$ on 5th and 7th day p.i. (Figure 1).

Figure 1. The IFN- γ level in BALF. The level observed was seen to be significantly increased in virus group (II) ($p < 0.001$) on 3rd, 5th and 7th day p.i. as compared to control group (I). Administration of rTGF- β 1 down regulated IFN- γ significantly $p < 0.05$ on 3rd day and $p < 0.001$ on 5th and 7th day p.i.



Lung Tissue iNOS mRNA. Expression of mRNA for iNOS was examined with RT-PCR by normalization of level of iNOS mRNA to GAPDH mRNA levels. The amplified fragments were 223 bp and 192 bp for mouse GAPDH and iNOS, respectively. The level of iNOS mRNA after influenza infection increased markedly. Increased levels of iNOS mRNA was detected in virus group (II) of mice on days 3, 5, and 7 p.i.. In rTGF- β administered infected mice (group III) iNOS expression was inhibited significantly on 3, 5 and 7th day p.i. (Figure 2A). iNOS mRNA was undetectable in the lungs of control group (I) of mice on days 3, 5 and 7 p.i. (Figure 2B).

Figure 2. RT-PCR analysis of iNOS mRNA expression after infection. GAPDH, (glyceraldehyde-3-phosphatedehydrogenase), level of iNOS mRNA after normalization to GAPDH mRNA levels. The level of induction of iNOS after influenza infection increased markedly. In rTGF- β 1 administered infected mice (group III) iNOS expression was inhibited significantly on 3, 5 and 7th day p.i.



Discussion

The mice lacking functional IFN- γ genes [6] allowed the assessment of the role of this cytokine during infection with influenza virus [7]. Mice lacking IFN- γ did not display a reduced ability to recover from infection with the A/JAP/57 (H2N2) strain of influenza virus and mounted cytotoxic T-lymphocyte (CTL) activity comparable to that of their wild-type counterparts. When CTL clones of IFN- γ mice, adoptively transferred into wild-type recipients previously challenged with influenza virus, mediated effective recovery from infection [7]. In our study attempts were made to minimize the inflammatory response without obliterating virus clearance. rTGF- β 1 is an immunomodulatory cytokine which has the ability to down regulate the IFN- γ and its down stream genes which are having deleterious effects in an overshooting immune response. The data presented in the study showed that there was an increase in level of IFN- γ in BALF on 3rd, 5th and 7th day p.i. and maximum level was observed on 5th day p.i. (Figure 1). Treatment with rTGF- β 1 decreased IFN- γ significantly $p < 0.05$ on 3rd day and $p < 0.001$ on 5th and 7th day p.i. IFN- γ is associated with the induction of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO), which plays an important role in the tissue damage. Elevated NO production is associated with pulmonary pathology after influenza infection. [8,10]. iNOS mRNA was elevated in infected animals on days 3, 5, and 7 p.i. (Figure 2A) compared to control mice. rTGF- β 1 treated infected animals did not show similar high levels of iNOS mRNA, particularly on the 5th day p.i. (Figure 2B). Expression of iNOS leads to production of NO at the site of infection. The higher levels of iNOS correlated with increased lung injury (data not shown), implying that pulmonary injury may be, in part, a consequence of excessive NO and/or NO-derived production. This is further emphasized

by the significant differences in iNOS expression when rTGF- β 1 administered mice were compared with infected mice (Figure 2B). So, rTGF- β 1 administration significantly attenuated influenza induced expression of iNOS. Thus NO or NO-derived species act to enhance influenza-associated pathology. The use of NOS inhibitors during infection with murine cytomegalovirus [9]. or the influenza A/Kumamoto/Y5/67 (H2N2) mouse-adapted strain [10] has shown reduced mortality. Inhibition of iNOS fully protected mice from herpes simplex virus-1 dependent lung injury despite an increase in viral titers [11]. The rTGF- β 1 administration significantly attenuated IFN- γ induced expression of iNOS during influenza virus infection in mice.

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Proceedings Topic #13

Clinical Guidance and Policies

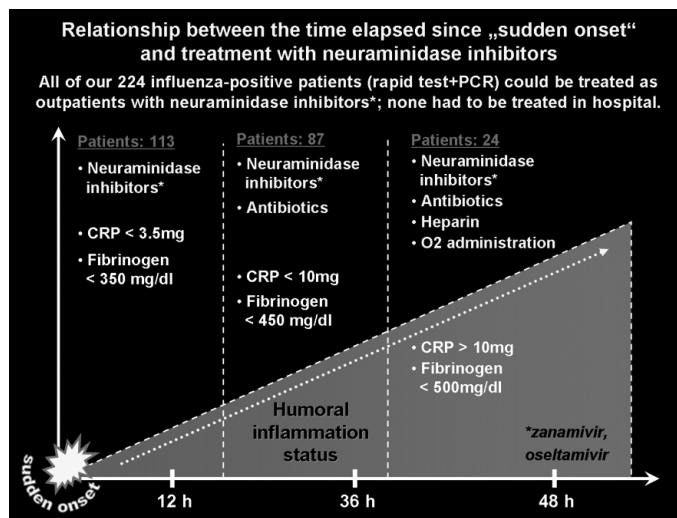
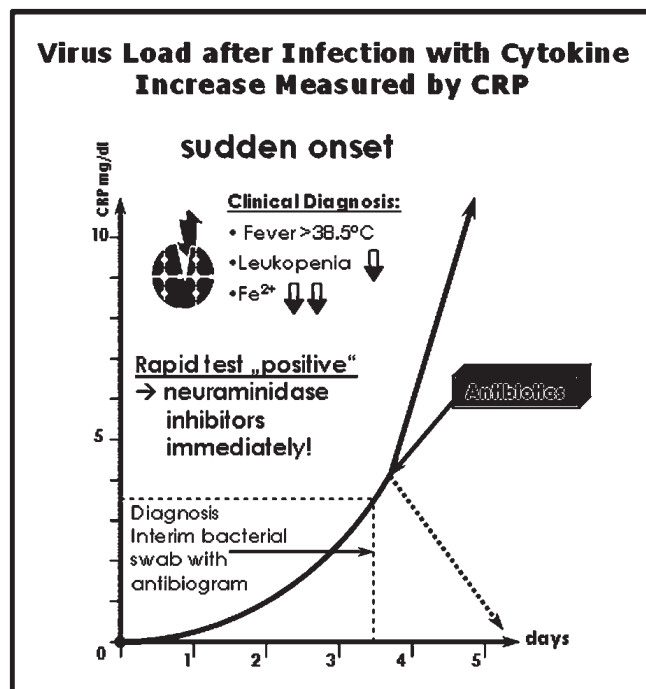
Poster Presentations

Etiotropic Treatment of Influenza: 7 1/2 years of Clinical Experience Using Neuraminidase Inhibitors

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Influenza has been placed at the head of a list of the most dangerous infectious pathogens in Germany drawn up in March 2007 by the leading German health authority, the Robert Koch Institute (RKI, www.rki.de). Before 1999, the year in which we first attempted to treat a patient with sialidase inhibitors (zanamivir, Jan 29,1999) influenza was not even a disease that had to be notified to the authorities. "If you understand Influenza you understand virology completely". We have followed this guiding principle from 1950s consistently in particular since Mark von Itzstein's discovery of neuraminidase inhibitors (Nature, 1993). After 7 1/2 years of clinical experience with this antiviral treatment, which was new at the time, we now have clinical data on 205 patients of both sexes and all ages, including infants. (In October 2002 we changed over from the inhalant zanamivir to the orally administered oseltamivir, which could also be applied as a suspension for infants and children and via nasal intubation for not fully conscious patients.) The clinical suspicion that a patient was infected with influenza was gained through the "art of viewing", as Dr. David Mueller from the Mount Sinai Hospital in New York refers to it. An objective examination of this suspicion was then achieved through a rapid test confirmed by PCR at the Health Authority Hannover (Landesgesundheitsamt Hannover, Dr. Dr. Rolf Heckler, Rolf.Heckler@niga.Niedersachsen.de). Even with our first patient, a 96-year-old male who had been unsuccessfully immunized against influenza, we recognized that time is the crucial factor in this new treatment. At the beginning we had no rapid tests and had to await the PCR results by telephone. In the meantime, the exponential replication of the virus continues undisturbed. Cells are destroyed, and released cytokines cause the clinical symptoms. According to N. HEIMBURGER's Leitmotiv that every infection acts via the vessels (the endothelium) and hence via coagulation, we objectivized our diagnosis through the humoral inflammation status. This led to the realization that the sudden onset is caused by the cytokine increase resulting from the virus load. It is even possible to see the extent of the continuing cytokine increase just by taking a clinical look at the increasingly fearful physiognomy of the patient reflecting the premonition of an impending threat.



The main topic today no longer revolves around the sceptical comment that "they only help in the first 48 hours", a view often expressed at the beginning with regard to sialidase inhibitors. Because that its use as early as possible prevents the threatening "cytokine storm" this comment has to be reversed: From the sudden onset, which has a lasting impression on the patient, we have 48 hours in which to start etiotropic countermeasures aimed at the source to rescue the patient. One in five influenza infections can lead to a complication (Arnold Monto). In "Influenza Virus, Inflammation, Arteriosclerosis" (Vogel et al., Conference "Cytokines and Inflammation", San Francisco, January 27-28, 2005) we were able to show that influenza virus is the cause of the most common diseases among the

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population, namely: 1. Community acquired pneumonia, 2. heart attack, and 3. stroke. This is supported by a recent study with extensive data (Mohammad Madjid et al. *Eur.Heart J.*, May 2007; 28: 1205-1210). Our conclusion from this is: The main duty of the primary care physician is to apply all the latest knowledge in treating his patients. The analysis of our 205 case studies clearly shows: Prior to a bacterial infection there is always viral damage to the cell with cytokine release. We can witness this viral-bacterial transition through the humoral inflammation status (sum of the CRP and fibrinogen levels). In comparison to a similar patient group treated before sialidase inhibitors became available, this parameter, which is decisive in the progression of arteriosclerosis, was reduced by 50%. We then gain time for an early bacterial swab with an antibiogram. This is supported by "It Pays to have a Rapid Test" (Falsey AR et al. *Arch Intern Med* 2007; 167: 354-360). In future all physicians must be given the possibility of fulfilling the needs of the moment: by applying antibiotics specifically and sparingly. The frequent consideration of viral infections and "colds" to be banal and self-limiting leads to the sometimes fatal consequences of the resulting treatment. Moreover the inadequate differentiation between colds and influenza is resulting in an excessive and often harmful use of antibiotics. A Canadian study reported that antibiotics were prescribed for 84% of outpatients diagnosed with influenza (McGeer I., *ICAAC* 2006). The evaluation of our 188 influenza patients showed a reduction in the necessity of antibiotic administration to below 50%. Following a antibiotic treatment we prescribe probiotics with *Semen psylli*, in order to bring the mucosa of the small intestine back to normal. The socioeconomic costs of other virally caused diseases totalling 40 billion Dollars annually in the USA alone (without influenza) are enormous. Through applying our influenza strategy in treating these patients we have learned a great deal and have been able to avoid a lot of unnecessary antibiotic administration. This is why we fully agree with the view in Brandeins (www.brandeins.de 8. Jahrgang Heft 04 April 2006) that Mark von Itzstein's discovery (*Nature* 1993) is a milestone in the history of medicine. Our diagnostic objectivization has to be extended to toddlers and children because they are the fuel for all influenza epidemics. Compared to the era prior to 1999, when we could only observe the viral damage and wait, today we are in a position to launch an offensive against it. If we take the simulated study of a pandemic reported in the Midas Program of Longhini and Fergusson (www.nigms.nih.gov/Initiatives/MIDAS) as a basis and apply this to the primary care physician, who is in a position to act, the statement "act locally, stop globally" can serve as a positive message for the future where an influenza epidemic and pandemic are concerned.

Clinical Effectiveness of Zanamivir for Both Influenza A and B Patients

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Recently, we reported that oseltamivir was less effective against influenza B than against influenza A. However, the effectiveness of another neuraminidase inhibitor, zanamivir, has not been compared between influenza A and influenza B infection. The effectiveness of zanamivir was investigated in the patients with influenza A and influenza B infection in the 2001/2002, 2002/2003, 2003/2004 and 2005/2006 seasons. The mean duration of fever from the first inhalation of zanamivir was 30.6 hours in patients with influenza A and 34.9 hours in patients with influenza B. The frequency of patients afebrile at 24 hours after the first inhalation was 46.6 % in patients with influenza A and 38.0 % in patients with influenza B. The frequency of patients afebrile at 48 hours after the first inhalation were 84.8 % and 78.9 % in patients with influenza A and influenza B, respectively. These results suggested that the clinical effectiveness of zanamivir is quite comparable for both influenza A and influenza B infection.

Introduction

Recently, patients with influenza are usually diagnosed using influenza antigen detection kits at clinics and treated with anti-influenza drugs in Japan. Oseltamivir, an oral neuraminidase inhibitor, has become widely prescribed in Japan for the treatment. The clinical effectiveness of oseltamivir in the treatment of influenza A has been well documented [1-3]. Recently, we reported that oseltamivir was less effective against influenza B than against influenza A [4]. It was also reported that oseltamivir was less effective against influenza B than against influenza A in children [5]. However, the effectiveness of another neuraminidase inhibitor, zanamivir, has not been compared between influenza A and influenza B infection. We investigated the effectiveness of zanamivir in the patients with influenza A and influenza B infection, diagnosed by commercial antigen detection kits and determined viral subtypes by virus isolation, in the 2001/2002, 2002/2003, 2003/2004 and 2005/2006 seasons.

Patients and Methods

Family doctors, pediatricians, and physicians at clinics participated in the study. Patients reporting to clinics with influenza like illness manifesting symptoms such as body temperature being over 37.5 C, upper respiratory tract symptoms, and systemic symptoms were diagnosed as influenza A or B by commercial antigen detection kits. Among the influenza patients positive for antigen detection kits, those

who received zanamivir within 48 hours of onset were registered with informed consent as a part of PMS study of GSK Japan. The date and time of the onset of fever, the first inhalation of zanamivir, and the disappearance of fever were recorded by the physician, patient, or an attending family member. The first time that a patient reported a fever of over 37.5 C was defined as onset. Patients were asked to measure body temperature at least three time a day, at 8:00 am, 2:00 pm, and 8:00 pm, and the time at which a body temperature of under 37.5 C was attained was defined the time the patient became afebrile. Virus isolation was done with throat swabs, nasal swabs, or nasal aspirates by standard methods using Madin-Darby canine kidney cells.

Results

The effectiveness of zanamivir in the treatment of 232 patients with influenza A and 105 patients with influenza B infection were analyzed. Among influenza A patients, 29 cases were influenza A/H1N1 and 203 cases were influenza A/H3N2. The mean duration of fever from the first inhalation of zanamivir was 30.2±21.1 hours in patients with influenza A and 35.1±24.4 hours in patients with influenza B. The mean duration of fever from the onset was 63.8 hours in patients with influenza A and 71.2 hours in patients with influenza B. Among influenza A patients, the mean duration of fever from the first inhalation of zanamivir was 30.6 hours in patients with influenza A/H1N1 and 34.9 hours in patients with influenza A/H3N2. The percentage of patients afebrile at 24 or 48 hours after the first inhalation of zanamivir was analyzed as a parameter of the effectiveness. The frequency of patients afebrile at 24 hours after the first inhalation was 46.6 % in patients with influenza A and 38.0 % in patients with influenza B. The frequency of patients afebrile at 48 hours after the first inhalation were 84.8 % and 78.9 % in patients with influenza A and influenza B, respectively. There was no significant difference between the patients with influenza A and influenza B.

Discussion

In our previous study, the duration of fever after the first dose of oseltamivir was 31.2±23.7 and 47.1±30.8 hours, and the duration of fever from the onset of fever was 47.9±26.0 and 65.4±32.8 hours in patients with influenza A and influenza B, respectively [4]. We also reported the more frequent isolation of influenza B than influenza A virus after oseltamivir therapy. Thus, oseltamivir is less effective for influenza B than influenza A in the clinical setting. In this study, the duration of fever and frequency of afebrile patients were not significantly different in influenza A and influenza B patients treated with zanamivir. Studies of the *in vitro* antiviral activity of oseltamivir or zanamivir against laboratory strains of influenza virus that used culture and enzymatic assays have suggested that the influenza B virus is less susceptible than is the influenza A virus to oseltamivir and zanamivir [6]. The reported difference of the mean IC50 between influenza A and B viruses was less for zanamivir (2.09 nM vs 4.15 nM) than for oseltamivir (0.73 nM vs 11.53 nM). These findings may explain our results, in a clinical setting, showing

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that oseltamivir is less effective against influenza B infection than against influenza A and that zanamivir is equally effective against both. The effectiveness of oseltamivir and zanamivir can not be compared directly because of the difference in the patients age in our previous study and this study. Zanamivir was not used in children of age under five years old. However, these results suggested that zanamivir is more effective than oseltamivir for the treatment of influenza B infection.

Figure 1. The mean duration of fever from the first inhalation of zanamivir was 30.2 ± 21.1 hours for patients with influenza A and 35.1 ± 24.4 hours for patients with influenza B. There was no significant difference between patients with influenza A and influenza B.

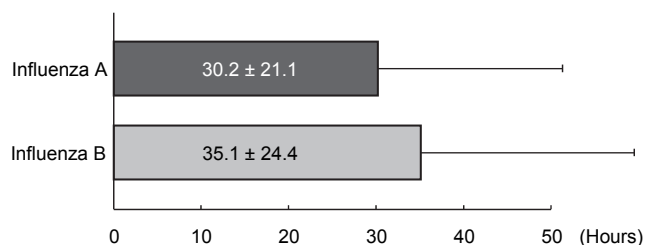
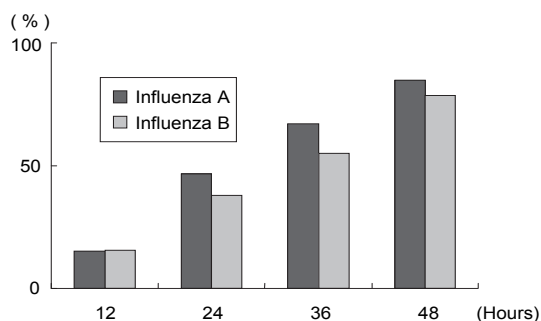


Figure 2. At 24 hours after the first inhalation of zanamivir, 46.6% of the patients with influenza A and 38.0% of the patients with influenza B had become afebrile. The respective frequencies of patients afebrile at 48 hours after the first inhalation were 84.8% and 78.9%. There was no significant difference between patients with influenza A and influenza B.



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The Impact of Rapid Antigen Testing for Influenza on Paediatric Admissions in a Large UK Hospital: A Retrospective Observation

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Introduction

Influenza-related hospital admissions in children are responsible for significant seasonal and unpredictable demands on the delivery of paediatric healthcare¹. As the majority of febrile children assessed in secondary care are managed without specific knowledge of the aetiology of their illness, influenza-related assessments are likely to lead to unnecessary admission, investigations and antibiotic treatment as opposed to outpatient treatment. The neuraminidase inhibitors offer targeted antiviral treatment and are licenced for use in children². Early treatment of influenza in children with oseltamivir is associated with significant reductions in complications such as otitis media and time to return to normal activities³. In many health care settings, clinical confidence in the use of antiviral therapy is dependent on confirmed diagnosis of influenza. However, near patient testing for respiratory pathogens including influenza in UK emergency departments is limited, in part due to resource constraints and a perception among clinicians that they offer little clinical benefit. In order to identify children presenting with influenza for enrolment into an oseltamivir treatment study, we introduced rapid antigen testing for influenza into the paediatric emergency unit at Leicester Children's Hospital and observed a reduction in influenza-related admissions compared to that seen in previous years.

Methods

The Leicester Children's Hospital assesses around 2800 children with acute respiratory illness during a typical winter season. Children attend the hospital admission unit as a result of direct referrals from primary care physicians or by self-presentation with their parents to the paediatric emergency department. Previously, during winters 2001/02 and 2002/03, we conducted a study to evaluate the burden of respiratory virus pathogens in children under 6 years of age presenting to secondary care with any febrile illness including respiratory, gastrointestinal, convulsion, and fever of unknown source⁴. Inclusion was not restricted to duration or severity of symptoms prior to presentation. Nose-and-throat or nasopharyngeal samples were obtained following written parental consent and later tested by RT-PCR for influenza A and B, respiratory syncytial virus and human metapneumovirus. Therefore, the result of molecular test was not available at the time of the assessment, and patients were managed by the paediatric admitting teams without knowledge of the underlying diagnosis. As a result

of this study, age-specific influenza-related assessment and admission hospitalisation rates for Leicester Children's Hospital are known for winters 2001/02 and 2002/03⁴. During winters 2005/06 and 2006/07, we introduced rapid antigen testing for influenza A and B (Roche Diagnostics) to the same paediatric emergency unit. The principal reason for near patient rapid testing was to identify influenza-infected children who could be approached for entry into an oseltamivir treatment study. Subjects offered oseltamivir were in accordance with licensed standard prescribing criteria in the UK and was therefore restricted to children whose symptom onset was <48 hours duration and who were aged >1 year. Exclusion criteria for rapid antigen test screening included contraindications to the use of oseltamivir such as use of probenecid, methotrexate or known allergy to oseltamivir. Importantly, no children were excluded from undergoing rapid test screening due to the severity of their presenting illness. The result of the near patient influenza rapid test was recorded in the clinical case-notes by the study nurse coordinating our treatment study. The normal emergency paediatric admission team remained responsible for all aspects of clinical management of the patient. Our study team had no role in clinical decisions including need for hospitalisation, investigations or antimicrobial therapy. We only approached parents of influenza-confirmed children to consider enrolment into a study evaluating the effect of oseltamivir on virus shedding. We were able to enroll children treated as either in and out-patients into the study. Both studies were approved by the Leicestershire ethics committee and Medicine Human Regulatory Agency as appropriate.

Results

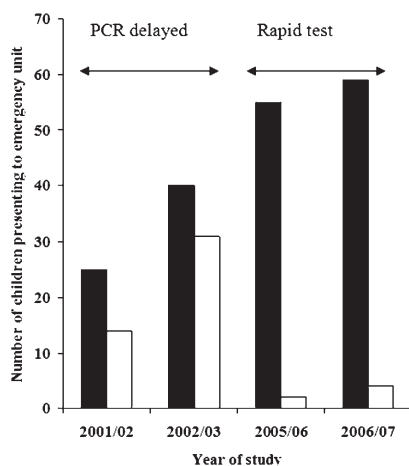
The number of influenza-confirmed admissions and attendances identified by PCR in winter 2001-03 or rapid antigen testing in winter 2005-07 are shown in Table 1 and Figure 1. There is a significant difference in % of influenza-confirmed children admitted to hospital between 2001-03 and 2005-07 (45/73, 62% vs. 6/114, 5%; $p < 0.001$). *Epidemiology surveillance study 2001-03*⁴. During 2001/02, 33 recruited children were subsequently diagnosed as presenting with influenza infection by PCR. Of these, 14 (42%) were hospitalised for a median duration of 2 days. During 2002/03, 40 children were subsequently diagnosed with influenza by PCR. Of these 31 (77%) were hospitalised for a median duration of 2 days. No specific antiviral treatment was prescribed for any of the influenza-confirmed children during 2001-03. For 2001-03, the frequency of influenza subtypes were H3 (73%), H1 (11%) and B (16%). *Oseltamivir treatment study 2005-07*. During 2005/6, 55 children screened positive for influenza by near patient rapid antigen test. Two (3.6%) were hospitalised. The remaining 53 were discharged; 47 received oseltamivir as part of the clinical study. During 2006/07, 59 children were positive for influenza by near patient rapid antigen test. Four (6.8%) were hospitalised. The remaining 56 were discharged; 32 received oseltamivir as part of the study. Reasons for non-inclusion into the oseltamivir treatment study

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Table 1. The number of influenza-related hospital admissions and assessment diagnosed by PCR or rapid antigen tests in winters 2001-03 and 2005-7 at Leicester Children's Hospital.

Winter season	Diagnostic test	Result available at time of presentation	Number of influenza assessments	Number of influenza admissions (% of assessments)	Median hospital stay (days)
2001-03	PCR	No	73	45 (62%)	2
2005-07	Rapid test	Yes	114	6 (5%)	3

Figure 1. The number of children with influenza attending and admitted from Leicester Children's Hospital by either PCR in 2001-03 or rapid antigen testing in 2005-07.



included <1 year of age, duration of symptoms >48 hours or parental decline of consent. Overall, for winters 2005-07, 72 children were enrolled in the oseltamivir treatment study and the distribution of virus subtypes for these patients was H3 (53%), H1 (16%), B (28%) and 2 (3%) mixed. The remaining samples (n=42) were from children who were not enrolled in the clinical study and who were tested by the near patient rapid test for influenza A or B only. However, most of these subjects would be expected to have been infected with influenza A/H3N2 reflecting its predominance in both local and national activity during 2006/07.

Discussion

The observed reduction in influenza-related hospitalisations from the emergency department of Leicester Children's Hospital following introduction of rapid testing for influenza is striking. It appears that diagnosis of influenza and the availability of specific antiviral treatment enables clinicians to safely discharge influenza-infected children presenting to secondary care. This reduction in hospital admissions is likely to decrease the potential for nosocomial transmission, unnecessary invasive investigations and antibiotic prescriptions. Previous cost-benefit analyses of near patient influenza testing have predominantly focused on their role in guiding treatment with antivirals rather than their role in reducing hospitalization rates. However, reductions in hospital stay, antibiotic use and number of microbiological tests per subject have been reported following the introduction of rapid antigen testing test into hospitals⁵⁻⁸. There

are a number of limitations to our observation as we did not formally assess the impact of near patient tests in a randomised study. We are comparing children hospitalisation rates from studies conducted in different influenza seasons. The annual impact of influenza varies considerably and is dependent on the prevalent circulating strain, the age-specific attack rates and the underlying herd immunity of the population in that winter. Influenza A/H3 viruses are generally considered to cause more severe illness with increased morbidity⁹. The impact of influenza B typically has greatest burden among school aged children¹⁰. Overall, the greatest burden of paediatric influenza falls among those aged <1 year¹⁴. In the oseltamivir treatment studies during 2005-07, we principally screened those over >1 year who were eligible for antiviral therapy. In contrast, these younger children were included in the epidemiology/burden of disease studies conducted in 2001-03. It is possible that there is a higher threshold for admissions among the very young children even if the aetiology of the febrile illness is known, particularly as antiviral treatment is not licensed in this population. We did not follow up those influenza-infected children who were discharged without antiviral treatment and do not know whether they attended primary care services for further evaluation or additional prescriptions. Despite these limitations, the observed reduction in influenza-related paediatric hospital admissions suggests that availability of near patient testing and antiviral treatment offers cost savings to the delivery of health care during periods of influenza activity.

Sources of Funding

The epidemiology studies (2001-03) were supported by Wyeth Pharmaceuticals. The oseltamivir treatment studies (2005-07) were supported by Hoffman-La Roche. The funders had no influence on data analysis, interpretation or conclusions. *Conflict of interest.* IS and KN have received funding from pharmaceutical industry for research support, speakers fees and travel to international meetings including from GSK and Roche who manufacture neuraminidase inhibitors.

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Diagnosis of Influenza By Clinical Symptoms: A Comparison With the Results By Antigen Detection Kits

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The clinical symptoms and the reliability of symptomatic criteria were evaluated in patients with influenza A or B diagnosed by commercial antigen detection kits. The percentage of patients with fever over 38°C was significantly lower, and with fever less than 37.6°C was significantly higher in influenza B than in influenza A in adults. In all age groups, the percentage of patients with cough was over 80% for both influenza A and B. The reliability of symptomatic criteria proposed by the Ministry of Health, Labor and Welfare in Japan (J-criteria) and by Monto, et al (M-criteria) was compared. For influenza A, the sensitivity and accuracy were significantly higher for the M-criteria (81.4% and 75.6%, respectively) than for the J-criteria (66.1% and 65.9%, respectively, both $p < 0.001$). However, even by the M-criteria, the sensitivity and accuracy were significantly lower for influenza B than for influenza A. And the accuracy was prominently lower for the J-criteria than for the M-criteria in the age group of over 64 years (51.9% and 75.6%, respectively, $p < 0.001$). An accurate symptomatic diagnosis is rather difficult and commercial antigen detection kits would seem to be necessary for the diagnosis of influenza B or to differentiate influenza A from influenza B infection.

Introduction

Commercial antigen detection kits have made it possible to easily differentiate influenza A and influenza B infection (1). A precise diagnosis of influenza in outpatients clinics is necessary before prescribing anti-influenza drugs, e.g. neuraminidase inhibitors (2,3). However, clinical symptoms and the reliability of symptomatic criteria have not been fully compared with influenza A and influenza B infection diagnosed by commercial antigen detection kits. We did a multi-center study among outpatients in Japan to evaluate the clinical symptoms and the reliability of symptomatic criteria.

Method

Clinical symptoms were compared between 1,408 patients with influenza A (749 females and 659 males, mean age 26.7 years) and 737 patients with influenza B (380 female and 357 male, mean age 16.3 years) diagnosed by commercial antigen detection kits at 25 clinics in the 2002-2003 influenza season in Japan (2). For all patients with influenza, the highest body temperature was recorded, and the presence or absence of each symptom (fever, cough, rhinorrhea, sore throat, headache, chilliness, myalgia, fatigue, loss of appetite,

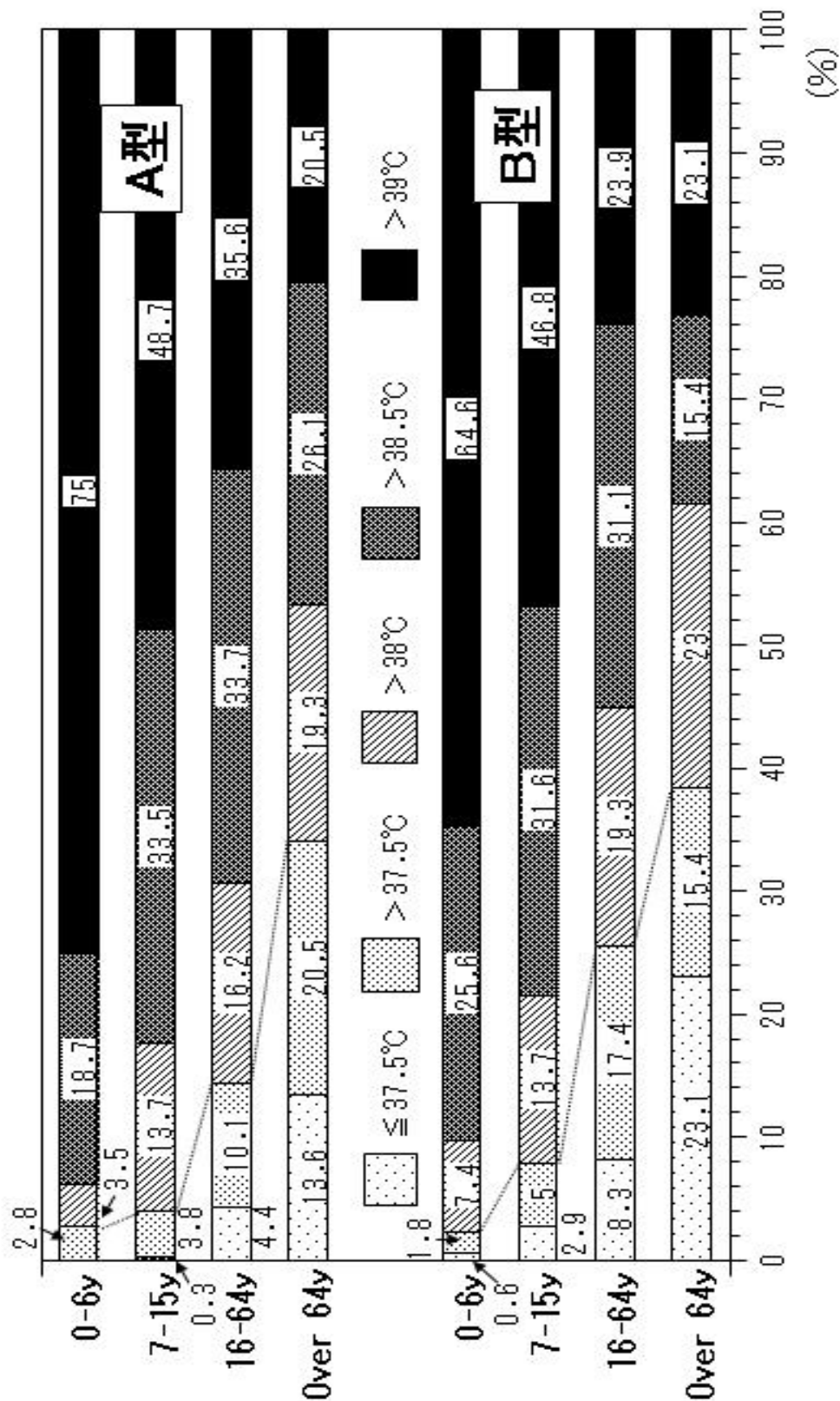
vomiting and diarrhea) was checked by their family doctor. The percentage of patients with each clinical symptom was calculated for the age cohorts 0-6, 7-15, 16-64 and over 64 years old, and also by influenza A and influenza B status. The reliability of symptomatic criteria proposed by the Ministry of Health, Labor and Welfare in Japan (J-criteria) was evaluated in the 2005-2006 season (1,611 patients with influenza A and 310 controls in 32 clinics). This criteria includes the presence of all of the following four major symptoms; sudden onset, fever over 38°C, upper respiratory infection (any of sore throat, rhinorrhea or cough, in this study) and general symptoms (any of general fatigue, loss of appetite, headache or myalgia, in this study). The reliability of symptomatic criteria by Monto, et al (M-criteria) was compared between patients with influenza A in the 2005-2006 season and patients with influenza B in the 2004-2005 season (2,479 with influenza B and 862 controls in 36 clinics). This criteria was the presence of cough and fever, with the fever criteria 37.8°C or over for patients under 65 years and 37.2°C or over for patients 65 years or older. Capilia FluA, B (2002/2003 and 2004/2005) and Capilia Flu A+B (2005/2006) were the most commonly used commercial antigen detection kits (1). For statistical analysis, the χ^2 -test was done to compare between group differences in the percentage, and a p value of < 0.05 was considered to be statistically significant.

Results

Fever (Figure 1). The respective percentages of patients with fever over 38°C in the 0-6, 7-15, 16-64 and over 64 years age groups were 97.2%, 95.9%, 85.5% and 65.9% in patients with influenza A and 97.6%, 92.1%, 74.3% and 61.5% in patients with influenza B. The percentage of patients with fever over 38°C was significantly lower in influenza B (74.3%) than in influenza A (85.5%) in adults aged 16-64 years ($p < 0.001$). The percentage of patients with fever less than 37.6°C was significantly higher in patients with influenza B (8.3%) than in patients with influenza A (4.4%) in adults ($p < 0.05$), and were 23.1% (influenza B) and 13.6% (influenza A) in patients aged 65 years or over.

Other symptoms. In all age groups, the percentage of patients with cough was over 80% for both influenza A (0-6 years: 88.5%, 7-15 years: 89.2%, 16-64 years: 83.5%, over 64 years: 83.0%) and influenza B (0-6 years: 84.1%, 7-15 years: 87.4%, 16-64 years: 82.1%, over 64 years: 84.6%). The percentage of patients with rhinorrhea was higher for children than for adults, both for patients with influenza A (0-6 years: 82.5%, 7-15 years: 73.7%, 16-64 years: 65.6%, over 64 years: 61.4%) and with influenza B (0-6 years: 85.4%, 7-15 years: 73.7%, 16-64 years: 70.2%, over 64 years: 53.8%). The respective percentages of patients with loss of appetite, vomiting, and diarrhea for each age group were 45.5-53.6%, 7.7-22.6% and 4.5-18.3% for influenza A, and 38.5-53.8%, 10.6-18.7% and 7.7-15.9% for influenza B. The respective percentages of influenza A infected patients 16-64 and over 64 years with other symptoms aged were 76.4 and 65.9% with general fatigue, 83.3 and 76.1% with chilliness, 74.9 and 60.2% with headache, 72.9 and 65.9% with myalgia, and 66.8 and 61.4% with sore throat. The respective percentages

Figure 1. The highest body temperature in each age group in patients with influenza A and influenza B (2002-2003 season).



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of influenza B infected patients aged 16-64 years and over 64 years were 73.9 and 92.3% with general fatigue, 73.9 and 46.2% with chilliness, 71.1 and 53.8% with headache, 63.8 and 53.8% with myalgia, and 71.6 and 61.5% with sore throat. The differences in the percentage of influenza A and influenza B infected patients with each symptom were minimal.

Reliability of the symptomatic criteria (Table 1). For influenza A, the sensitivity and accuracy were significantly higher for the M-criteria (81.4% and 75.6%, respectively) than for the J-criteria (66.1% and 65.9%, respectively, both $p < 0.001$). However, even by the M-criteria, the sensitivity and accuracy were significantly lower for influenza B (72.8% and 64.1%, respectively) than for influenza A infection (81.4% and 75.6%, respectively, $p < 0.001$).

Table 1. Reliability of each symptomatic criteria and accuracy in each age group.

Reliability of each criteria	(2004-2005 and 2005-2006 seasons)							
	Sensitivity		Specificity		PPV	NPV	Accuracy	
J-criteria for influenza A	66.1	1	64.8	1	90.7	26.9	65.9	1
		***		***				***
M-criteria for influenza A	81.4	J	45.2	J	88.5	31.9	75.6	J
	###			###			###	
M-criteria for influenza B	72.8	L	39.2	L	77.5	33.3	64.1	L
Accuracy in each age group								
	0-6y		7-15y		16-64y	over 64y	All ages	
J-criteria for influenza A	62.5	1	66.9	1	69.8	51.9	65.9	1
		***		***	*	*		***
M-criteria for influenza A	72.3	J	81.4	J	74.6	78.5	75.6	J
	###		###		###		###	
M-criteria for influenza B	60.2	L	64.8	L	66.1	67	64.1	L

* $p < 0.05$, *** $p < 0.001$ between J-criteria and M-criteria in influenza A

$p < 0.001$ between influenza A and influenza B in M-criteria

(Kawai N, et al)

There were no significant differences in PPV (Positive Predictive Value) or NPV (Negative Predictive Value) between the J-criteria and M-criteria in influenza A infection. However, PPV was significantly lower for influenza B (77.5%) than for influenza A infection (88.5%) by the M-criteria. In general, PPV was high (77.5-90.7%) and NPV was low (26.9-33.3%) for both symptomatic criteria. For influenza A, the accuracy was significantly lower for the J-criteria than for the M-criteria in all age groups: 0-6 years (62.5% and 72.3%, respectively, $p < 0.001$), 7-15 years (66.9% and 81.4%, respectively, $p < 0.001$), 16-64 years (69.8% and 74.6%, respectively, $p < 0.05$) and over 64 years (51.9% and 75.6%, respectively, $p < 0.001$). The difference was prominent in patients over 64 years. By the M-criteria, the accuracy was significantly lower for influenza B than for influenza A infection in the age groups 0-6 years (60.2% and 72.3%, respectively, $p < 0.001$), 7-15 years (64.8% and 81.4%, respectively, $p < 0.001$) and 16-64 years (66.1% and 74.6%, respectively, $p < 0.001$).

Discussion

The percentage of patients with a high fever, over 38°C, was higher in children and lower in elderly patients. This may be caused by the immaturity of the immune system or no prior exposure to any type of influenza in children. The percentage of patients aged 16-64 years with high fever was significantly

lower for influenza B than for influenza A infection. Seasonal variance of the influenza B virus was little in comparison with influenza A virus, indicating that adults may have some protection against high fever because of prior exposure of influenza B. It should be noted that a subfebrile highest body temperature, less than 37.5°C, was detected in 13.6% of the patients aged over 64 years with influenza A and 23.1% of such patients with influenza B. It may be difficult to diagnose influenza in these elderly outpatients without commercial antigen detection kits. Cough was constantly (over 80%) detected in all age groups and in both influenza A and influenza B patients. Cough would thus be a useful parameter for diagnosing influenza. However, no appropriate parameters were found that symptomatically differentiated influenza A and influenza B infection. The use of commercial antigen detection kits would seem to be necessary to differentiate influenza A and B infection. Our previous studies of oseltamivir therapy found duration of fever 2,3) and virus shedding 5) to be significantly longer in influenza B than in influenza A infection. To the contrary, no differences were shown in the effectiveness of zanamivir between influenza A and influenza B infection in our preliminary study, and zanamivir was recommended by our recent report as an effective anti-influenza drug for influenza B infection 6). For the evaluation of symptomatic criteria, the accuracy was extremely lower (51.9%) by the J-criteria (fever criteria of over 38°C in all ages) than by the M-criteria (78.5%, fever criteria of 37.8°C for patients less than 65 years or 37.2°C for 65 years or over) in patients over 64 years with influenza A. The accuracy was lower for influenza B than for influenza A. The percentage of patients with fever over 38°C was lower in patients with influenza B than in patients with influenza A. It may be necessary to change the fever criteria for influenza A and influenza B. For the symptomatic criteria, PPV was very high, however, NPV was low. The diagnosis of influenza by commercial antigen detection kits would seem to be necessary for patients with negative response to symptomatic criteria, especially in epidemics of influenza B7) and in the elderly.

Conclusion

The reliability of the J-criteria was lower than that of the M-criteria, especially for elderly patients. An accurate symptomatic diagnosis is rather difficult, thus commercial antigen detection kits would seem to be necessary for the diagnosis of influenza B infection and to differentiate influenza A from influenza B infection in mixed epidemics of influenza A and B infection.

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Vaccination of Children Reduces Hospitalization Rates Due To Influenza in the Elderly

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A growing body of research suggests that children may play an integral role in the transmission of influenza, not only to other children, but also to older members of the community. The elderly are especially vulnerable to the effects of influenza in terms of hospitalizations, medical complications, and deaths. We hypothesize that, in the absence of universal vaccination for all age groups, vaccinating children would have a beneficial effect on reducing pneumonia and influenza-related (P&I) hospitalizations in the elderly population (≥ 65). We abstracted approximately 7.7 million hospitalization records from the database of medical claims maintained by the Center for Medicare and Medicaid Services for six influenza seasons, 1998-99 through 2003-04. State-level influenza annual vaccination coverage in children age 6-23 months and related vaccines in children age 19-35 months were obtained from the National Immunization Survey, and influenza vaccination coverage in the elderly was obtained from the Behavioral Risk Factor Surveillance System for the same period. We estimated a single-year age distribution of rates of P&I hospitalization by state for each influenza season, defined as July 1 through June 30 of the following year. We observed an exponential acceleration in the P&I rates with age for each state and influenza season. State- and season-specific acceleration rates were regressed against the percentage of vaccinated children or elderly, or both. State-level vaccination coverage in children was significantly associated with acceleration of the P&I hospitalization in the elderly in 1998-99 ($\beta = -0.046$, $p = 0.011$) and in 2000-01 ($\beta = -0.042$, $p = 0.018$). Child influenza vaccination coverage was also negatively associated with elderly P&I hospitalizations in the 2003-04 season ($\beta = -0.096$, $p = 0.027$). However, state-level influenza vaccination coverage of the elderly was not significantly associated with the acceleration in P&I rates in any of the six seasons in any of the models. These results suggest that, on the state level, vaccination of children reduces hospitalization rates due to influenza in the elderly.

Introduction

Despite advancements in public health and medicine to reduce the global influence of infectious diseases, influenza still remains a significant threat to public health today. Children and elderly are particularly vulnerable to influenza. Hospitalization rates due to influenza in children range from 25 per 10,000 for children age 5 to 14 to as high as 600 per 10,000 in infants less than 6 months of age.¹ The elderly population experiences the most serious consequences of influenza, primarily due to the development of pneumonia following

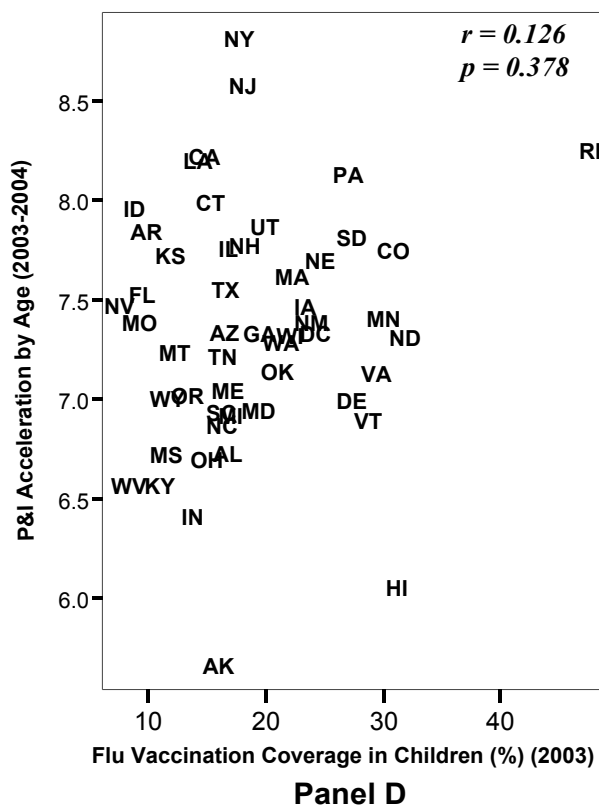
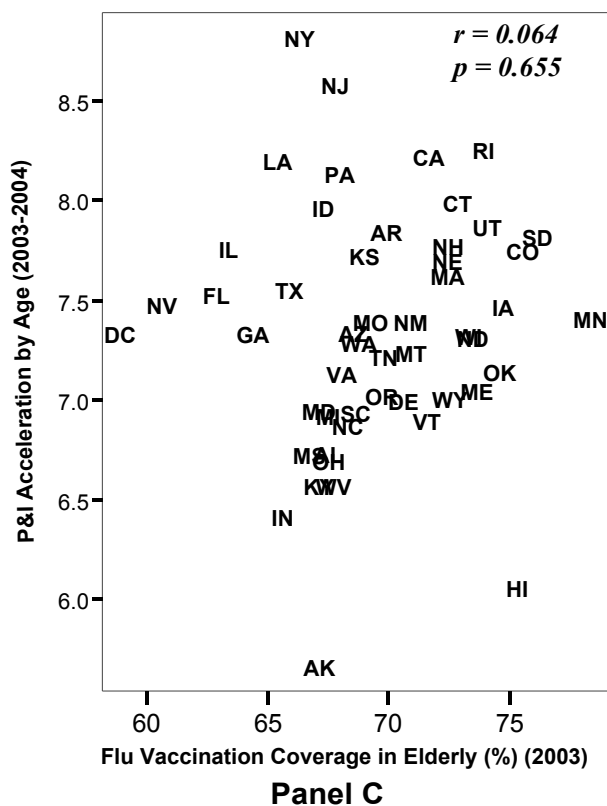
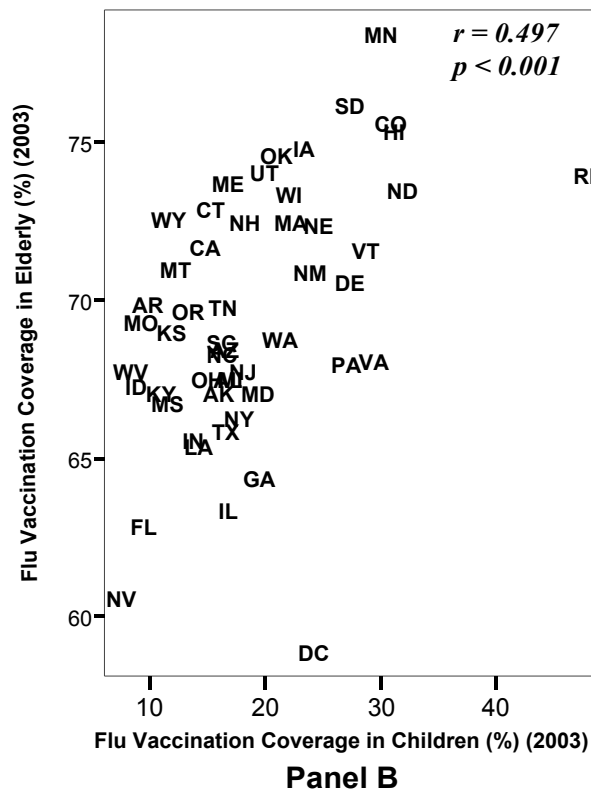
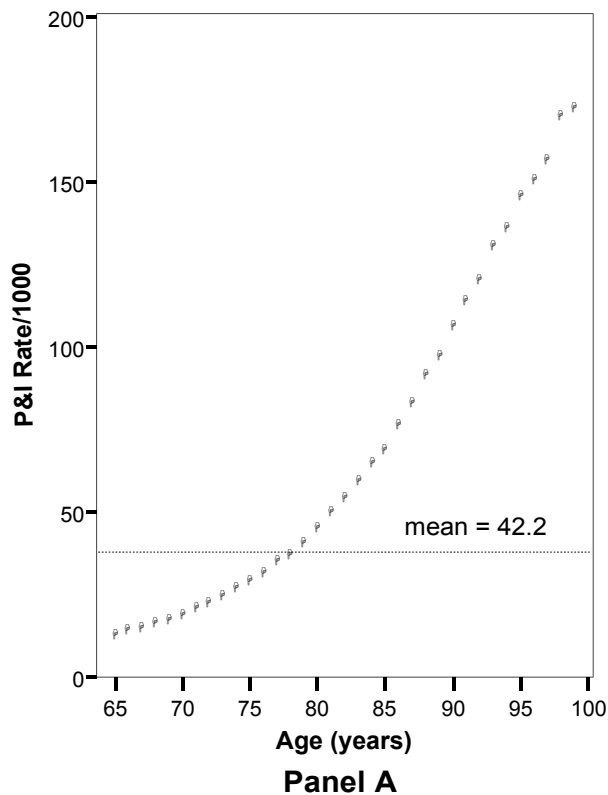
influenza virus infection.^{2,3} The age-specific mortality rate from P&I deaths is nearly 100 times higher in people aged 65 and over (22.1/100,000 person-years) than for children under 1 year of age, the group having the second-highest influenza mortality rate (0.3/100,000 person-years). From 1990 through 1998, 90% of influenza-associated deaths occurred among the population age 65 and older. There are interventions designed to alleviate the burden of influenza. Influenza control is primarily accomplished through annual vaccination. The Centers for Disease Control and Prevention (CDC) disseminate a listing of which population groups should be vaccinated, maintaining that certain population subgroups have priority in obtaining vaccinations. These guidelines generally emphasize the vaccination of those most exposed and most vulnerable to influenza, including health care workers, elderly, then children, and finally the general population. Vaccine efficacy is shown to be lowest in the elderly population, compared to children and non-elderly adults.^{4,5} There is a small but growing body of evidence suggesting that vaccinating children against influenza may not only benefit children themselves, but has indirect benefits for the elderly population with respect to reducing the influenza burden.^{6,7,8,9} The purpose of this study is to evaluate if and how influenza vaccination coverage in the elderly and children relate to influenza hospitalizations in the elderly population.

Methods

Outcome data. All claims records of hospitalizations associated with pneumonia and influenza (ICD-9 codes 480-487)^{10,11} were abstracted from the Centers for Medicare and Medicaid Services data bases for each of six influenza seasons from 1998-99 to 2003-04. For each state and each season the records were arranged as a single year age distribution, US Census 2000 population counts were obtained for the population age 65 to 99, and the P&I rates were estimated for each year of age, each state, and each season, where an influenza season is defined as July through June of the following year. Age-specific P&I rates increase approximately exponentially with age. To obtain summary-level statistics of this exponential acceleration of P&I rates in the older population, log-transformed age-specific rates were regressed against age for each state and season (see Figure 1, Panel A). The slope obtained from these regression models represented the degree of exponential increase, or acceleration, of P&I rates with age. This slope parameter will be referred to as the exponential acceleration of pneumonia and influenza rates with age (EAIR). The EAIR provides a measure of acceleration by age in the elderly population. For example, an EAIR of 0.02 can be interpreted as an exponential increase in the P&I rate of $\exp(0.02)$, or a 2.0% increase, with each year of age. For the age group 65-100, this could correspond to an increase of $\exp(0.02*35)$, or a 101% increase in P&I rates from age 65 to 100. This method was developed and used in a prior study to alleviate the problem of aggregation by age and to take advantage of the robustness of the CMS data.¹²

Poster Presentations: Clinical Guidance and Policies

Figure 1. P&I rates and vaccination coverage (2003-04)-US P&I rates by age (Panel A); child influenza vaccination coverage versus elderly influenza vaccination coverage (Panel B); P&I acceleration versus elderly influenza vaccination coverage (Panel C); P&I acceleration versus child influenza vaccination coverage (Panel D).



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Exposure data. Vaccination coverage in the elderly (65+) population was obtained through the Behavioral Risk Factor Surveillance System (BRFSS). The BRFSS is a random, digit-dial telephone survey administered by CDC to assess prevalence, trends, and spatial distributions of many disease and disease risk factors in the US and its territories.¹³ Vaccination status was asked of study participants age 65 and above and the aggregated state-level annual influenza vaccination coverage was abstracted for this analysis. Influenza vaccination coverage in children was provided by the CDC's National Immunization Program, which conducts the annual National Immunization Survey (NIS), a list-assisted, random digit-dialing telephone survey of homes with young children. Population characteristics were obtained from the US Census Bureau 2000 decennial census. This data was available for children age 6-23 months of age for two seasons, 2002-03 and 2003-04. Because of the limited availability of this data, and because the outcome data contains both influenza and pneumonia, we abstracted an additional set of data depicting vaccination of children age 19-35 months as a control for general coverage with childhood vaccines at the state level. This data was also obtained from the NIS and was assessed using the immunization covering 4:3:1:3, which includes four or more doses of DTP, three or more doses of poliovirus vaccine, one or more doses of any MCV, and three or more doses of *Haemophilus influenzae*.

Statistical Analysis. The EAIRs were regressed separately against state estimates of influenza vaccination coverage in elderly (Model 1) and in children (Model 3) for each of the size influenza seasons (1998-99 through and 2003-04). Model 2 was an extension of Model 1, including a term for the average P&I rate in each state. Model 4 was utilized for 2002-03 and 2003-04 only, and included only influenza coverage in children. Model 5 contained all of the aforementioned variables, plus an interaction term for influenza vaccination coverage in children and the average P&I rate, and was available only for 2002-03 and 2003-04. All models controlled for population density and average per capita income. All statistical analyses were conducted using SPSS version 14.0 (Chicago, IL).

Results

The results of the analysis are shown in Table 1. Median per capita income was found to be negatively associated with the average P&I rate for all seasons. Influenza coverage in children age 6-23 months was found to be positively associated with influenza coverage in the elderly population in the last two seasons (see Figure 1, Panel B). For the season 1998-99, states with higher 4:3:1:3 vaccination coverage in children tended to have lower acceleration in P&I rates in the elderly. This relationship held, even after including elderly influenza vaccination coverage and average P&I rate in the elderly in the model. In the 2000-01 season, a similar negative association was observed between 4:3:1:3 vaccination coverage in children and the elderly EAIRs, and this association remained significant in the model with elderly influenza vaccination coverage and average elderly P&I rate. No significant associations were

observed, however, between the vaccination coverage in the elderly and the elderly P&I curve slope in any of the six seasons, even after adjusting for other variables (see Figure 1, Panel C). In model 5 for 2003-04, P&I acceleration with age was negatively associated with influenza vaccination coverage in children and the average P&I rate (see Figure 1, Panel D). Panel A shows the national age-specific P&I rates against age. This illustrates the approximately exponential nature of the increase in P&I rates with age in the elderly population. Panel B shows the relationship between influenza vaccination coverage in children and in the elderly by state ($r = 0.497, p < 0.001$). This relationship is positive, suggesting that states with higher vaccination coverage in children also tend to have high coverage in the elderly. Panels C and D depicts the weak relationships between the EAIR and state influenza vaccination coverage in the elderly ($r = 0.064, p = 0.655$) or in children ($r = -0.126, p = 0.378$).

Discussion

These findings suggest that vaccination coverage in the elderly is not associated with P&I hospitalizations in the elderly. However, vaccinating children against influenza may potentially reduce the influenza burden in the elderly. This analysis is among the first to consider immunosenescence, as measured by acceleration of P&I hospitalizations with age, on the population level, and our results suggest that the elderly population might receive indirect benefits from vaccinating children potentially through indirect protection. The main contribution of this study is to compare vaccination coverage in both children and the elderly to influenza morbidity in the elderly both individually and simultaneously in population-based models. A second contribution of this study is the recognition and use of the exponential increase in P&I hospitalization rates in the elderly population with age. Not only did we fit the P&I acceleration curves with high precision to produce one summary-level measure of P&I acceleration,¹² but we also regressed curve estimates against population-level socioeconomic measurements that influence the underlying processes of both disease and population dynamics. The proposed characteristics of the exponential acceleration provides an overall assessment of how P&I morbidity changes with age in each state. These curves are generally distinct from one another, depending upon state and influenza season. The faster age-specific P&I hospitalization rates increase with age, the higher the acceleration is. This measure is reflective of the entire age spectrum of the 65 and over population and takes into account the richness of the age-specific data, while avoiding the problem of truncation and aggregation by age. There are several limitations of this study. In this study, only six years of data were examined, with only two years of influenza vaccination coverage in children. Additionally, the models did not account for vaccination effectiveness, which undoubtedly depend upon matching the vaccination strain to the prevailing strain of influenza in each season.¹⁴ The Centers for Medicare and Medicaid Services data represent nearly 100% of the hospitalizations in the elderly population; however, these records do not include influenza cases that did not result in

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Table 1. Summary statistics and parameter estimates and standard errors for slope and intercept models for four seasons and for the difference between seasons.

		1998-1999	1999-2000	2000-2001	2001-2002	2002-2003	2003-2004
Summary Statistics							
	Total number of cases	1,237,412	1,244,126	1,209,522	1,317,959	1,287,532	1,424,390
	Overall State Elderly P&I Rate (per 1,000)	37.9 (10.4)	36.5 (9.6)	39.6 (10.2)	39.4 (9.9)	38.9 (10.1)	42.2 (10.9)
	Exponential Acceleration of P&I Rate (EAIR)	7.79 (0.59)	7.87 (0.71)	8.01 (0.59)	8.01 (0.56)	7.62 (0.55)	7.36 (0.61)
	Predicted P&I at age 65 (per 1,000)	14.0 (4.2)	14.1 (4.2)	13.4 (3.9)	14.4 (4.0)	14.8 (4.3)	16.6 (4.8)
	Elderly vaccination coverage (EVC) (%)	67.3 (4.1)	67.6 (4.1)	67.9 (4.3)	68.2 (4.5)	69.0 (4.2)	69.6 (4.0)
	Child 4:3:1:3 vaccination coverage (CVC) (%)	79.8 (4.3)	79.6 (4.6)	77.5 (4.4)	78.1 (4.3)	77.9 (5.4)	81.7 (4.7)
	Child influenza vaccination coverage (CIVC) (%)					8.2 (4.8)	19.0 (7.8)
Correlation							
	CVC vs. EVC	0.068	0.064	0.195	-0.108	0.188	0.155
	CIVC vs. EVC					0.451**	0.497***
	CIVC vs. CVC					0.226	0.146
	CVC vs. EAIR	-0.284*	-0.170	-0.294*	-0.123	-0.122	-0.050
	CIVC vs. EAIR					0.285*	0.126
	EVC vs. EAIR	-0.119	-0.195	-0.123	-0.108	-0.077	0.064
	Median Per Capita Income and Average Rate	-0.553***	-0.559***	-0.480***	-0.466**	-0.471***	-0.420**
	Median Per Capita Income and EVC	-0.202	-0.179	-0.148	-0.020	-0.067	-0.131
	Median Per Capita Income and CIVC					0.231	0.248
Parameter Estimates- Beta (SE)							
Model 1†	Elderly vaccination coverage	-0.008 (0.020)	-0.016 (0.023)	-0.001 (0.019)	-0.003 (0.019)	-0.003 (0.020)	0.026 (0.021)
Model 2†	Elderly vaccination coverage	-0.016 (0.022)	-0.029 (0.026)	-0.010 (0.021)	-0.018 (0.019)	-0.016 (0.019)	0.023 (0.022)
	Average Rate	-0.012 (0.010)	-0.008 (0.002)	-0.011 (0.010)	-0.004 (0.009)	-0.004 (0.009)	-0.002 (0.009)
Model 3†	Child vaccination coverage	-0.044 (0.017)*	-0.018 (0.021)	-0.039 (0.017)*	-0.011 (0.019)	-0.023 (0.014)	-0.011 (0.018)
Model 4†	Child flu coverage	--	--	--	--	0.026 (0.016)	0.005 (0.011)
Model 5†	Child vaccination coverage	-0.046 (0.019)*	-0.030 (0.023)	-0.042 (0.020)*	-0.008 (0.022)	-0.022 (0.015)	-0.027 (0.019)
	Child flu coverage	--	--	--	--	-0.028 (0.066)	-0.096 (0.042)*
	Elderly vaccination coverage	-0.006 (0.021)	-0.022 (0.027)	0.003 (0.021)	-0.014 (0.021)	-0.034 (0.022)	0.045 (0.028)
	Average Rate	-0.007 (0.010)	-0.002 (0.012)	-0.004 (0.010)	-0.003 (0.010)	-0.020 (0.016)	-0.045 (0.021)*
	Child flu coverage*average rate	--	--	--	--	0.002 (0.002)	0.002 (0.001)*
Circulating virus strain¹⁴							
	H1N1	Bayern/7/95, Beijing/262/95	New Caledonia/20/99, Bayern/7/95	Bayern/7/95, New Caledonia/20/99	New Caledonia/20/99	New Caledonia/20/99	New Caledonia/20/99
	H3N2	Sydney/5/97	Sydney/5/97, Moscow/10/99, Panama/2007/99	Moscow/10/99, Panama/2007/99	Moscow/10/99, Panama/2007/99	Panama/2007/99	Panama/2007/99, Fujian/411/2002
	B	Beijing/184/93, Victoria/2/87	Beijing/184/93	Beijing/184/93, Sichuan/379/99	Sichuan/379/99, Shizouka/15/01, Hong Kong/330/01	Hong Kong/330/01	Hong Kong/330/01, B/Sichuan/379/99, B/Victoria/2/87

Boldface indicates match with recommended vaccine strain for that season.

*p<0.05

**p<0.01

***p<0.001

†Adjusted for population density and median state income.

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hospitalizations, nor the complications of influenza resulting in non-pneumonia hospitalizations. The exposure data, vaccination coverage in two population subgroups, are based on surveys, and, as is the case with other surveys, are subject to potential sampling error and bias. As far as we know, the National Immunization Survey is validated by provider contact and a review of a random sample of immunization records on children included in the survey, while the BRFSS is not validated. Likewise, the vaccination data in children is based solely on children age 6 to 23 months. It is possible that older, school-aged children may also play a more important role than infants and toddlers in spreading influenza from children to the elderly, and this prospective feature is not adequately captured in this study. Also, the results of this study are from the first 2 years of influenza vaccine "encouragement" for children age 6-23 months, but not yet recommended and coverage was quite low. Additional years of data where vaccine coverage among children has increased will need to be evaluated to validate the results of our study. Despite these limitations, this study is among the first to assess the relationship between vaccination coverage in children and the elderly and pneumonia and influenza outcomes in the elderly on the population level. Though preliminary, these findings suggest that one effective strategy to lessen influenza in the vulnerable elderly population may be to immunize children against the disease. Our future research will address some of the limitations discussed above and consist of a more detailed longitudinal analysis of P&I morbidity and vaccination coverage in population subgroups, and the impact of various socioeconomic factors on the population level, including income, access to care and availability of hospital beds, that may mitigate or mediate the relationship between vaccination distribution and influenza outcomes at the population level.

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**Preclinical Vaccines and Other
Intervention Strategies**

**Poster
Presentations**

Cellular Immunity and Cross Protection Studies in BALB/C Mice Immunized With Vero Cell Derived H5N1 A/Vietnam/1203/2004 (Clade 1) and Indonesia/05/2005 (Clade 2) Inactivated Whole Virus Pandemic Influenza Vaccines

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Introduction

It is widely believed that the emergence of a new influenza pandemic caused by avian strains is only a matter of time [1]. Vaccines are considered the most effective means of controlling influenza pandemics [2]. The most common strategy for vaccine production makes use of genetically attenuated reassortants to manufacture vaccine in embryonated eggs [3]. There are however several draw backs to using this strategy: (a) significant time may be required for generation and safety testing of such reassortants; (b) the vaccine may also not provide an optimal antigenic fit with the wild-type circulating virus as it is derived from a reassortant which contains only the modified HA and N genes from the wild-type virus; (c) egg supplies could be endangered by H5N1 infections of chicken flocks; and (d) recent clinical trial with H5N1 split vaccine formulations have demonstrated that very high antigen doses are required to induce seroconversion in immunized subjects [4, 5]. An alternative strategy has been developed to avoid the delay and potential antigenic mismatch mentioned above. This involves use of wild-type virus to produce vaccine antigen in Vero cell culture, one of the most advanced cell culture systems for production of influenza viruses [6]. Candidate vaccines based on clade 1 (A/Vietnam/1203/2004 (H5N1) and clade 2 (A/Indonesia/05/2005 (H5N1)) strains were developed and demonstrated to be highly immunogenic in animal models [7]. The vaccines induce cross-neutralising antibodies and protect CD1 mice in a nasal challenge model not only against the homologous virus but against other H5N1 strains including those from another clade. These data indicate that this strategy allows the rapid high yield production of a pandemic vaccine and that these inactivated whole virus vaccines, based on the wild-type virus, have the potential to induce broadly protective humoral immune responses. However it is also known that influenza specific T cell responses play a role in diminishing the lethal effects of highly pathogenic strains of Influenza and in speeding up recovery from influenza infection [8]. In the studies presented here, we further characterise the two candidate vaccines using the BALB/c inbred mouse strain model which is more suitable for T cell studies. In these initial studies we were particularly interested in looking at the extent and type of homologous T helper cell responses and potential

heterologous cross-stimulation responses the inactivated whole virus vaccines could induce. We were also interested in seeing whether these responses were associated with cross protection among various strains from different clades in BALB/c mice.

Materials and Methods

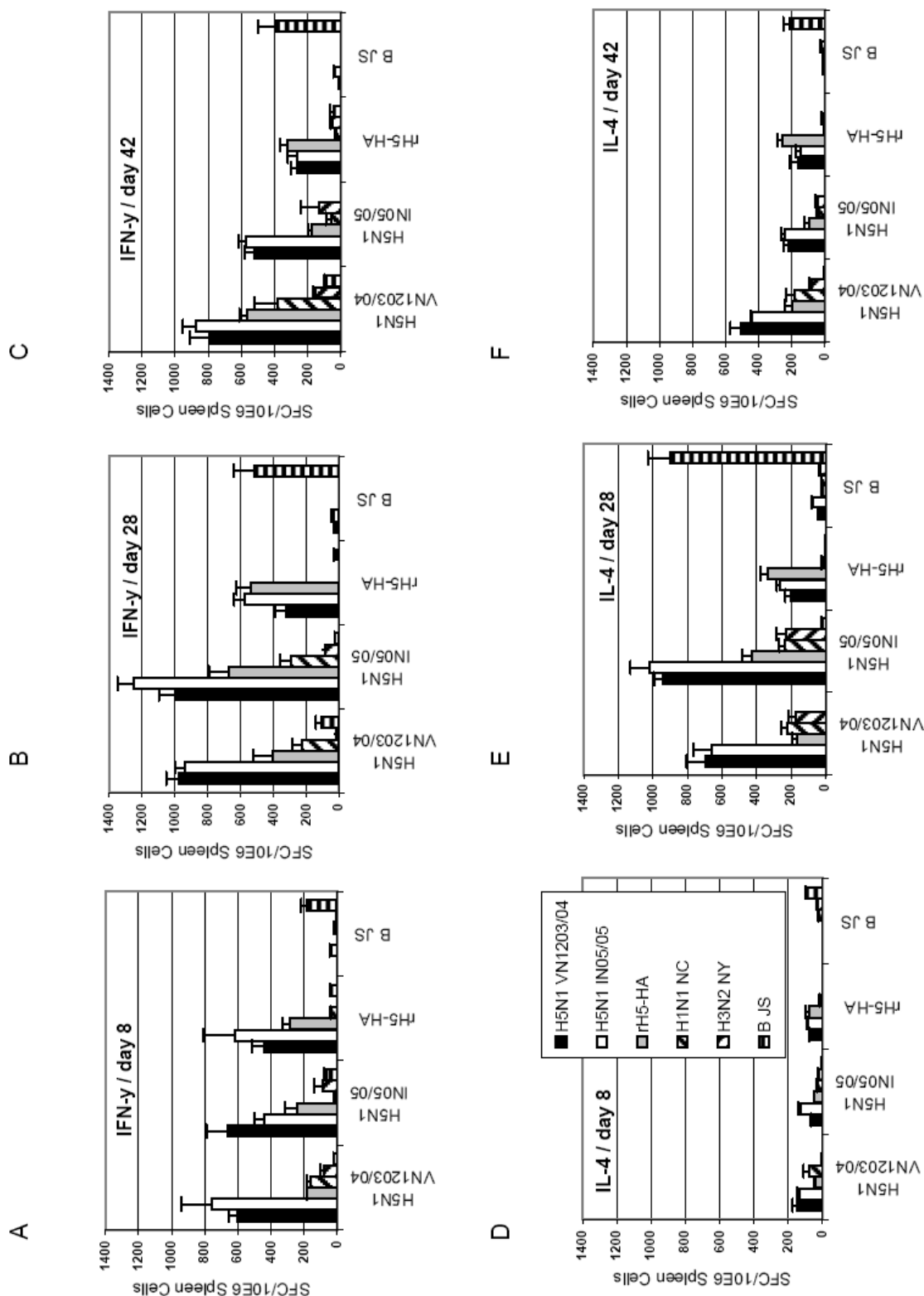
Immunization and challenge. Groups of BALB/c mice were immunized with 1,5µg HA/dose of non-adjuvanted and alum adjuvanted (0.2%) H5N1 A/Vietnam/1203/2004 and A/Indonesia/05/2005 whole viral vaccines, a B/Jiangsu/10/2003 strain whole viral vaccine, or rH5-HA antigen using a prime (day 0) / booster (day 21) regime. The whole virus vaccines were prepared as previously described [7]. Mice from all group were sacrificed on days 8, 28 and 42 and splenocyte preparations were pooled and stimulated *in vitro* with various non-adjuvanted whole viral or recombinant antigen preparations (see below). Additional groups of 30 mice, similarly immunized with either the two H5N1 whole viral vaccines without and with alum, were challenged on day 42 with either the live H5N1 clade 1 A/Vietnam/1203/2004 strain (10 mice) or the live H5N1 clade 2 A/Indonesia/05/2005 strain (10 mice) or the live clade 3 A/Hong Kong /156/1997 strain using a challenge dose of 1×10^5 PFU. and monitored for survival after 14 days. Viruses were provided by the Center for Disease Control and Prevention (CDC, Atlanta, USA) or the National Institute for Biological Standards and Control (NIBSC, UK).

ELISPOT Assay. The frequency of interferon-gamma (IFN- γ) or interleukin-4 (IL-4) secreting cells was analysed using mouse IFN- γ and IL-4 ELISPOT kits (Mabtech AB, Nacka, Sweden). ELISPOT plates (Milipore Corp. Bedford, MA) were coated with purified anti-IFN- γ or anti-IL-4 monoclonal antibodies. After overnight incubation at 4°C the plates were washed with PBS and blocked by addition of complete culture medium for 2h at 37°C. Serial dilutions of spleen cells were added to the wells ranging from 6×10^4 to 2×10^5 cells per well. The following whole viral (A/Vietnam/1203/2004 (H5N1); A/Indonesia/05/2005 (H5N1); A/New Caledonia/20/99 (H1N1); A/New York/55/04 (H3N2) and B/Jiangsu/10/2003) and recombinant baculovirus-derived rH5-HA (Protein Sciences: A/Vietnam/1203/2004 (H5N1) preparations were added at a concentration of 0,1 µg/ml. The plates were then incubated overnight at 37°C and 5% CO₂. The next day, the plates were washed with PBS and bound IFN- γ or IL-4 was detected by conjugated cytokine-specific antibodies. Spots were counted using an automated ELISPOT reader (AID, Strassberg, Germany). The number of spots observed in wells containing no antigen was subtracted from the number of spots observed in wells containing specific antigen and the results were expressed as spot forming cells (SFC) per 10^6 spleen cells.

Results

T cell ELISPOT Analysis. The *ex vivo* T cell responses of splenocytes of mice immunized with the non-adjuvanted whole viral vaccines and rHA-H5 antigens and sacrificed 8, 28 and 42 days after initial immunization are shown in Figure 1.

Figure 1. Results of the ELISPOT analysis of number of IFN- γ (A,B,C) And IL-4 (D,E,F) secreting T cells for the splenocytes of BALB/C mice isolated days 8, 28 and 42 on days after the mice were immunized with non-adjuvanted vaccines and Rha-H5 antigen at day 0 And 21. The splenocytes were stimulated for 16 hrs with the following inactivated whole virus vaccines H5N1 VN1203/04, H5N1 IN5/05, H1N1 NC, H3N2 NY, and B JS or with Rha-H5 antigen.



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Large numbers of INF- γ secreting T cells (a marker, although not exclusively, for T helper type 1 cells) were observed after antigen stimulation on day 8 and 28, respectively (see Fig 1A and B). In contrast, the number of IL-4 secreting T cells (a marker for T helper type 2 cells) were initially weak on day 8, but increased markedly after the second immunization on day 28. (see Fig. 1D and E). The numbers of both INF- γ and IL-4 secreting T cell diminished slightly on day 42 compared to that seen on day 28. In general it can be stated that a mixed INF- γ and IL-4 secreting T cell response was observed after the booster injection, i.e. on days 28 and 42. (see Fig. 1 B-F). A similar picture was observed for the T cell response for mice immunized with the alum-adjuvanted whole virus vaccines and rHA-H5 antigen (data not shown). The number of INF- γ secreting cells was higher for the mice immunized with non-adjuvanted preparations, while a greater number of IL-4 secreting cells were observed for the alum-adjuvanted vaccine and recombinant antigen immunized mice (data not shown). The mice immunized with the two H5N1 whole viral vaccines not only showed good stimulation responses with the H5N1 whole viral and rH5-HA antigen preparations but also with the H1N1 and H3N2 whole viral preparations. Whereas, no cross stimulation was observed after stimulation with the H1N1 and H3N2 whole viral preparations for the rH5-HA immunized mice. The mice immunized with the whole virus B JS preparation gave a highly B strain specific stimulation responses.

Mouse Challenge Studies. The results of the mouse challenge studies using H5N1 virus strains of different clades are shown in Table 1. Immunization with the H5N1 Vietnam 1203 (Clade 1) or Indonesia/05/05 (Clade 2) derived vaccines protected BALB/c mice to a similar degree against challenge with H5N1 Vietnam 1203 Clade 1 strain. The non-adjuvanted H5N1 Vietnam 1203 homologous vaccine gave complete protection while one mouse died in the group immunized with adjuvanted counterpart. In contrast the adjuvanted Indonesia strain vaccine gave full protection while the non-adjuvanted afforded 80% protection.

Table 1. Protection studies using different clades of H5N1 challenge viruses.

Vaccine	Alum	No. of mice surviving / No. of mice challenged		
		Challenge strain		
		A/ Vietnam 1203/04 (clade 1)	A/ Indonesia 05/05 (clade 2)	A/ Hong Kong/156/97 (clade 3)
A/ Vietnam 1203/04 (clade 1)	-	10 / 10	8 / 10	9 / 10
	+	9 / 10	8 / 10	9 / 10
A/ Indonesia 05/05 (clade 2)	-	8 / 10	10 / 10	8 / 10
	+	10 / 10	10 / 10	10 / 10
Buffer Control	-	0 / 10	2 / 10	0 / 10
	+	0 / 10	0 / 10	0 / 10

Mice challenged with the H5N1 Indonesia/05/05 clade 2 strain were fully protected against death when immunized with the homologous vaccine and were protected to 80% when immunized with the heterologous H5N1 Vietnam /1203 vaccine. In general, there was no difference in the actual protection level observed for the non-adjuvanted or alum adjuvanted vaccines. Mice immunized with either of the inactivated whole vaccines (Vietnam 1203 or Indonesia/05/05) were also protected against

lethal challenge with H5N1 Hong Kong/156/1997 (Clade 3 strain).

Discussion

The studies presented here show that Vero cell derived H5N1 inactivated whole virus vaccines are capable of inducing large numbers of both INF- γ and IL-4 secreting T cells, which can be cross stimulated by whole viral preparations of type A Influenza strains of the HA-H5 subtype of different clades, and by strains of different HA subtypes e.g. H1 and H3. The fact that no cross stimulation with the H1N1 and H3N2 whole viral preparations was observed for the rH5-HA immunized mice suggests that common antigens to influenza type A strains, such as the nucleoprotein or matrix protein, contribute to the T cell cross stimulation response induced by the inactivated whole viral H5N1 vaccines. The challenge experiments demonstrated that immunization with either of the two Vero cell derived inactivated H5N1 whole virus vaccines (Vietnam VN1203 or Indonesia IN5/05) also gave good homologous and cross clade protection against death from H5N1 strains of clades 1 (A/Vietnam/1203/2004), clade 2 (A/Indonesia/05/2005) and clade 3 (A/Kong/156/1997) in Balb/c mice. The contribution of the T cell responses to the overall homologous and cross clade protection induced by the inactivated whole virus vaccines is currently being evaluated.

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A Cross Border Initiative for Addressing Surveillance and Laboratory Issues Related to Avian and Pandemic Influenza in North America

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A Laboratory and Surveillance Technical Working Group was formed in 2006 between public health departments in Canada, Mexico and the United States to provide technical support for avian and pandemic influenza preparedness under the trilateral Security and Prosperity Partnership (SPP) initiative. The working group membership includes representatives from: the Ministry of Health of Mexico, the Undersecretariat of Health Promotion and Prevention, General Directorate of Epidemiology, Institute of Epidemiological Diagnosis and Reference, General Direction of Health Promotion; the United States Department of Human Health Services; the Centers for Disease Control and Prevention; and the Public Health Agency of Canada. The group aims to address laboratory, surveillance and epidemiological issues related to the development of a coordinated and evidence-based approach to plan and respond to an influenza pandemic and human health issues associated with the introduction of H5N1 avian influenza virus into North America.

Methods

Within the framework of the SPP initiative, two face-to-face meetings of key stakeholders were held in order to address issues related to avian and pandemic influenza. Conference calls, web-based communications and the remote exchange of information were used to develop and complete the following working group activities: outline operational procedures and tools for the communication of laboratory, surveillance and epidemiological information across borders to allow for consistent interpretation of data and translation into key, common messages; share H5N1 virus case definitions and surveillance approaches; exchange information and collaborate on joint preparedness and response activities for slowing the introduction and spread of H5N1 virus into the three countries; share laboratory protocols and information reporting procedures; identify areas of technical and mutual assistance, including a review of Federal-Province/Federal-State and State-Local interactions; review the real time application of epidemiologic and laboratory information to inform and evaluate vaccine and antiviral use; and develop a trilateral document to serve as guidance during a regional response in North America.

Results

Following the establishment of the working group's membership, a terms of reference and workplan were developed. Communication through identified points of contact has been established and key issues are being addressed, such as the initial development of communication procedures for sharing laboratory, surveillance and epidemiological information, including identification of what information will be shared, when, how, and with whom. The group has provided technical input into the development of the North American Plan for Avian and Pandemic Influenza and collaborated on joint preparedness and response activities to promote the exchange of information through cross border projects, including the Early Warning Infectious Disease Surveillance project (EWIDS). In addition, the group has shared current case definitions and surveillance approaches for human H5N1 cases, and identified challenges for sharing laboratory specimens and materials across borders including transportation procedures. Finally, a secure on-line discussion forum for sharing information with working group members through the Canadian Network for Public Health Intelligence (CNPHI) has been developed. The working group will begin addressing other activities including: sharing surveillance strategies for novel influenza viruses; establishing approaches for collaborating on North American outbreak investigations and response efforts; establishing a definition for a binational H5N1 case; reviewing Federal-Province/Federal-State and State-Local interactions; identifying privacy issues as potential barriers to sharing information across jurisdictions; and, strengthening operating procedures/processes for the sharing of laboratory information before and during an emergency.

Challenges

Language barriers and scheduling teleconferences for a multi-disciplinary group across the three countries has been one of the important challenges realised by the working group. Also identified were: limited resources for implementing activities, particularly resources for travel, translation and human resources for administrative functions; country-specific competing work priorities; and, establishing linkages with various provincial/territorial/state/regional cross-border surveillance initiatives.

Conclusions

The work of this multi-national group will strengthen the capacity in North America to detect influenza strains of pandemic potential. It will help to address the very high demand for real time epidemiologic and laboratory information needed to respond to an influenza pandemic. This approach for pandemic influenza preparedness in the North American region serves as a model addressing cross-border mutual assistance arrangements, protocols, and/or agreements for responding to public health emergencies of international concern.

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Immune Responses to Live Attenuated H5N1 Influenza Vaccines in Ferrets

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Live attenuated influenza H5N1 vaccines have been produced by reverse genetics using the six internal genes from the cold adapted A/Ann Arbor/6/60 strain and the NA and cleavage site-modified HA of the H5N1 strains isolated from humans in 1997, 2003 and 2004. Three candidate H5 vaccine strains all have the characteristic cold adapted (*ca*), temperature sensitive (*ts*) and attenuation (*att*) phenotypes that are conferred by the internal genes of *ca* A/Ann Arbor/6/60 donor virus. Immune responses elicited by each of these three H5N1 candidate vaccines were evaluated in ferrets by serologic assays including HAI, ELISA and Western blotting. In addition, virus antigen-specific B cell recall responses were examined directly *ex vivo* using an antibody secreting cell (ASC) ELISpot assay. Our data demonstrate that live attenuated H5N1 candidate vaccines elicit protective immune responses in the ferret model and these vaccines could potentially be used for pandemic preparedness against H5N1 infection.

Introduction

A number of avian influenza virus subtypes, including H5, H9, and H7 have caused infection in humans. Among those, avian H5N1 infection caused over 160 deaths during the past five years. Live, attenuated viral vaccines are being developed with the goal of preventing severe disease and death caused by pandemic influenza. Live attenuated influenza vaccines are attractive for the prevention of the pandemic influenza in humans because of the following properties: 1) stimulation of durable mucosal and systemic immunity in influenza A virus-naïve individuals following a single dose of vaccine, 2) induction of cross-reactive immune responses, 3) efficient production, and 4) safety for fully susceptible seronegative individuals due to poor transmissibility and genetic and phenotypic stability. To produce live attenuated H5N1 pandemic influenza vaccine candidates, the multibasic amino acids at the proteolytic cleavage site between HA1 and HA2 were removed from H5N1 viruses isolated from humans in 1997, 2003 and 2004. Modified HA and NA gene segments from these H5 viruses were combined with the six gene segments encoding the internal proteins of the cold adapted A/Ann Arbor/6/60, the master donor virus for FluMist[®] vaccines, using reverse genetics (Jin et al., 2004). The resultant candidate H5 vaccine strains all have the characteristic cold adapted (*ca*), temperature sensitive (*ts*) and attenuation (*att*) phenotypes. When evaluated in mice and ferrets for vaccine efficacy, these H5N1 candidate vaccines provided complete protection against lethal challenge of

homologous and heterologous wild-type (wt) H5N1 viruses (Suguitan et al., 2006). Among the three candidate H5N1 vaccines, *ca* A/VN/1203/04 induced the lowest serum HAI antibodies after a single dose of vaccine in mice (Suguitan et al., 2006). In this report, the immune responses elicited by these candidate H5N1 vaccines in ferrets were evaluated by serologic assays and by an antibody secreting cell (ASC) ELISpot assay to examine virus-specific B cell responses.

Materials and Methods

Ferrets and virus infection. Groups of three sero-negative ferrets (7-9 weeks of age) from Triple F farms (Sayre, PA) were each intranasally inoculated with 10⁷ PFU of H5N1 vaccine virus, 2005-06 FluMist[®] vaccine, or vehicle on Day 0. In some experiments, a second equivalent dose of homologous or heterologous vaccine virus was administered intranasally on Day 28. For serologic studies, serum samples were collected three weeks after the first dose, and two weeks after the second dose. For ELISpot studies, ferrets were sacrificed 7-10 days after the first dose and 3-5 days after a second dose. All procedures were performed in accordance with IACUC approved protocols.

2.2. HAI and Western blotting assays. Serum HAI antibodies were tested by a standard method using 4 HA units of virus in V-bottom 96-well microtiter plates with 0.5% turkey erythrocytes (tRBC). For Western blotting, MDCK cells were infected with H1N1 (*ca* A/New Caledonia/99), H3N2 (*ca* A/Wyoming/03), H5N1 (*ca* A/HK/491/1997, *ca* A/HK/213/2003, or *ca* A/VN/1203/2004) at an moi of 5 and incubated at 33°C for 5-6 h. The infected cell lysates were electrophoresed on polyacrylamide gels containing 10% SDS and then transferred to PVDF membranes. After blocking, membranes were incubated with 1:50 diluted ferret serum followed by incubation with HRP-conjugated anti-ferret antibodies. Antibody-antigen complexes were captured on X-ray film after treating the membrane with a chemiluminescent substrate.

B cell ELISpot assay. Ninety-six-well PVDF plates (PALL, Ann Arbor, MI) were coated overnight at 4°C with 50 µL/well PBS containing BPL-inactivated *ca* A/Ann Arbor/6/60, *ca* H5N1 A/VN/1203/2004 or *ca* A/HK/213/2003 each diluted to 2,000 HA unit/mL or 50 µL/well PBS containing recombinant HA derived from A/VN/1203/2004 (Protein Sciences, Meriden, CT) at 10 µg/mL. Plates were washed with PBS and blocked with RPMI-1640 + 10% FBS for 2 h at 37°C prior to the addition of cells. Peripheral blood and peritracheal lymph nodes of ferrets were collected immediately following euthanasia and each was processed to prepare single-cell suspensions of PBMC or lymphocytes, respectively, in media (RPMI-1640 with 10% FBS, L-glutamine, and penicillin/streptomycin). Cell suspensions were added to triplicate wells (100µL/well) at a concentration of 3 x 10⁶/mL for PBMC or 10⁶/mL for lymph node cells and incubated at 37°C and 5% CO₂ for 5 h. After washing with PBS-T (PBS + 0.05% Tween-20), goat anti-ferret IgG (Bethyl Laboratories, Montgomery, TX) diluted in PBS-T/1%BSA was added to the plates, which were then stored overnight at 4°C. After washing with PBS-T, HRP-labeled rabbit anti-goat Ig (Dako, Carpinteria, CA) diluted in

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Table 1. Serum HAI antibodies elicited in ferrets following a single dose of H5N1 *ca* vaccine.

Virus administered ^a	GMT of HAI antibodies ^b to indicated antigen ^b		
	<i>ca</i> VN04	<i>ca</i> HK03	<i>ca</i> HK97
<i>ca</i> VN04	<8	12	<8
<i>ca</i> HK03	<8	203	16
<i>ca</i> HK97	<8	10	51

^aThe indicated viruses were administered intranasally to 3 ferrets at a dose of 10⁷ pfu and sera were collected 28 days later.

^bSerum antibody titers were measured by hemagglutination inhibition assay (HAI) using turkey red blood cells and the titers are expressed as geometric mean titers (GMT).

PBS-T/BSA (100 µL/well) was added to the plates, which were then incubated at 37°C for 1 h. Plates were then washed again with PBS-T and then developed with AEC substrate (Vector Labs, Burlingame, CA) for 10 min. Spots in each well were counted with an ImmunoSpot plate reader (Cellular Technologies, Ltd., Cleveland, OH).

Results

Immunogenicity of H5N1 vaccines. Immunogenicity of the three H5N1 candidate vaccines was evaluated by HAI assay using post-infection ferret sera collected three weeks after intranasal vaccination. As shown in Table 1, HAI antibodies were not detected in ferrets immunized with a single dose of *ca* A/VN/1203/2004, while *ca* A/HK/213/2003 induced highest serum antibody titer (GMT of 203) and *ca* A/HK/491/1997 induced a moderate level of HAI antibodies against homologous viruses. However, neither *ca* A/HK/213/2003 nor *ca* A/HK/491/1997 induced HAI antibodies that cross-reacted well with each other. To determine whether *ca* A/VN/1203/2004 induced an antibody response in ferrets, Western blotting was performed against virus proteins using post-infection ferret sera (Figure 1). Under these conditions, sera from *ca* A/VN/1203/2004 immunized ferrets were able to recognize H5-specific HA in a manner comparable to sera from *ca* A/HK/491/1997 and *ca* A/HK/213/2003 infected ferrets. In addition, anti-NP antibodies were elicited by each of the three H5N1 vaccines in ferrets and anti-M1 antibodies were also detected following immunization that also reacted to the NP and M1 proteins produced in H1N1 (Figure 1, lane 1) or H3N2 (Figure 1, lane 2) infected cells. The level of anti-M1 antibody in *ca* A/VN/1203/2004 immunized ferret was probably too low to detect after a single dose, but was detected after a second dose. These data indicate that *ca* A/VN/1203/2004 does in fact elicit H5-specific immune responses after a single dose, even though antibody responses were not detected by the conventional HAI assay.

An HA receptor variant of A/VN/213/2004 increased the sensitivity of the HAI assay. HAI antibodies induced by H5N1 vaccines were evaluated following two doses of vaccine. Groups of three ferrets received *ca* A/VN/1203/2004 or *ca* A/HK/213/2003 intranasally, and 4 weeks later, a second intranasal vaccination of either a

Table 2. HAI titers of post-infection ferret sera tested against homologous and heterologous H5N1 vaccine viruses.

Virus(es) administered ^a		GMT of HAI antibodies to indicated antigen ^b		
Dose 1	Dose 2	<i>ca</i> HK03	<i>ca</i> VN04	<i>ca</i> VN04 variant
<i>ca</i> VN04	<i>ca</i> VN04	181	<u>64</u>	512
<i>ca</i> HK03	<i>ca</i> HK03	<u>256</u>	8	128
<i>ca</i> HK03	<i>ca</i> VN04	512	64	512
<i>ca</i> VN04	<i>ca</i> HK03	256	32	256

^a Three ferrets per group received the indicated viruses intranasally; dose 2 was administered 28 days after dose 1 and sera were collected 28 days later.

^b Serum antibody titers were measured by hemagglutination inhibition assay (HAI) using turkey red blood cells and geometric mean titers (GMT) from three ferrets are presented. Homologous titers are underlined.

homologous or heterologous vaccine was administered. Serum samples were obtained two weeks after the second dose and assayed by HAI against *ca* A/HK/213/2003 and *ca* A/VN/1203/2004. In addition, an HA variant that was isolated from MDCK cell culture through several passages and was identified to preferentially bind to α-2'6 linked sialic acid was also used in the HAI assay. As shown in Table 2, after two doses of vaccination with *ca* A/VN/1203/2004, the geometric mean HAI antibody titer against the homologous antigen was 64, however, the HAI titer against heterologous *ca* A/HK/213/2003 was higher than the homologous titer. The HAI titer was 8-fold higher against the receptor variant of *ca* A/VN/1203/2004. Antibodies elicited by two doses of *ca* A/HK/213/2003 also reacted to the A/VN/1203/2004 HA variant better than with A/VN/1203/2004. Prime/boost with two different H5N1 vaccine strains generated HAI antibody titers that cross-reacted slightly better than two doses of homologous vaccine.

H5 antibody secreting cells are boosted by prior vaccination with FluMist vaccine. Influenza H5-specific IgG secreting B cells were examined by ELISpot assay using PBMC samples and lymphocytes isolated from peritracheal lymph nodes of ferrets vaccinated with a single dose of *ca* A/VN/1203/04 or *ca* A/HK/213/2003 using purified H5 HA. The number of H5-specific ASC measured was much lower than H3 or H1-specific ASC induced by *ca* H3N2 or *ca* H1N1 vaccination, respectively (data not shown). However, the number of H5-specific antibody secreting B cells greatly increased following a second homologous dose of *ca* H5N1 vaccine, from approximately 5 ASC/10⁶ cells to about 200 ASC/10⁶ cells for *ca* A/VN/1203/2004 infected ferrets. Most people have pre-existing immunity against human influenza strains either through natural infection or vaccination. To mimic this situation, ferrets were vaccinated with commercial FluMist[®] vaccines that contained a trivalent blend of H1N1, H3N2 and B strains. After one month, ferrets were vaccinated with either H5N1 *ca* A/VN/1203/2004 or *ca* A/HK/213/2003 virus. In contrast to the control animals that did

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not receive influenza vaccine and showed few if any H5-specific ASC 3-5 days after vaccination, ferrets that received FluMist® vaccine had significant numbers of H5-specific ASC in both PBMC and peritracheal LN samples. Average H5-specific ASC numbers from these ferrets were even comparable to those measured in samples from ferrets given two doses of H5N1 *ca* vaccine. These data suggest that vaccination with seasonal influenza vaccine might enhance protection against H5N1 infection.

Discussion

In this study, we clearly demonstrated that *ca* H5N1 vaccine induced virus specific immune responses in ferrets, the protective immunity that conferred complete protection against wt H5N1 challenge (Suguitan et al., 2006). The level of HAI antibodies after a single dose vaccination is low but greatly enhanced by a second dose vaccination. In addition, we show that the use of a *ca* A/VN/1203/2004 HA variant, which binds preferentially to α -2'6 linked sialic acid instead of α -2'3 linked sialic acid, significantly increases HAI titers in the HAI assay. We also report that previous vaccination with seasonal influenza vaccines could potentially increase H5-specific immune response as determined by the B cell ELISpot analysis. This response is likely caused by heterosubtypic immunity conferred by an influenza virus from a different subgroup. More studies are needed to confirm whether heterosubtypic immunity could offer any protection against H5N1 infection.

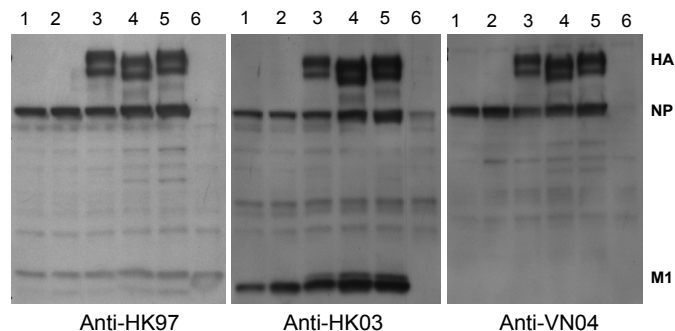
Acknowledgements

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Figure 1. MDCK cells in 6-well plates were infected With H1N1 A/New Caledonia/20/1999 (Lane 1), H3N2 A/Wyoming/03/2003 (Lane 2), H5N1 *ca* A/HK/491/1997 (Lane 3), *ca* A/HK/213/2003 (Lane 4), *ca* A/VN/1203/2004 (Lane 5) or Uninfected (Lane 6). After 6 h incubation at 37°C, cell lysates (5%) were prepared and electrophoresed on SDS-PAGE and processed for western blotting using postinfection ferret sera from ferrets immunized with the indicated *ca* H5N1 vaccines.



MDCK Cells as a Tool for Screening Temperature Sensitive Live Attenuated Influenza Vaccine Candidate Reassortants

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Live cold-adapted trivalent Russian influenza vaccine is regularly prepared in embryonated hens' eggs. The determination of temperature sensitive (*ts*) and cold-adapted (*ca*) phenotype of reassortants for a live influenza vaccine has usually been conducted using eggs. As attempts are made to move away from eggs, it is important to develop an equivalent cell based assay. It has been previously shown that the Russian *ca*, *ts* and attenuated master donor virus (MDV) A/Leningrad/134/17/57 (H2N2) showed a significantly greater temperature sensitive phenotype in MDCK cells than in eggs. The cut off temperature for type A influenza viruses in MDCK cells was determined to be 37 degrees C since at this temperature there is a clear and definite difference in growth between MDV and currently circulating non-*ts* influenza viruses. In this study we selected current influenza A and B viruses that appear to be naturally temperature sensitive in eggs and have evaluated the use of MDCK cells as a tool for determining their *ts* phenotype. Also, we compared this assay with the same assay performed in eggs. Methods: Naturally occurring temperature sensitive current influenza A(H1N1), A(H3N2) and B viruses were grown both in eggs and MDCK cells at the following temperatures: 32, 37, 38, 39 and 40 degrees C and were compared with A and B MDVs. Viral titers were determined by either EID₅₀ or TCID₅₀. Results: It was shown that current influenza A and B viruses appear to be naturally temperature sensitive to reproduction in embryonated eggs and demonstrated strong non-*ts* phenotype in MDCK cells at 37 and 38 degrees C respectively. Conclusion: These findings can be used to support live influenza vaccine production. It is important for evaluation of the *ts* phenotype of currently circulating influenza viruses as potential candidates for a reassortant influenza vaccine. Determination of *ts* phenotype in MDCK cells provides a new universal attenuation marker for primary screening of *ts* candidate reassortants for live influenza vaccine prior to their evaluation for genome composition.

Introduction

Live attenuated (*att*) influenza vaccine (LAIV) is currently prepared in embryonated hens' eggs by the method of genetic reassortment between currently circulating wild type virus and cold-adapted (*ca*), temperature sensitive (*ts*) AND attenuated master donor virus (MDV). After co-infection 6:2 reassortants which inherited 2 genes encoding the surface antigens from wild type virus and 6 internal genes from the MDV are selected. For preparing Russian *ca/ts* LAIV, A/Leningrad/134/17/57 (H2N2) (influenza A vaccine) and B/USSR/60/69 (influenza B vaccine)

are used as MDVs. The A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 master donor viruses were obtained from wild-type parental viruses A/Leningrad/134/57 (H2N2) and B/USSR/69 respectively after sequential passages in embryonated hens' eggs at low temperature (25°C). In the process of their passaging these strains acquired *ts* and *ca* phenotypes as determined in eggs and became attenuated for humans. All 6:2 vaccine strains inherit these properties from the MDVs. *ts* and *ca* phenotypes of reassortants have been widely used as attenuation markers for primary screening, but these markers can be used only when the epidemic parent strain have a non-*ts* phenotypes. Unfortunately, some currently circulating wild-type influenza A and B strains have a *ts* phenotype in eggs (Kiseleva e.a., 2002; Larionova e.a., 2004). The development of cell culture LAIV is a public health priority. When the vaccine strains are produced in a new substrate, attenuation markers for screening of 6:2 reassortants in cell culture will be required. Previously we reported that A/Leningrad/134/17/57 (H2N2) MDV showed a significantly greater *ts* phenotype in MDCK cells than in eggs (Kiseleva, 2004). Also MDCK cells were shown to confirm the non-*ts* phenotype of epidemic influenza viruses, naturally non-*ts* in eggs. The cut off temperature for type A and B influenza viruses in MDCK cells was determined to be 37°C and 38°C respectively since at these temperatures clear and definite difference in replication between MDVs and currently circulating non-*ts* influenza viruses was shown. But it still remained uncertain whether the *ts* marker would work in MDCK cells with wild-type parental viruses which showed strong temperature sensitive phenotype in hens' eggs. In this study we selected current influenza A and B viruses that appear to be naturally temperature sensitive in eggs and have evaluated the use of MDCK cells as a tool for determining their *ts* phenotype. Also, we compared this assay with the same assay performed in eggs.

Viruses. The following wild-type A and B influenza viruses showing *ts* phenotype in eggs were used: A/Saint Petersburg/11/01 (H1N1), A/Astrakhan/68/01 (H1N1), A/Malaysia/01/04 (H3N2), A/Voronezh/511/00 (H3N2), B/Beijing/184/93, B/Pennsylvania/02/96, B/Washington/01/96, B/Wisconsin/03/96, B/Iowa/03/96, B/Jilin/01/03, B/Arkhangelsk/312/99, B/Hawaii/13/04. The epidemic viruses with *ts* phenotype were compared with those having non-*ts* phenotype in eggs: A/PR8/34 (H1N1), A/New Caledonia/20/99 (H1N1), A/Sydney/5/97 (H3N2), A/Leningrad/134/57 (H2N2), A/California/7/04 (H3N2), B/Lee/40, B/Russia/69, B/Shandong/07/97, B/Hong Kong/330/01, B/Johannesburg/05/99. Cold-adapted A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 MDVs and egg-derived 6:2 reassortant vaccine strains based on them were used as well.

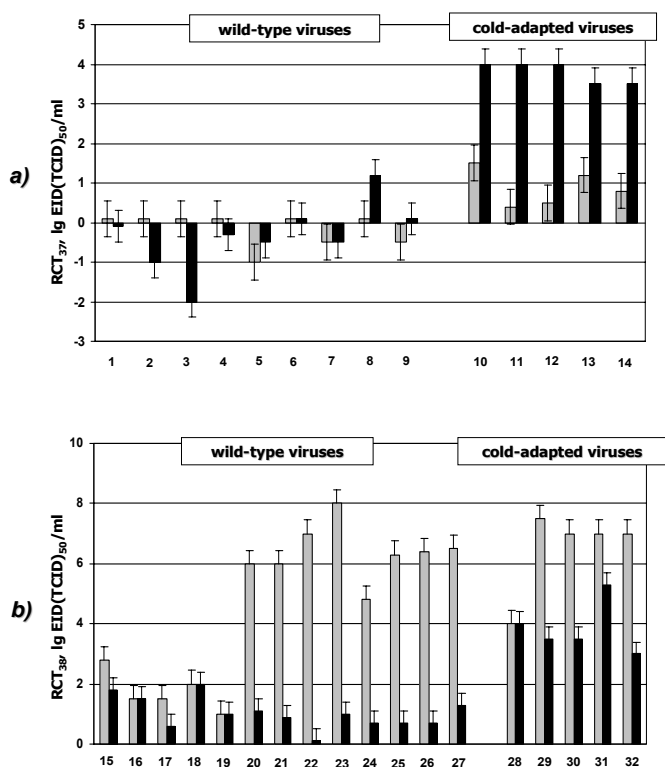
Methods

Influenza A and B viruses were grown both in eggs and MDCK cells at the following temperatures: 26, 32, 37, 38, 39 and 40°C. Viral titers were determined by either titration in eggs (EID₅₀) or in MDCK cells (TCID₅₀). RCT characteristics (reproductive capacity at different temperatures) were used to determine virus *ts* and

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ca phenotypes. EID_{50} and $TCID_{50}$ calculation was based on Reed and Muench (1938). $RCT_{40} (39, 38, 37 \text{ or } 26) = EID_{50}/TCID_{50}$ at $32^{\circ}\text{C} - EID_{50}/TCID_{50}$ at 40°C (39, 38, 37 or 26°C).

Figure 1. Comparative study of the infection titers of wild-type and cold-adapted influenza A and B viruses in chicken eggs and MDCK cells at optimal and high temperatures.



1 – A/PR/8/34 (H1N1); 2 – A/New Caledonia/20/99 (H1N1); 3 – A/Leningrad/134/57 (H2N2); 4 – A/Sydney/5/97 (H3N2); 5 – A/California/7/04 (H3N2); 6 – A/St.Petersburg/11/01 (H1N1); 7 – A/Astrakhan/68/01 (H1N1); 8 – A/Malaysia/01/04 (H3N2); 9 – A/Voronezh/511/00 (H3N2); 10 – A/Leningrad/134/17/57 (H2N2); 11 – A/Leningrad/134/47/57 (H2N2); 12 – A/17/New Caledonia/99/145 (H1N1); 13 – A/17/Malaysia/04/11 (H3N2); 14 – A/17/California/04/6 (H3N2).
15 – B/Lee/40; 16 – B/Russia/69; 17 – B/Shandong/07/97; 18 – B/Hong Kong/330/01; 19 – B/Johannesburg/05/99; 20 – B/Beijing/184/93; 21 – B/Jilin/01/03; 22 – B/Arkhangelsk/312/99; 23 – B/Hawaii/13/04; 24 – B/Pennsylvania/02/96; 25 – B/Washington/01/96; 26 – B/Iowa/03/96; 27 – B/Wisconsin/03/96; 28 – B/USSR/60/69; 29 – B/60/Panama/90/4/E; 30 – B/60/Petersburg/95/20; 31 – B/60/Hong Kong/01/22; 32 – B/60/Jilin/03/1.

Results and Discussion

Phenotype of influenza A and B viruses in eggs and MDCK cells. Comparative studies of virus replication in eggs and MDCK cells revealed that influenza A viruses with a *ts* phenotype in eggs displayed a different level of temperature sensitivity in MDCK cells. The A/Leningrad/134/17/57 (H2N2) MDV appeared to be significantly more temperature sensitive in MDCK cells than in

eggs, whereas all wild-type influenza A viruses showed stronger *non-ts* phenotype in MDCK cells. The reproductive capacity of epidemic viruses in eggs at 38-39°C was much lower than in MDCK cells. Viruses with *non-ts* phenotype in eggs were able to replicate well in MDCK cells at high temperature. The same results were observed for wild-type influenza B viruses. Viruses which were able to replicate well in chicken eggs at high temperature (38°C) displayed a strong *non-ts* phenotype in MDCK cells. Epidemic B strains that showed a *ts* phenotype in eggs also had the ability to replicate in MDCK cells at 38°C. Thus, all studied wild-type viruses showed the ability to replicate well in MDCK cells at elevated temperatures. Interestingly, B/USSR/60/69 MDV had similar *ts* profiles in both substrates studied.

Ts attenuation marker for screening cold-adapted LAIV reassortants. Differences in the ability of both substrates to determine *ts* phenotype of wild-type and cold-adapted viruses indicated that MDCK cells are a more sensitive system to determine temperature sensitivity, and indirectly attenuation, compared to eggs. MDCK cells were shown to be able to differentiate cold-adapted influenza viruses from any epidemic strains, both of which were undistinguishable when using eggs. The reduced ability of influenza A vaccine viruses to replicate in MDCK cells at temperatures above 37°C can be successfully used as a “cell culture” *ts* marker. Figure 1-a clearly demonstrate that in MDCK cells the RCT_{37} value for each epidemic A strain was significantly lower than that of cold-adapted viruses, while in eggs wild-type and attenuated viruses behaved similarly at 37°C. An analogous *ts* marker was also found for type B influenza viruses (Fig.1-b). All cold-adapted viruses showed restricted reproductive activity in MDCK cells at 38°C while all wild-type viruses replicated to high titers in cells at this temperature. As for influenza A viruses, we failed to distinguish cold-adapted and epidemic viruses using chicken eggs at this temperature.

Conclusion

Wild-type and cold-adapted influenza A and B viruses were shown to display different degree of temperature-sensitivity in two substrates studied. All epidemic strains had strong *non-ts* phenotype in MDCK cells regardless their phenotype in hens' eggs while cold-adapted viruses were more temperature-sensitive in MDCK cells than in eggs. Determination of the *ts* phenotype in MDCK cells provides a universal attenuation marker for primary screening of *ca/ts* live attenuated influenza vaccine candidate reassortants prior to their evaluation for genome composition.

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Development of Candidate H7N3 Live Attenuated Cold-Adapted Influenza Vaccine

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To prepare candidate live influenza vaccines (LAIV) for a potential future pandemic we used classical genetic reassortment with non-pathogenic avian viruses and the cold-adapted (*ca*) H2N2 master donor strain (MDS) A/Leningrad/134/17/57. In the present study we evaluated a 6:2 reassortant derived from A/Leningrad/134/17/57(H2N2) MDS (Len17) and low-pathogenic avian A/Mallard/Netherlands/12/00(H7N3) virus (H7N3-wt). The vaccine candidate A/17/Mallard/ Netherlands/00/84(H7N3) demonstrated cold-adapted (*ca*) and temperature-sensitive (*ts*) phenotypes similar to those of Len17. The antigenic profile of the reassortant strain was similar to that of the H7N3-wt parent strain as well as of human H7N7 isolates from the Netherlands including the virus isolated from the fatal case. The reassortant virus was shown to be attenuated for chickens and mice. Mice receiving 10^6 EID₅₀ of A/17/Mallard/ Netherlands/00/84(H7N3) raised virus-specific serum IgG and mucosal IgA antibody responses.

Introduction

A number of confirmed cases of human infection caused by avian influenza viruses demonstrated that influenza viruses of different HA subtypes may acquire the ability to spread among humans. The outbreak of highly pathogenic (HP) avian influenza H7N7 in the Netherlands during 2003 resulted in more than 80 human cases of conjunctivitis and mild respiratory illnesses and 1 fatal case [1, 2]. Since similar H7 viruses are still circulating among wild birds, generation of vaccine candidates the of appropriated subtype is ongoing [3]. To prepare a LAIV candidate we used classic genetic reassortment with a non-pathogenic avian virus H7N3-wt and the cold-adapted (*ca*) H2N2 MDS Len17 that is currently used in Russia for preparing licensed live reassortant vaccines. Human H1N1 and H3N2 reassortant viruses possessing the six internal genes from Len17 were shown to be safe, genetically stable and effective in protecting adults (including the elderly with chronic disorders), children 3-14 years old [4, 5, 6]. Previously it was shown the Len17-based 7:1 reassortant with a non-pathogenic avian H5N2 influenza virus was attenuated for chickens and mice providing cross-protection in mice against HP H5N1 viruses of different antigenic sublineages [7, 8]. In the present study a 6:2 reassortant A/17/Mallard/Netherlands/00/84(H7N3) was developed and characterized.

Methods

Viruses. The A/17/Mallard/Netherlands/00/84(H7N3) reassortant virus (Len17/H7N3) was obtained using classical genetic reassortment between H7N3-wt and MDS Len17 as described previously [9]. RFLP analysis [10] and/or nucleotide sequencing of PCR-amplified DNA copies of all genes were used for genome composition analysis of Len17/H7N3. Viruses were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs (CE) at 34°C for 2 days (Len17/H7N3 and Len17) or at 37°C for 26 h (H7N3-wt virus).

Antigenic specificity. Hemagglutination-inhibition (HI) test with a panel of ferret antisera to different H7 avian and human viruses was performed using the standard method [11].

Pathogenicity for chickens. Chickens received the standard 0.2 ml of a 10^{-1} dilution of either parent viruses or Len17/H7N3 reassortant virus intravenously (i.v.) and were observed daily for 14 days for clinical signs and death. To determine infectivity, chickens were inoculated intranasally (i.n.) with 10^6 EID₅₀ of each virus. On day 3 post inoculation (p.i.), oropharyngeal and cloacal swabs were collected from each chicken and virus replication was assessed in CE. The chickens were observed for clinical signs of disease and death for 21 days, at which time serum samples were harvested and tested for presence of antibodies by agar gel immunodiffusion (AGID) test.

Mouse study. 10 weeks old BALB/c mice were inoculated with 10^6 EID₅₀ per mouse of Len17 MDS, Len17/H7N3, H7N3-wt or received PBS as a negative control. Nasal turbinates and lungs were collected from 3 mice per group on day 3 p.i. and titrated in CE. Sera and nasal washes were collected from 5 mice per group on day 28 p.i. and tested in enzyme-linked immunosorbent assay (ELISA) as previously described [12] using 100 HAU/0,1 ml of whole purified H7N3-wt virus for coating plates.

Results and Discussion

The Len17/H7N3 reassortant virus demonstrated high growth capacity in CE at optimal (34°C) temperature and demonstrated *ca*- and temperature-sensitive (*ts*) phenotypes similar to those of Len17. Titers of Len17/H7N3 in CE at 25°C were 6.2 ± 0.8 log₁₀ EID₅₀/ml compared to 9.3 ± 0.3 log₁₀ EID₅₀/ml at the optimal temperature (34°C). Corresponding titers for Len17 were 7.0 ± 0.1 (25°C) and 9.5 ± 0.3 log₁₀ EID₅₀/ml (34°C). Titers of Len17/H7N3 at 40°C did not exceeded 1.8 ± 0.2 log₁₀ EID₅₀/ml in contrast to those for H7N3-wt that grew at 40°C up to 8.7 ± 0.2 log₁₀ EID₅₀/ml. The HA nucleotide sequence of the Len17/H7N3 reassortant strain was identical to that of the H7N3-wt parental strain. According to the HI test with a panel of ferret antisera to different avian and human H7 viruses the antigenic profile of the reassortant virus was similar to that of the H7N3-wt parent strain as well as human isolates including fatal case (data not shown). Thus, no molecular or antigenic changes between Len17/H7N3 reassortant virus and H7N3-wt parent strain occurred during the reassortment and selection procedure. Both Len17/H7N3 and Len17 parental strain was shown to be attenuated for chickens whereas H7N3-wt virus caused 60% mortality after i.v. inoculation. The H7N3-wt parent virus was

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detected in respiratory and intestinal tracts of chickens after i.n. inoculation in low titers (Table 1). Evidence of infection with H7N3-wt virus was detected by presence of antibodies. The Len17/H7N3 reassortant virus similar to Len17 MDS failed to replicate in chickens after i.n. inoculation. This data suggest that acquisition of six "internal" genes from Len17 MDS completely attenuated avian H7N3 virus for chickens as was previously shown for another reassortant of H5N2 subtype [7]. Thus, use of the reassortants in the manufacturing of human vaccines will not pose a threat to the poultry industry.

Table 1. Pathogenicity and infectivity data for chickens.

Virus	I.V. pathogenicity test (n=8)		I.N. pathogenicity and infectivity data (n=5)				
	Morbidity	Mortality	Virus isolation on day 3 p.i.		Serology (AGID)	Morbidity	Mortality
			Oropharyngeal swabs	Cloacal swabs			
Len17/H7	0	0	0	0	0	0	0
Len17	0	0	0	0	0	0	0
H7N3-wt	5	5	2 (10 ^{1.1})*	1 (10 ^{0.91})*	5	0	0

*Mean virus titers (EID₅₀/0.1 ml)

Like the Len17 MDS, Len17/H7N3 was completely attenuated for mice. After intranasal inoculation with 10⁶ EID₅₀ Len17/H7N3 the virus replicated well in nasal turbinates of mice but did not replicate in mouse lungs. Titers of H7N3-wt in mouse lungs were as high as 10^{6.4} EID₅₀/ml (Table 2).

Table 2. Pathotyping and immunogenicity in mice.

Virus	Virus isolation 3 days p.i. (log ₁₀ EID ₅₀ /ml)		ELISA antibody 28 days p.i.(log ₁₀)	
	Lungs	Noses	Serum IgG	Local IgA
Len17/H7N3	≤1.5	3.7±0.3	2.7±0.2	2.3±0.5
Len17	2.3±0.5	2.7±0.2	2.7±0.4	1.3±0.5
H7N3-wt	6.4±0.2	1.7±0.0	3.5±0.3	2.7±0.9
PBS	≤1.5	≤0.8	≤2.0	≤1.0

After one i.n. dose the Len17/H7N3 reassortant strain induced detectable serum levels of virus-specific IgG to whole H7N3-wt virus. Despite the lack of Len17/H7N3 replication in mouse lungs virus-specific neutralising antibodies was detected in sera of immunized animals in titers 1:20-1:80. The Len17 parent strain was shown to induce cross-reactive serum IgG and a low level of local IgA antibodies to H7N3-wt virus. In previous study with the *ca* reassortant strain of H5N2 subtype it was shown that Len17 also induced cross-reactive H5 specific serum and local antibodies and provide 20-80% protection against lethal challenge with HP H5N1 viruses. However for complete protection it was necessary to use the vaccine strain of the same H5 subtype. [7]. The Len17/H7N3 reassortant induced local virus-specific IgA in levels comparable to that of H7N3-wt virus. Conclusion: The high-growth Len17/H7N3 reassortant strain possessing six internal genes proteins from Len17 demonstrated *ts*- and *ca*- phenotype that correlated with *att*- phenotype for chickens and mice. In mice, the Len17/H7N3 reassortant given as a LAIV raised systemic and local virus-specific antibody responses. Our data suggest that classical genetic reassortment in CE of antigenically appropriate non-pathogenic avian influenza viruses with *ca* Len17 MDS can be

used as an approach for preparing and evaluating LAIV prior to the widespread circulation of a HP virus in the population.

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Emergency Production of Avian Flu Vaccines

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Introduction

Vaccines are the best prevention method against diseases and “prevention is the best weapon to reduce health care cost”. Potential pandemic influenza caused by the high pathogenic avian influenza virus (HPAIV) is now around the corner and the current influenza vaccine production facilities would not be able to produce sufficient vaccines for the demand [1,2]. Countries that currently do not have vaccine manufacturing facilities will be the most vulnerable. The Vaccine R&D Center at National Health Research Institutes (NHRI) Taiwan was assigned to develop strategic plans for emergency production of avian influenza vaccines in 2005. In this presentation, we described how to equip a P2+ facility containing a positive pressure cell culture room, and two negative pressure rooms for virus growth and downstream purification processes. We have successfully developed the production processes for growing the WHO vaccine strain NIBRG-14 in MDCK cell using roller-bottle technology and found that alum phosphate formulated inactivated virus could elicit both high virus neutralizing antibody titers in different animal models and protect mice from the wild type H5N1 challenges.

Materials and Methods

The vaccine center's MDCK cells were purchased from Food Industry Research and Development Institute (FIRDI), Taiwan. The source of these MDCK cells is American Type Culture Collection (ATCC), CCL-34™. The NIBRG-14 H5N1 vaccine strain was kindly given from WHO reference laboratory (NIBSC) to Taiwan Centers for Disease Control. The NIBRG-14 is a reassortant of H5N1 virus strain A/Vietnam/1194/2004 and PR8 using reverse genetics technology. The NIBRG-14 virus had been cultivated for 4 generations in eggs at Taiwan CDC and then transferred to NHRI vaccine center. The manufacturing facility is an independent space with an area approximately 100 square meters. This space is specified as P2+ facility with one class 100,000 standard cell culture room, one class 10,000 standard virus culture room, one class 10,000 virus purification room, two air-lock rooms and two buffer rooms. There are ten stages to produce H5N1 vaccine bulk using serum-free media, MDCK plus (Cesco, Taiwan) as shown in Figure 1. The essential

QC tests were transferred from the Influenza Reference Laboratory at the National Institutes of Infectious Diseases of Japan, and performed according to WHO recommendations that included viral inactivation test by the plaque assay, HA titer of virus solution using turkey blood method, HA protein content using single radial diffusion (SRD) method (HA working standard obtained from NIBSC), total protein content by BCA method, residual DNA content using hybridization method according to the US Pharmacopoeia, residual host-cell protein content by ELISA and western blot, formaldehyde content by colorimetric analysis. Mouse immunogenicity test were performed to determine the potency of the vaccine candidates formulated with and without different adjuvants. The immune responses were evaluated using hemagglutinin-inhibition (HI) and virus neutralization tests.

Results

Cell culture and virus growth. The roller bottle technology that has been used for rotavirus vaccine and biologics production, is currently the fastest and cheapest way to develop cell-culture based viral vaccines. MDCK cells adapted and grew well in serum-free media. We had screened most commercial available serum-free media and found a media, MDCK Plus manufactured by local company Cesco to be the best for cell growth and virus yield.

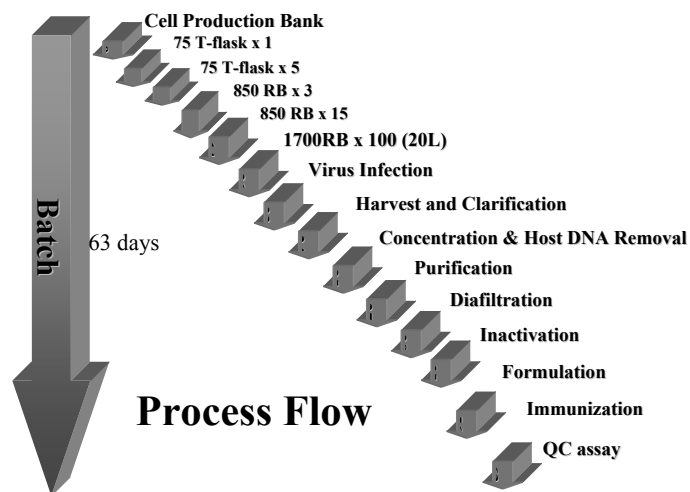
Downstream Processes Development. As shown in figure 1, the total time for the 60L/batch manufacturing process was about 63 days starting from the cell bank to the completion of QC tests of vaccine candidate. We also found the best time for harvest to be 3 days after virus inoculation and expansion. We found formalin inactivation could be completed within 24 hours at 37°C. The results from different production batches indicated that 7,000 to 8,000 doses (15µg of HA antigen) of vaccine bulk could be generated from a 60 L virus culture. Based on the current production processes, we could produce one 60 liters batch in about 21 days.

Characterization of vaccine bulk. For safety issues, residual DNA test using the Threshold system (Molecular Devices) was performed to determine whether the benzonase treatment was effective to remove the host DNA fragments from the cell lysate. The residual DNA was consistently found to be below 10 ng per dose. We had performed plaque and 50% tissue culture infectious dose (TCID50) assays to evaluate whether there was incomplete formalin inactivation during storage. After the sterile filtration, the preliminary stability studies using SRD method indicated that the vaccine bulk could store at 4°C for more than 6 months. Electron micrograph revealed the inactivated virus was intact virion. The vaccine bulk mainly contained viral proteins as shown by both SDS-PAGE and Western blot analyses.

Immunogenicity studies. A recent review by Subbarao and Joseph [1] concluded that H5N1 split vaccines were poorly immunogenic in human clinical trials, and most of these vaccines required over 90 µg of HA antigen and multi-doses to elicit acceptable immune responses. Therefore, strong adjuvant

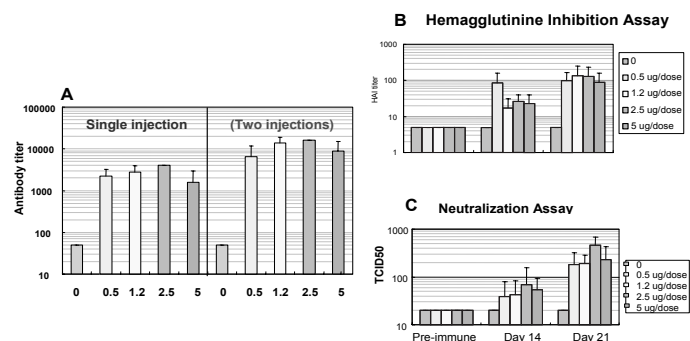
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Figure 1. A schematic diagram describing the H5N1 flu vaccine candidate manufacturing processes flow using the cell culture based roller bottle technology.



that could elicit strong immune responses is necessary for inactivated H5N1 to be effective as a vaccine candidate. Different adjuvant formulations could induce different immune responses in immunized animals. To assess the immune responses elicited by inactivated H5N1 virions, we used guinea pigs, rat, BALB/c mouse and C56BL/6 mouse as models to evaluate the immunogenicity of vaccine formulated with different adjuvants, or PBS alone. After 2 intramuscular immunizations with 5µg of HA antigen, or above, we observed strong virus neutralizing antibody response even in the absence of adjuvant in all animal models. At low doses (0.1 to 0.5µg) of HA antigen, we observed the importance of the adjuvant effect in the mouse models. In addition, we found BALB/c mice to be more sensitive to the antigen dosages. A typical experiment result was shown in Figure 2.

Figure 2. Mouse immunogenicity study was performed as groups of 6 C56BL/6 mice were immunized twice intramuscularly with respective vaccine candidate containing various amount of HA antigens (Ranging 0.1 To 5µg) in two weeks apart. From day 0, the mice were bled every two weeks for immune responses evaluations. (A) Igg antibody responses determined by ELISA; (B) The functional antibody responses were measured by HI assay; (C) The functional antibody responses were measured by virus neutralization assay using TCID₅₀ method.



In the presence of AlPO₄ (alum), the optimum dose was found to be in between 0.5 to 1µg of HA antigen. In both mouse models the vaccine bulk containing 0.5 µg of HA antigen formulated with alum could elicit HI antibody titers of >300 and virus neutralizing antibody titers of >800 based on TCID₅₀. Protection studies. Mouse protection model was established in the P4 facility at the Animal Health Research Institute (AHRI), Taiwan. A wild H5N1 virus strain isolated from a smuggled duck from China (A/duck/China/E319-2/03), had been adapted to infect mice through intranasal route. The LD₅₀ was found to be 1 x 10^{8.5} pfu. Mouse protection studies were performed with two different batches of bulk vaccine formulated with and without alum. Each BALB/c mouse was immunized twice intramuscularly with respective vaccine candidate two weeks apart. About 1 week after the second dose, the mice were moved to P4 animal challenge facility at AHRI and inoculated with 10^{8.5} pfu of virus in 100 the microliter by intranasal route. The results were shown in Table 1. It was interesting to see 0.1µg dose was more effective to protect the mice than those immunized with the 0.5µg. The vaccine candidates provided about 70% protection rate, while the control mice immunized with alum alone were not protected from the virus challenge.

Table 1. Mouse protection studies.

Vaccine Candidates	Group1	Group 2	Group 3	Group 4	Group 5
Vaccine bulk lot #	A537	A537	A532	A532	No vaccine
Ha antigen (µg/dose)	0.5	0.1	0.5	0.1	0
Alum (µg/ dose)	300	300	300	300	300
Mortality (live/total)	2/6	4/6	2/5	4/6	0/5
Protection (%)	34	67	40	67	0

Discussion

Subbarao and Joseph [1] had pointed out that the current flu vaccine production facilities would not be able to produce sufficient vaccines for the demand if a pandemic happened now. The countries that currently do not have vaccine manufacturing facilities are the most vulnerable and should consider developing their own manufacturing capability. The above strategic plans for emergency production of H5N1 flu vaccines has been successfully developed by the Vaccine Center of NHRI. We found it was possible to renovate and equipped a P2+ facility containing a positive pressure cell culture room, and two negative pressure rooms for virus growth and downstream purification processes within two months and at less than 1 millions US dollars.. Within 6 months from the ground zero, we had successfully (1) developed the production processes for growing the WHO vaccine strain NIBRG-14 in MDCK cell at

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60 liters scale; (2) established all basic QC tests; (3) performed mouse immunogenicity studies and found out that the novel adjuvant formulated inactivated virus at 0.1 µg of HA antigen could elicit high virus neutralizing antibody titers in mice; (4) demonstrated the vaccine candidate formulated in alum could protect mice from the wild type H5N1 challenges. With the current P2+ facility we can produce about 7,500 doses of avian flu vaccines containing 15 µg of HA antigen every month. To enhance the virus growth and yield, we are currently screening different serum-free media in the presence of different culture media supplements. The production processes using micro-carriers bio-reactor technology are now being developed. To prove our vaccine candidate will be safe and effective, we are currently embarking on pre-clinical studies, such as cell bank and virus seed validation, acute and chronic toxicology studies, a vaccine stability study and preparing the dossiers for filing Investigation New Drug (IND). In summary, our strategies could be generally applicable to those countries that currently do not have influenza vaccine manufacturing facilities.

Acknowledgements

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Mucosal or Systemic Delivery of VLPs Induces Protective Immunity Against Influenza Virus

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Recurrent outbreaks of highly pathogenic avian influenza virus pose the threat of pandemic spread of lethal disease and make it a priority to develop safe and effective vaccines. Influenza virus-like particles (VLPs) have been suggested to be a promising vaccine approach. We developed VLPs containing influenza A/PR8/34 (H1N1) or A/Aichi (H3N2) hemagglutinin (HA) and matrix (M1) proteins, and investigated their immunogenicity and protective efficacy in mice. Intranasal or systemic immunizations with VLPs containing A/PR8 HA protected mice from lethal challenges with mouse-adapted PR8 or WSN viruses, but not the heterosubtypic A/Philippines (H3N2) strain. Influenza VLPs containing A/Aichi HA provided the immunized mice with complete protection against lethal challenge with homologous strain A/Aichi but not heterologous strain A/Philippines (H3N2). Mice immunized intramuscularly with a mixture of VLPs containing H1 HA (A/PR8) and VLPs containing H3 HA (A/Aichi) were protected against lethal challenges with either A/PR8 (H1N1) or A/Aichi (H3N2). Influenza HA strain specific immune responses induced by influenza VLPs provide further evidence that anti-HA antibodies are primarily responsible for providing protective immunity against influenza viruses that match the vaccine strains. Taken together, these results indicate that non-replicating influenza VLPs represent an effective alternative to the current influenza vaccine as well as a promising strategy for the development of a safe vaccine to control the spread of lethal influenza viruses from birds.

Introduction

Currently, embryonated hen's eggs provide the main substrate for influenza virus isolation and vaccine manufacture. The influenza A subtypes are adapted to growth in embryonated eggs, or may be reassortant viruses containing genes which encode the internal proteins of A/PR8/34 (H1N1) virus which confer high growth capacity in eggs. During recent years, shortfalls in vaccine supply in response to the influenza season raised concerns about the current methods of vaccine production. Local or systemic allergic reactions to vaccine components can occur in some individuals due to residual egg protein. The H5 avian influenza strains responsible for recent epizootic outbreaks in Asia are lethal to chicken eggs (4, 6). Thus, there exist potential problems for growing pathogenic avian influenza virus in embryonated eggs because of the pathogenic nature of the H5 and H7 avian influenza viruses. In addition, diseases that affect chicken flocks due to an

avian influenza virus infection could easily disrupt the supply of eggs for vaccine manufacturing. These factors as well as the requirement for biosafety level 3 or higher containment facilities for safe handling of pathogenic avian influenza viruses warrant the urgent need to develop a new influenza vaccine modality. Virus-like particles (VLPs) have been generated and tested as vaccine candidates for a variety of viruses. It was recently reported that influenza VLPs can be an alternative approach for developing a safe and effective influenza vaccines (1-3, 5). However, the immune responses induced by influenza VLPs are not well characterized, and the memory responses and cross-protective immunity are unknown for VLP immunization. We developed VLPs for influenza virus A/PR8 (H1N1), for which the challenge system and immune epitopes are well-defined in a mouse model. We also generated H3 (A/Aichi) HA VLPs to test the cross-protective immune responses of influenza vaccines since current influenza vaccines contain different subtypes of circulating strains. Intranasal or muscular immunizations of mice with these influenza VLPs containing HA induced protective immunity which was subtype or strain specific-protection against lethal challenges.

Methods

For generation of recombinant baculoviruses (rBVs), cDNAs encoding A/Aichi H3 HA (Dr. David Steinhauer, Emory University) and A/PR8 H1 HA (Dr. Yumiko Matsuoka, CDC) were cloned into the pFastBac plasmid, a BV transfer vector. Recombinant Bacmid baculovirus DNAs (rAcNPV) containing Aichi HA or PR8 HA were isolated from transformed DH10Bac cells and transfected into Sf9 insect cells following the manufacturer's instructions (Invitrogen). Influenza VLPs were produced and purified from culture supernatants of insect SF9 cells infected with rBVs expressing HA (H1 or H3) and M1 as described (5). Groups of mice (Balb/c) were intranasally or intramuscularly immunized with 5 or 10 µg of influenza VLPs (A/PR8 HA or A/Aichi HA or both) at week 0 and 3 (Table 1). Blood serum samples were collected at 2 weeks after immunization, and binding and neutralizing antibodies determined by ELISA as described (5). At 4 weeks after the second immunization, immunized and naïve control mice were challenged with a lethal dose (10 x LD50) of following influenza viruses, and monitored daily for morbidity and mortality. Mouse-adapted pathogenic influenza viruses, A/PR8 and A/WSN were described in our previous study (5). Lethal challenge doses of influenza viruses A/Aichi and A/Philippines were pre-determined prior to challenge experiments.

Results and Discussion

HA containing VLPs were produced and released into the culture supernatants of insect cell co-infected with rBVs expressing M1 and HA without neuraminidase co-expression, which is consistent with a previous study (2). Influenza VLPs produced in insect cells were characterized by western blot and hemagglutination activity assays as described (5, 7). To determine the biological function and content of HA incorporated into VLPs, hemaggluti-

Table 1. Protective efficacy of immunization with influenza VLPs¹.

Mouse groups ²	Route	Virus dose for challenge	Clinical signs ³	Survivors	% of protection
Naïve mouse controls	i.n.	A/PR8 (H1N1) (10LD50)	Sick (+++)	0/6	0
	i.n.	A/WSN (H1N1) (10LD50)	Sick (+++)	0/6	0
	i.n.	A/Aichi (H3N2) (10LD50)	Sick (+++)	0/6	0
	i.n.	A/Phil (H3N2) (10LD50)	Sick (+++)	0/6	0
H1 HA (PR8) VLPs (5 µg)	i.n.	A/PR8 (H1N1) (10LD50)	Healthy	6/6	100
	i.n.	A/WSN (H1N1) (10LD50)	Healthy	6/6	100
	i.n.	A/Phil (H3N2) (10LD50)	Sick (+++)	0/6	0
H1 HA (PR8) VLPs (10 µg)	i.m.	A/PR8 (H1N1) (10LD50)	Healthy	6/6	100
	i.m.	A/WSN (H1N1) (10LD50)	Healthy	6/6	100
	i.m.	A/Phil (H3N2) (10LD50)	Sick (+++)	0/0	0
H3 HA (Aichi) VLPs (10 µg)	i.m.	A/PR8 (H1N1) (10LD50)	Sick (+++)	0/6	0
	i.m.	A/Aichi (H3N2) (10LD50)	Healthy	6/6	100
	i.m.	A/Phil (H3N2) (10LD50)	Sick (+++)	0/6	0
H1 (PR8) + H3 (Aichi) HA VLP (10 µg + 10µg)	i.m.	A/PR8 (H1N1) (10LD50)	Healthy	6/6	100
	i.m.	A/Aichi (H3N2) (10LD50)	Healthy	6/6	100

¹Mice were immunized intranasally (i.n.) or intramuscularly (i.m.) with influenza VLPs containing either H1 HA (PR8) or H3 HA (Aichi) or a mixture of VLPs containing H1 (PR8) HA and VLPs containing H3 (Aichi) HA at week 0 and 2. At 4 weeks after the last immunization, naïve and immunized mice were infected or challenged with a lethal dose (10x LD₅₀) of mouse-adapted homologous or heterologous viruses. Each challenge group contains 6 mice. ²Mice with +++ signs showed severe illness. Clinical signs were determined by body weight losses and mouse sick symptoms. +++ indicates losses in body weight between 30% to 40%.

nation units were determined using chicken red blood cells (7). Based on HA (A/PR8) units of influenza VLPs, the HA content in VLPs produced using pFastbac with the BV Polyhedrin promoter were 3 to 4 fold higher than those in previously reported VLPs produced using the pc/pS1 BV vector with nucleocapsid hybrid promoter (5). Although quantitative comparison of HA in influenza VLPs remains to be determined, influenza VLPs containing functional HA (A/PR8 or A/Aichi) were successfully produced for studies on the immunogenicity and protective efficacy of influenza VLP vaccines. Mice immunized intranasally with PR8 VLPs (5 µg) two times at week 0 and 3 showed complete protection against a lethal challenge dose (10x LD50) of mouse-adapted pathogenic A/PR8 strain (Table 1). Also, PR8 HA VLP immunized mice were protected against a heterologous strain A/WSN (Table 1). This protective efficacy was similar to that previously observed with 3 intranasal immunizations with 10 µg of PR8 HA VLPs (5), which reflects the improved incorporation of HA into VLPs. Most chemically inactivated whole influenza virus vaccines are administered intramuscularly to humans. The composition of the vaccine is determined based on the strains of virus that are expected to be circulating in the human population, and currently contain two influenza A subtypes (H1N1 and H3N2) and one variant of influenza B virus. Therefore, to test influenza VLPs as an alternative influenza vaccine, we intramuscularly immunized mice with A/PR8 (H1 HA) VLPs or A/Aichi (H3 HA) or a mixture of both VLPs as described in Table 1. Mice immunized with PR8 VLP showed A/PR8 (H1N1) strain specific immune responses in binding and neutralizing antibodies (data not shown) resulting in complete protection against A/PR8 virus challenge, and did not show cross-reactivities or protection against A/Aichi (H3N2) (Table 1). Similarly, immune

responses induced by immunization with Aichi HA (H3) VLPs were specific to A/Aichi virus and were protective against the homologous virus, but did not show cross-reactivity against A/Philippines (H3N2) within the same subtype (data not shown) and were not protective against A/Philippines (Table 1). Groups of mice immunized with a mixture of both VLPs showed immune responses specific to both vaccine strains and conferred complete protection against lethal challenges of either A/PR8 or A/Aichi viruses (Table 1). These results indicate that antibody responses specific to influenza HA play a major role in providing strain-specific protective immunity, similar to inactivated influenza vaccines. However, it remains to be determined whether other additional influenza components such as NA or nucleoprotein may contribute to the breadth of protective immunity. These results suggest that influenza VLPs are a promising alternative to egg-based influenza vaccines, and an attractive approach for developing safe and effective vaccine for avian influenza viruses.

Acknowledgements

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Broad-Spectrum and Extremely Early Protection Provided By Live Influenza Vaccine

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A live attenuated influenza vaccine (H3N2) has been evaluated as fast-acting prophylaxis against influenza infection from both heterologous (A/New Caledonia/99, H1N1) and (B/Shandong/97) infection. We found: (1) vaccination one to four days prior to infection results in immediate protection, 2) broad-spectrum protection can be achieved even after challenge with a lethal dose of heterologous influenza A or heterotypic B viruses, and 3) protection is achieved in a dose-dependent manner. The observed protection is in part mediated by genetic interference between vaccine and challenge viruses. These results support a novel use of live influenza vaccine especially for curbing unexpected influenza outbreaks.

Introduction

The potential threat of pandemic by various influenza subtypes demands advanced vaccine strategies. Various approaches were developed to enhance efficacy and safety of influenza vaccine [1, 2]. However, conventional vaccines have limitations in providing immediate protection, because a certain duration is required before developing virus specific immune responses. Furthermore, adaptive immunity is not as effective when the HA subtype is not matched between vaccine and the circulating virus [3, 4]. Previous studies have attempted to develop antiviral strategies using live influenza vaccine [5, 6]. When mutant virus and wild-type virus are infected simultaneously, interference occurs during their replication cycle [7]. This interference is also observed between influenza A and B virus [8-10]. Several studies on interference by cold-adapted (*ca*) live vaccine strain were performed and the *ca* virus suppressed the virulence of concurrently infecting virus [6, 11]. The dominance in replication provided a mechanism that attenuates overall virulence [12, 13]. Here we evaluated a cold-adapted live vaccine strain (PTIV-1 (H3N2)), isolated from cold-adapted X-31 virus (reassortant of A/PR/8/34(H1N1) and A/Aichi/2/68(H3N2)). Besides previously known therapeutic effects, we found that prior vaccination one to four days before infection results in good protection from lethal challenge. The prophylaxis is mediated without antibody responses and results in broad-spectrum protection from lethal challenge of heterologous influenza A or heterotypic B viruses.

Materials and Methods

Viruses. A live vaccine strain (PTIV-1) was isolated by serial plaque purification from the X-31 *ca* virus as previously described [3]. A/NC/99 (H1N1) and B/SD/97 was mouse-adapted and used for lethal challenge. Viruses were cultured using the 11-days-old embryonated chicken eggs. Eggs infected with

virulent viruses were incubated at 37°C while eggs infected with *ca* virus were maintained at lower temperatures (25°C). **Immunization and challenge.** Five to 6-week-old BALB/c mice were used for vaccination. To investigate the overall interfering effect and reassortant virus generation, a group of mice were vaccinated every day by the intranasal route with 1.0×10^6 p.f.u. of PTIV-1 from four days before challenge. The mice received a lethal dose of A/NC/99 or B/SD/97 (5.0×10^5 and 3.0×10^5 p.f.u., respectively) for challenge. Mice in the 'mixed group' were infected simultaneously with vaccine virus and virulent virus. For the evaluation of *trans*-acting attenuating property of the vaccine, a lethal dose of A/NC/99 was introduced simultaneously with various titer of PTIV-1, 2.0×10^6 , 1.0×10^6 , 5.0×10^5 , and 2.5×10^5 p.f.u., respectively. After 24 hours post infection, viruses were recovered from lung homogenate and propagated in embryonated chicken eggs. Then virus obtained from each group was re-introduced to mice in the same titer (5.0×10^4 p.f.u.).

Results

Vaccination provides early protection from heterologous and heterotypic lethal challenge. To evaluate immediate protection by live influenza vaccine, mice were vaccinated with PTIV-1 prior to virulent virus challenge. A group of mice received 1.0×10^6 p.f.u. of vaccine virus by intranasal route 1-4 days before lethal infection with A/NewCaledonia/20/99 (A/NC/99) or with B/Shangdong/7/97 (B/SD/97) (Table 1).

Table 1. Early protection against heterologous and heterotypic challenge of mice by vaccination with PTIV-1.

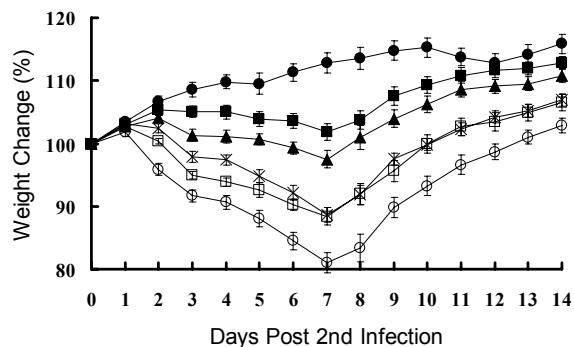
Group	A/NC/99 challenge		B/SD/97 challenge	
	Maximum weight loss (%)	Survival rate (%)	Maximum weight loss (%)	Survival rate (%)
-4d	9.0	100	10.6	100
-3d	19.7	100	12.9	100
-2d	21.7	87.5	29.0	75
-1d	32.3	87.5	26.1	75
Mix	35.2	25	28.2	75
Placebo	37.6	0	34.4	0

The vaccination resulted in marked improvement in clinical signs associated with A/NC/99 infection. The mortality was greatly reduced in groups vaccinated at least 1 day before challenge. Survival was 87.5% for vaccination groups of -1 and -2 day whereas 100% survival was achieved for the -3 and -4 day groups. Even in mixed infection, 25% mice survived while all mice succumbed in non-vaccinated group. The vaccine was also evaluated for protection against lethal influenza B virus challenge. Similarly, 100% survival and reduced morbidity was shown in mouse vaccinated -3 and -4 days before challenge, while 75% survival for the -1, -2 day and mixed groups. In general, better protection is provided with increased intervals between vaccination and challenge.

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Collectively, the data showed that administration of the live vaccine conferred immediate protection minimizing morbidity and mortality associated with influenza infection. **Dose-dependent protection by live vaccine.** To further investigate the observed early protection, we tested the dose-dependent effect in a simultaneous infection. The mice were vaccinated with PTIV-1 at a dose of 2.0×10^6 , 1.0×10^6 , 5.0×10^5 , and 2.5×10^5 p.f.u., respectively, as a mixture with a fixed dose 5.0×10^5 p.f.u. of the challenge virus A/NC/99 (1st infection). After 24 hours, viruses were obtained from lung homogenate of infected mice and cultured in embryonated chicken eggs. Then, mice were re-infected with the cultured virus at a titer of 5.0×10^4 p.f.u. uniformly for all infection groups (2nd infection) and the body weight was monitored for 2 weeks. As compared to control mice challenged with A/NC/99, the vaccinated group resulted in significant reduction of clinical signs and faster recovery. The attenuating effect strongly correlated with the amount of the vaccine virus in the 1st infection (Figure 1).

Figure 1. Dose dependent attenuation of virulent infection by co-administration of PTIV-1. Mice were simultaneously infected with A/NC/99 and various titer of PTIV-1, 2.0×10^6 (■), 1.0×10^6 (▲), 5.0×10^5 (×), 2.5×10^5 (□) p.f.u. After 1 day, viruses were harvested from the lung of infected mice and were used for fresh infection. A control group of mice were infected with A/NC/99 (○) or PBS (●). Body weight change was recorded daily ($n = 10$).



The data suggest that vaccine virus compete with A/NC/99 in mixed infection in a dose dependent manner, and the interference is provided directly by vaccine virus.

Discussion

The protection provided by the live vaccine is not only immediate (within a day before infection) but broad-spectrum as well. The broad-spectrum protection was extended to homologous challenge with the parental X-31 (H3N2) virus and heterologous challenge with A/PR/8/34 (H1N1) or A/Japan/305/57 (H2N2) (data not shown). The immediate and broad spectrum protection is achieved in the absence specific antibody response and is mediated by interference at genetic level and innate immune response through TLR receptor(s) (data not shown). We now suggest that these unique properties of broad protection could be extended to avian strain and for the control of influenza pandemics. Recently, a number of

sequence changes observed in the 1918 H1N1 pandemic-like virus have been identified in the recent H5N1 avian strains as well [14]. Although oseltamivir, a potent neuraminidase inhibitor, proved effective in the inhibition of 1918-like virus [15], there are no other effective options to block further spread of the current H5N1 virus. Classic vaccine should be administered well ahead of infection to elicit specific adaptive immune responses. Moreover, antivirals mainly provide therapeutic effects only after infection: there are so far no effective prophylactic strategies available to control immediate infection. This issue is important especially for avian influenza, where outbreak and the speed of spread among human population are unpredictable. The limitation of the current influenza vaccine therefore calls for the development of novel vaccine and prophylactic strategies. Ideally, an efficient vaccine would provide both (1) broad-spectrum immediate prophylaxis against variety of influenza viruses of unknown identity, and in addition (2) delayed but specific protection against circulating virus of which the identity has been predicted when used well ahead of infection, preferably 2-4 weeks before infection following generally recommended vaccination schedule. A broad-spectrum and immediate prophylaxis with the live vaccine warrants further investigation for controlling currently circulating human influenza and potential avian influenza pandemics. These results support a novel use of live influenza especially for curbing unexpected influenza outbreaks.

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Influenza A Virus Containing A Mutant M2 Protein With a 22-Amino-Acid Deletion in its C-Terminus as a Live Attenuated Vaccine Against H5N1 Virus

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Outbreaks of highly pathogenic influenza H5N1 viruses in avian species began in Asia and have since spread to other continents. Concern regarding the pandemic potential of H5N1 viruses in humans is clearly warranted, as is the need to develop effective vaccines against these viruses. Previously, we showed that A/WSN/33 (H1N1) M2 cytoplasmic tail deletion mutants grow less efficiently than wild-type virus *in vitro* (Iwatsuki-Horimoto *et al.*, 2006, *J. Virol.*, 80: 5233-5240). Here, we demonstrate that A/Vietnam/1203/04 (H5N1) containing a mutant M2 protein in which 22 amino acids were deleted from its C-terminus (the H5 M2del22 virus) protected mice against challenge with a lethal dose of H5N1 virus. Our results suggest that the H5 M2del22 virus has potential as a live attenuated influenza vaccine against H5N1 infection.

Introduction

Highly pathogenic avian influenza A virus (H5N1 subtype) is enzootic in Asia and has recently emerged in Europe and Africa. The continued circulation of this virus in birds provides ample opportunity for infection of humans by this virus. Indeed, H5N1 viruses have overcome host species barriers and infected humans, with devastating outcomes. Of over 300 reported cases of human infection by H5N1 viruses, approximately 60% have been fatal. Concerns of an H5N1 virus pandemic are clearly warranted, and there is an urgent need to develop effective vaccines against these viruses. Recently, we demonstrated that a 22-amino-acid deletion from the C-terminus of the M2 protein of A/WSN/33 (H1N1) influenza A viruses cause growth defects in cell culture (Iwatsuki-Horimoto *et al.*, 2006). We, therefore, tested the feasibility of using an H5N1 virus with a similar deletion in its M2 protein (the H5 M2del22 virus) as a live attenuated vaccine. The H5 M2del22 virus protected mice from a lethal challenge of H5N1 virus, suggesting its potential as a live attenuated influenza vaccine.

Materials and Methods

Cells. 293T human embryonic kidney cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and in minimal essential medium (MEM) containing 5% newborn calf serum, respectively. MDCK cells constitutively

expressing M2 from A/Puerto Rico/8/34 (H1N1; M2CK) were cultured in MEM supplemented with 10% fetal calf serum and 0.15 mg/ml of hygromycin (Iwatsuki-Horimoto *et al.*, 2006). All cells were maintained at 37°C in 5% CO₂, *Viruses*. Human H5N1 virus [A/Vietnam/1203/2004 (VN1203)] was used. Mutant VN1203 virus with an M2 protein containing a 22-amino-acid deletion from its C-terminus (the H5 M2del22 virus) was generated by reverse genetics (Neumann *et al.*, 1999). All experiments with live viruses and with transfectants generated by reverse genetics were performed in a biosafety level 3 containment laboratory approved for such use by the CDC and the U.S. Department of Agriculture. **Immunization and protection tests.** BALB/c mice (4-week-old female) were intranasally immunized with the H5 M2del22 virus. One month after vaccination, the mice were challenged intranasally with 100 MLD₅₀ of wild-type VN1203 virus and monitored daily for survival and body weight over the next 14 days.

Figure 1. Survival of mice immunized with the H5 M2del22 virus following lethal challenge with H5N1 VN1203 virus. Groups of three mice immunized with 3x10³, 3x10⁴, 3x10⁵, or 3x10⁶ PFU of the H5 M2del22 virus were challenged with 100 LD₅₀ of VN1203 (H5N1) virus. Survival was monitored for 14 days post-challenge. Watanabe *et al.*

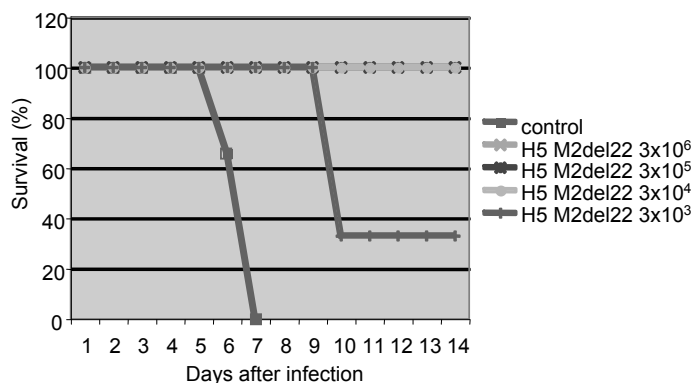
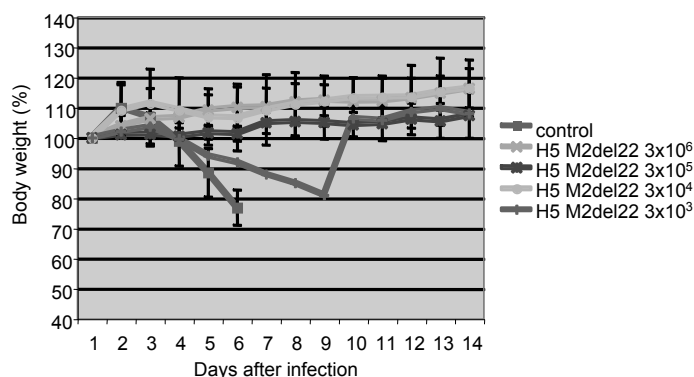


Figure 2. Body weights of immunized mice after challenge with wild-type VN1203 virus. Control mice and mice immunized with the H5 M2del22 virus (containing a mutant M2 in which 22 amino acids were deleted from the C-terminus) were challenged with 100 LD₅₀ doses one month post-vaccination. Values are mean weights (± standard deviations). Watanabe *et al.*



Results and Discussion

To characterize the attenuated phenotype of the H5 M2del22 virus in mice, we determined the 50% mouse lethal dose (MLD₅₀) of the wild-type VN1203 and the H5 M2del22 viruses. The MLD₅₀ of the H5 delM2del22 virus was 3.8×10^6 PFU, whereas that of the wild-type virus was 2.1 PFU, indicating that the H5 M2del22 virus was highly attenuated in mice. Next, to evaluate the vaccine efficacy of the H5 M2del22 virus, mice were immunized intranasally with 3×10^3 , 3×10^4 , 3×10^5 , or 3×10^6 PFU of the H5 M2del22 virus. As a control, mice were inoculated with PBS intranasally. One month after vaccination, the mice were challenged intranasally with 100 MLD₅₀ of wild-type VN1203 virus and monitored daily for survival and body weight for 14 days. Unlike the control mice, mice immunized with high doses (3×10^4 – 3×10^6 PFU) of the H5 M2del22 virus were protected against a lethal challenge with highly pathogenic VN1203 virus. All of the mice vaccinated with high doses of the H5 M2del22 virus survived (Figure 1). Moreover, their body weights were not appreciably affected by virus challenge, in contrast to the control group, whose weights decreased rapidly post-challenge (Figure 2). Even among the three mice immunized with a low dose (3×10^3 PFU) of the H5 M2del22 virus, one survived and partially recovered from the infection (Figures 1 and 2). Taken together, these results show that the H5 M2del22 virus is highly attenuated in mice and is protective against a lethal challenge of VN1203 virus. Promising live virus vaccine candidates must satisfy the following criteria: growth to high titers in a suitable preparative medium, attenuation in the host, and consistent immunogenicity. Although the H5 M2del22 virus did not grow well in MDCK cells, it replicated efficiently in M2CK cells that stably express the M2 protein (Watanabe *et al.*, unpublished data). Here, we demonstrated that the H5 M2del22 virus was highly attenuated in mice and was clearly immunogenic in our animal model, indicating its potential as a live virus vaccine against highly pathogenic H5N1 influenza viruses.

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Use of BALB/c Mice to Measure Cross-Reactive Antibody Responses and Degrees of Cross-Protection Between Pre-Pandemic Vaccines and A H5N1 Antigenic Variant

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We have used the BALB/c mouse model in an effort to better understand the potential of two H5 pre-pandemic vaccines, A/Duck/Singapore-Q/F119-3/1997 (Dk/Sing, H5N3) and A/Hong Kong/213/2003 (HK/213, H5N1) to provide cross-reactive antibodies and cross-protection against an A/Vietnam/1203/2004 (VN/1203) H5N1 virus. Mice were immunized intramuscularly (i.m.) with one dose of either inactivated whole virus vaccine. The extent of antibody cross-reactivity between H5 vaccines and H5 viruses in post-vaccination sera was assessed using a horse red blood cell hemagglutination-inhibition (HHI) and micro-neutralization (MN) assay, and an enzyme-linked immunosorbent assay (ELISA). Both vaccines elicited substantial HI and MN antibody responses to homologous viruses. The HI and MN antibodies induced by HK/213, but not Dk/Sing vaccine showed low cross-reactivity with VN/1203 virus. However, both vaccines induced substantial cross-reactive HA-specific IgG, provided cross-protection from death and systemic infection, but failed to protect animals from illness and respiratory tract infection. Mice that received HK/213 vaccine, which is genetically closer to VN/1203 virus, exhibited less weight loss compared with mice that received Dk/Sing vaccine following a high dose lethal challenge with VN/1203 virus. These data suggest that certain parameters of the BALB/c mouse model may be used to monitor the degrees of cross-protection afforded by pre-pandemic vaccine candidates for newly emerging H5N1 viruses with pandemic potential.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 viruses may acquire the ability to cause the next pandemic. Since these viruses first emerged in humans in 1997, multiple pre-pandemic vaccine strains have been generated and stockpiling of egg-based vaccines has begun in several countries [1]. Optimal influenza vaccines are those that are antigenically closely matched with the circulating disease-causing strain. Vaccine produced from the actual pandemic strain will not be available in the early pandemic phase because the production of such a vaccine may take approximately 5 months [2]. Unfortunately, pre-pandemic vaccine candidates and/or stockpiled vaccines will very likely not be an exact antigenic match with the actual pandemic strain, because of constant viral evolution. Therefore, timely monitoring of the cross-reactivity of antibody responses

and cross-protection between pre-pandemic vaccines and newly emerging H5N1 antigenic variants with pandemic potential is an ongoing need. In this study, we evaluate whether or not the BALB/c mouse could be used to monitor the likely degree of cross-protective antibody responses and cross-protection afforded by pre-pandemic vaccine candidates for newly emerging H5N1 variants with pandemic potential. Inactivated whole virus vaccines were prepared using A/Duck/Singapore-Q/F119-3/1997 (Dk/Sing, H5N3), the non-pathogenic "surrogate" vaccine candidate for the 1997 H5N1 outbreak, and A/Hong Kong/213/2003 (HK/213, H5N1), a HPAI virus isolated from a human in 2003, that was used as a HA and NA genes donor for the 2003 H5N1 vaccine candidate [3, 4]. We evaluated the ability of these vaccines to induce cross-reactive antibody and cross-protect against the genetic and antigenic HPAI H5N1 variant that arose in 2004, A/Vietnam/1203/2004 (VN/1203) virus. The HA1 region of Dk/Sing and HK/213 shared 91.2% and 97% amino acid identity, respectively, with the comparable region in the VN/1203 virus. **Materials and methods: Viruses.** Dk/Sing, HK/213 and VN/1203 viruses were used. The 50% egg infectious dose (EID₅₀), 50% mouse infectious dose (MID₅₀), and 50% lethal dose (LD₅₀) were determined as described previously [5].

Vaccine preparation and immunization of mice. Dk/Sing and HK/213 viruses were concentrated from allantoic fluid, purified on a linear sucrose gradient, and inactivated with 0.025% formalin [5, 6]. Six-week-old BALB/c mice were injected i.m. with one dose of 10 µg of inactivated whole virus vaccine (≈3 µg of HA protein) in a volume of 0.1 ml.

Serologic assays. Sera were treated with receptor-destroying enzyme before testing for antibodies. The HI was performed using 4 hemagglutinating units of virus and 1% horse red blood cells [7].

Table 1. Homologous and heterologous antibody titers induced by H5 vaccines.

Groups	HI titers			MN titers			ELISA anti-H5 HA IgG titers (log ₁₀)
	Dk/Sing	HK/213	VN/1203	Dk/Sing	HK/213	VN/1203	
Dk/Sing	80	46	< 10	256	< 20	< 20	4.2
HK/213	< 10	279	30	24	422	23	4.8
Unvaccinated	< 10	< 10	< 10	< 20	< 20	< 20	2.0

Note: Antibody titers are expressed as the GMT of 5 mice per group. Values in bold text represent titers to the homologous virus. The antibody titers given for the unvaccinated group of mice represent the limit of detection for each antibody assay.

Titers of neutralizing antibody were determined by the MN assay [8]. HA-specific IgG was detected by ELISA [9] using purified baculovirus-expressed recombinant hemagglutinin (rHA) from VN/1203 virus to coat plates (Protein Sciences Corporation, Meriden, CT, USA).

Challenge experiments. Mice were infected intranasally (i.n.) with 50 µl of virus one month after immunization. Organs

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were harvested 3 and 6 days post-infection (p.i) and titrated in embryonated eggs [5]. The remaining mice were observed daily for weight loss (WL) and death for 14 days p.i.

Statistical analysis. Statistical significance of the data was determined by using Student's *t* test.

Results

Measuring cross-reactive antibody responses in vitro. Sera collected one month after vaccination were used to measure antibody responses against homologous and heterologous H5 viruses. As shown in Table 1, unvaccinated mice did not show any antibody response. The Dk/Sing vaccine induced substantial HI and MN antibodies to the homologous virus, but did not induce antibodies that cross-reacted with VN/1203 virus. Similarly, the HK/213 vaccine induced high HI and MN antibodies to homologous virus, but only minimal antibody cross-reactive for heterologous VN/1203 virus was detected. In contrast to the low levels of cross-reactive functional antibodies measured by the HHI and MN assays, both vaccines induced high levels of cross-reactive IgG antibody against a VN/1203 rHA. Overall, when comparing antibody responses to the homologous strain, HK/213 vaccine was more immunogenic than Dk/Sing vaccine. Furthermore, the HK/213 vaccine induced 6-fold higher HI, 2-fold higher MN, and 4-fold higher HA-specific IgG ($p = 0.005$) antibodies to VN/1203 virus compared with that induced by Dk/Sing vaccine.

Measuring degrees of cross-protection in vivo. Mice immunized once with H5 vaccine were challenged i.n. with 200 LD₅₀ ($=10^{4.5}$ EID₅₀) of VN/1203 virus one month after vaccination. The degree of cross-protection was measured as protection from death, illness as measured by weight loss (WL) or viral replication. As shown in Table 2, unvaccinated mice died 5 to 9 days post-challenge with VN/1203 virus, with a mean maximum weight loss (MMWL) of 19.1%. In contrast, all immunized mice survived the lethal challenge, but exhibited some degree of WL, a marker for illness. However, mice that received HK/213 vaccine showed significant less WL compared to those of mice received Dk/Sing vaccine ($p < 0.01$). Unvaccinated mice had high titers of virus in both respiratory and other organs on day 3 and 6 p.i. In contrast, the virus was only present in respiratory tissues, and not other organs in mice immunized with either the Dk/Sing or HK/213 vaccine. Hence, both vaccines failed to provide cross-protection from respiratory infection against the antigenically distinct VN/1203 virus. Although virus was isolated from respiratory tissues in all mice tested on 3 days p.i., virus titers in lungs were reduced by 100-fold in mice receiving Dk/Sing vaccine and 251-fold in mice receiving HK/213 vaccine compared to unvaccinated mice ($p < 0.01$). Furthermore, both vaccines promoted comparable clearance of virus from the respiratory tract by day 6 p.i. Although the Dk/Sing vaccine was marginally less protective for reducing VN/1203 viral load than HK/213 vaccine, this was not statistically significant at either time point p.i.

Table 2. Cross-protection induced by H5 vaccines against a lethal infection with VN/1203 virus.

Group	Mean virus titers						No. protected/ No. challenged	
	Day 3 p.i.			Day 6 p.i.			Infection	Death
	Lung	Nose	Spleen	Lung	Brain	%MMWL		
Dk/Sing	5.2±0.7	3.2±0.5	≤ 0.8	1.1±0.4	≤ 0.8	7.0	0/5	9/9
HK/213	4.8±0.9	3.1±0.3	≤ 0.8	1.0±0.2	≤ 0.8	2.8	0/5	9/9
Unvaccinated	7.2±0.9**	4.0±0.5*	3.1±1.5*	6.1±0.9**	4.1±1.0**	19.1**	0/5	0/9

Note: The virus titers are expressed as the mean log₁₀EID₅₀ ±S.D. of 5 mice per group.* $p < 0.05$ or ** $p < 0.01$, unvaccinated mice vs vaccinated mice.

Discussion

As HPAI H5N1 viruses infecting birds and humans become ever more genetically and antigenically diverse, there is a growing need to assess the degree of cross-protection that H5 pre-pandemic vaccines may confer on newly emerging H5N1 virus variant viruses. Our results demonstrated that, in the BALB/c mouse model, the degree of cross-protection from death and systemic infection was equivalent regardless of the closeness of the genetic match between the two H5 vaccines and the challenge strain. Furthermore, these levels of cross-protection were observed in immunized mice that did not display any substantial cross-reactive HI and MN antibodies to the challenge virus, even though significantly elevated HI and MN antibodies (≥ 40) to homologous viruses were detected. In contrast, high levels of cross-reactive IgG (≥ 4.3 log₁₀) to challenge VN/1203 virus HA were detected in mice immunized with either vaccine. Therefore, these levels of cross-protection may, in part, be associated with the induction of non-HI and non-MN antibodies that are cross-reactive for H5N1 HA. Alternatively, functional antibody that is neutralizing but below the detection limits of our assays may also play a role. Other studies have also demonstrated a lack of correlation between *in vitro* functional antibody responses and *in vivo* cross-protection in mice [5, 10]. Wit et al. reported that immunization with an antigenically related H7N3 vaccine did not protect mice against a lethal infection with HPAI H7N7 virus, although high levels of cross-reactive HI antibody were induced [10]. Interestingly, although neither vaccine provided complete cross-protection from illness (WL), mice that received HK/213 vaccine showed significantly less WL following VN/1203 challenge compared with mice that received Dk/Sing vaccine. These results suggest that protection from WL may be one useful marker to discriminate between the cross-protective efficacies of pre-pandemic vaccines in mice. Viral shedding in the respiratory tract early after challenge may be another useful parameter with which to estimate the levels of cross-protection in mice, even though our results only demonstrated a trend and no significance in distinguishing the vaccines used in this study by this criteria. The use of a lower vaccine dose may increase the sensitivity of this approach in future studies. Also, it should be noted that the HK/213 vaccine is suggested to be superior in immunogenicity to other H5 vaccine candidates in animal models [11] and this may contribute to the enhanced cross-protection from illness

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and virus shedding observed here. In conclusion, the levels of cross-protective immunity between a pre-pandemic vaccine and a new emerging virus should be evaluated using multiple *in vitro* serologic assays as well as *in vivo* studies. Our data suggest that some parameters of the influenza mouse challenge model, including antibody titers against homologous and heterologous virus, WL, and the viral shedding early after challenge, may be useful to monitor the degree of cross-protective immunity afforded by pre-pandemic vaccine candidates for a newly emerging H5N1 variant with pandemic potential.

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A Study on the Immunogenicity of a H5N1 Vaccine Based on the Hemagglutinin of A/Indonesia/CDC625/2006

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Since 1997, highly pathogenic avian influenza A (H5N1) viruses have infected humans with lethal outcomes. Originating from Asia, the viruses have spread to other continents and increasing numbers of human cases have been recorded in recent years. These events have raised concerns that the viruses will eventually acquire the ability to be transmitted directly from human-to-human, leading to a pandemic. The availability of a pandemic vaccine thwarts this threat and many innovative methods have been developed to address this need. Using reverse genetics, our laboratory has generated an inactivated H5N1 vaccine containing a modified hemagglutinin (HA) gene from the A/Indonesia/CDC625/2006 (H5N1) and the rest of the seven genes from the A/Puerto Rico/8/34 (H1N1). The size and the appearance of the recombinant virus were similar to the parental strain which was tiny with a diffused edge. The growth of the recombinant virus was well-supported by 10-day old embryonated chicken eggs with up to 5×10^8 plaque forming unit (pfu) per mL, although this was still about 4-fold less than the parental strain. The hemagglutinin activity could be readily detected by both turkey and horse red blood cells. Mice injected subcutaneously with formalin-inactivated whole virion elicited good antibody activity 3 weeks after immunization. This response could be further enhanced by the addition of an external adjuvant (Figure 1, p value < 0.05). Using a murine immature dendritic cell (DC) line, D1 cells, we show that the virus is capable of activating DC, including up-regulating the mRNA expression of certain chemokines and cytokines, such as IP-10, IL-12, MIP3 and CD40L. Compared with A/Puerto Rico/8/34, the 7+1 viruses, containing the HA gene from either A/Indonesia/CDC625/2006 or A/Vietnam/1203/2004, are more potent in inducing up-regulation of mRNA expression of these molecules. Inactivation of the viruses using ultraviolet light did not affect their ability to activate DC. Based on these findings, we concluded that although the vaccine virus can activate the innate immune system, including a human-compatible adjuvant into the pandemic vaccine might be an essential step to potentiate the immunogenicity of the vaccine. Currently, several experimental adjuvants are in trial to examine their adjuvanting property on our H5 vaccine.

Figure 1. Enhancement of Antibody Responses of Formalin-Inactivated Whole Virion Vaccine Using External Adjuvant. Groups of 5 mice were inoculated with 3 micrograms of inactivated virus subcutaneously at the base of the tail, either admixed with complete Freund's adjuvant or in PBS alone. On day 21, sera were collected and the mice received a booster inoculation. On day 42, sera were collected again. The antibody titre against the virus was determined by an ELISA assay. The antibody titres in the primary and secondary response are represented by the black dots and red dots respectively. The difference between the two groups in the secondary response is statistically significant (p value < 0.05).

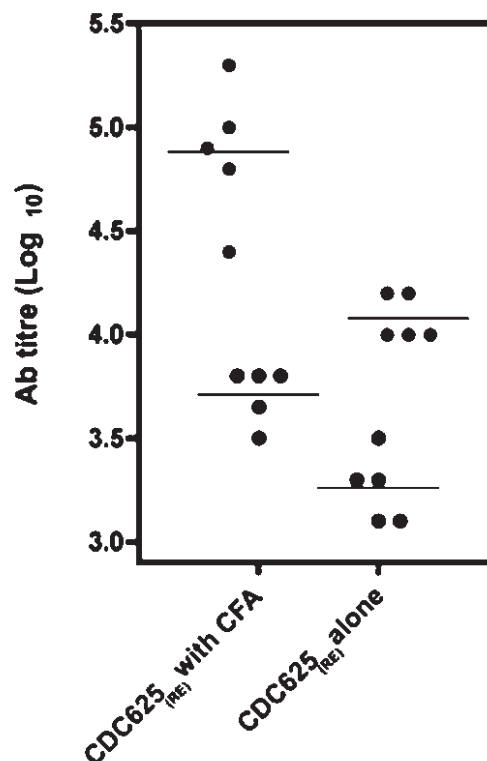
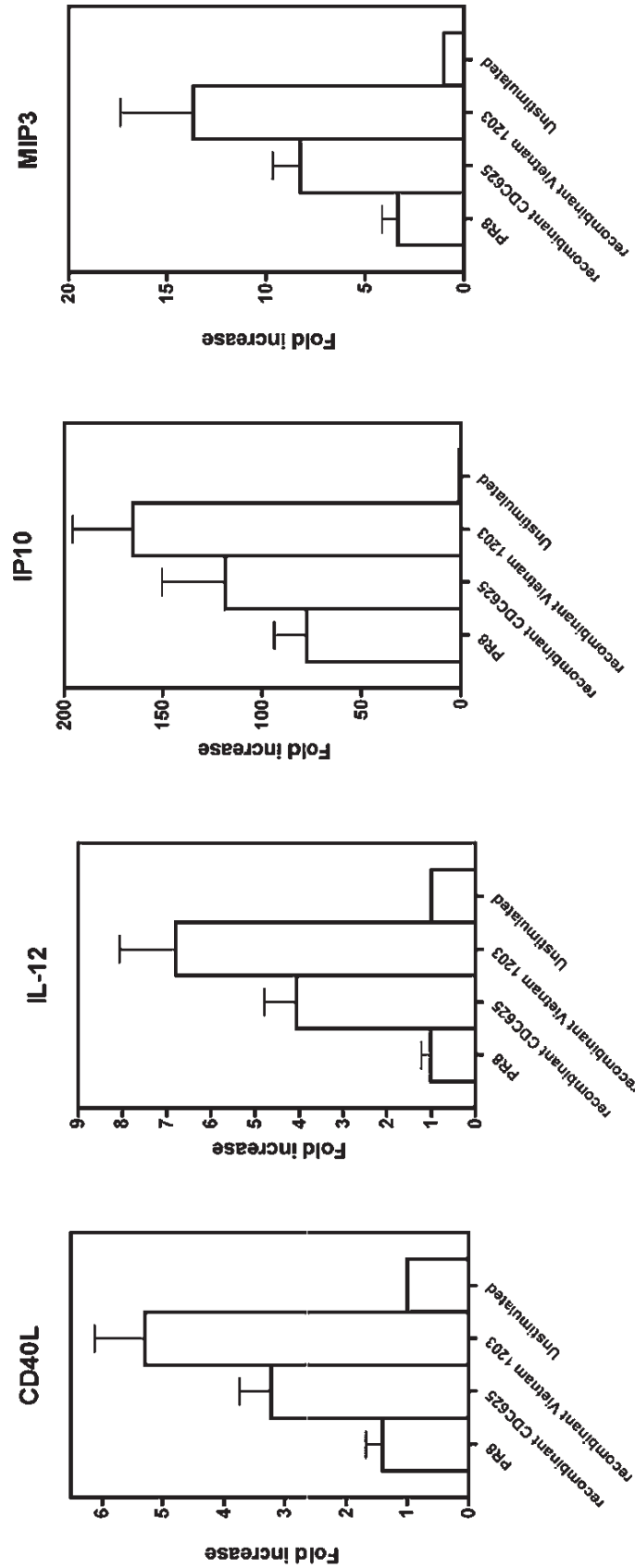


Figure 2. Differential Capability of Various Viruses in Activating Cytokine or Chemokine Genes of Dendritic Cells. Splenic immature murine dendritic cells, D1 cells, derived from C57/BL6 were infected with the indicated viruses at MOI 100. Cells were harvested 16 hours post-infection followed by mRNA harvesting. The mRNA was reverse-transcribed into cDNA. The various cytokine expression levels were determined by real-time PCR and were normalized using a house-keeping gene, beta-actin. The bars and error bars present the mean and standard deviation of duplicate samples. This is a representative of two independent experiments.



Cross-Protective Efficacy of Reverse-Genetics-Based H5N1/PR8 Reassortant Vaccines Against Challenge Infection With Homologous and Heterologous Clade Viruses In Mice

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To mitigate the impact of a pandemic by rapid deployment of vaccine, we have produced several reverse genetics-derived pre-pandemic vaccine candidate viruses for various subtypes and antigenic variants. Reassortant viruses containing attenuated hemagglutinin (HA) and intact neuraminidase (NA) genes from A/Vietnam/1203/04 (clade 1), A/Vietnam/Hanoi/30408/04 (clade 1) and A/Indonesia/05/05 (clade 2.1) and 6 internal genes from A/Puerto Rico/8/34 (PR8) viruses were produced and designated as VN1203/PR8, VN30408/PR8 and Indo05/PR8, respectively. VN1203/PR8 and Indo05/PR8 were produced in compliance with Good Laboratory Practice (GLP). All reassortant viruses showed an attenuated pathogenic phenotype in chickens and mice. Mice immunized with inactivated whole virion vaccine prepared from any of the three reassortant viruses were completely protected from lethal challenge infection with both clade 1 and clade 2 viruses.

Introduction

Since 2003, highly pathogenic H5N1 avian influenza A viruses have caused outbreaks in poultry and human infections in 11 countries. H5N1 influenza virus may cause a pandemic if it acquired the ability to transmit among humans [1]. H5N1 influenza viruses isolated during the past three years are categorized into two different clades based on genetic and antigenic characteristics of the hemagglutinin (HA). The majority of currently circulating H5N1 viruses belongs to clade 2 and this group of viruses is further divided into subclades. It is important to assess the efficacy of the vaccine against viruses from a different clade since the preparation of vaccine and its approval for human use will take several months while viruses are continuously evolving. The pandemic vaccines we developed include strains from these two different clades. The safety of the reassortant viruses was evaluated by inoculation into chickens and mice. Formalin inactivated whole virion vaccines against different clades of H5N1 viruses were evaluated by studying antibody responses and protective efficacy in mice.

Materials and Methods

Viruses. A/Puerto Rico/8/34 (PR8), A/Vietnam/1203/04, A/Vietnam/Hanoi/30408/04, A/Indonesia/05/05 and reassortant viruses were propagated in the 10-day-old embryonated chicken eggs.

Laboratory facility. All experiments using infectious H5N1

viruses and reassortant viruses, including work with animals, were conducted using BSL3-enhanced containment procedures. Investigators wore appropriate respirator equipment (RACAL Health and Safety Inc., Frederick, MD). *Generation of the reassortant viruses.* Reassortant viruses were generated by reverse genetics as reported previously [2, 3]. *Pathogenicity studies in chickens.* The standard chicken pathogenicity virulence test was conducted at Southeast Poultry Research Laboratory as recommended by the US Animal Health Association [4]. *Pathogenicity and protective efficacy in mice.* Fifty percent mouse lethal dose (MLD₅₀) of each of these viruses and titers of virus replication in the lungs of mice were determined as described previously [2, 3, 5]. Challenge studies and serological studies were conducted as previously reported [2, 3, 5].

Results

Pathogenicity of reassortant viruses in mice. The fifty percents mouse lethal dose (MLD₅₀) was determined by inoculating groups of six 6-8 week-old female BALB/c mice intranasally with serial 10-fold dilutions of the reassortant viruses and their parent wild-type viruses. All reassortant viruses exhibited low pathogenicity with more than 10^{6.5} MLD₅₀ (>10^{6.85}, >10^{6.5}, >10^{7.2} 50% egg infectious dose (EID₅₀) for VN1203/PR8, VN30408/PR8 and Indo05/PR8, respectively). On the other hand, parent wild-type viruses exhibited high pathogenicity with MLD₅₀ range from 0.57 to 2.5 EID₅₀. Pathotyping and replication in chickens. Eight 4-week-old White Rock chickens were inoculated with test viruses at a standard dose (0.2 ml of a 1:10 dilution of stock virus) by the intravenous route (i.v.). Oropharyngeal and cloacal swabs were collected on day three p.i. for virus isolation. All 8 chickens survived from infection with reassortant viruses while all died with wild-type virus infection.

Protective efficacy of the formalin-inactivated reassortant vaccines. Mice immunized with 10 µg of each formalin inactivated vaccine with or without Alum twice at two week interval and were challenged with three different viruses 4 weeks after the second immunization. All mice immunized with reassortant vaccines were completely protected from lethal challenge infection with clade 1 or clade 2 viruses.

Table 1a. Protective efficacy of the formalin-inactivated H5N1 vaccines against VN1203 (Clade 1) virus infection.

Vaccine	Alum	Survival Rate	Virus isolation ^a		
			Lung	Turbinates	Brain
PBS	-	0	3/3	3/3	2/3
	+	0	3/3	2/3	0/3
VN1203/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3
VN30408/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3
Indo05/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3

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Table 1b. Protective efficacy of the formalin-inactivated H5N1 vaccines against VN30408 (Clade 1) virus infection.

Vaccine	Alum	Survival Rate	Virus isolation ^a		
			Lung	Turbinate	Brain
PBS	-	0	3/3	1/3	1/3
	+	0	3/3	1/3	0/3
VN1203/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3
VN30408/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3
Indo05/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3

Table 1c. Protective efficacy of the formalin-inactivated H5N1 vaccines against Indo-5 (Clade 2) virus infection.

Vaccine	Alum	Survival Rate	Virus isolation ^a		
			Lung	Turbinate	Brain
PBS	-	0	3/3	1/3	1/3
	+	0	3/3	1/3	0/3
VN1203/PR8	-	100	0/3	2/3	0/3
	+	100	0/3	0/3	0/3
VN30408/PR8	-	100	1/3	3/3	0/3
	+	100	0/3	0/3	0/3
Indo05/PR8	-	100	0/3	0/3	0/3
	+	100	0/3	0/3	0/3

^aMice were challenged i.n. 4 weeks later with 100 MID₅₀ of viruses. Virus titers in lungs were determined on day 3 p.i. Virus titers were calculated by the method of Reed and Muench and are expressed as the mean log₁₀EID₅₀/ml ±S.D. of six mice per group.

Immune response of mice. Collection of sera was performed 1) before immunization, 2) 1 week after the 1st immunization, 3) 1 week after the 2nd immunization and 4) 4 days after challenge infection. Hemagglutination inhibition (HI) test with sera from immunized mice was performed. No HI titer was detected in sera from before immunization or 1 week after the 1st immunization. However, mice developed protective levels of HI antibody titer (titer>40) against the immunizing strain after the second immunization. Sera from mice immunized with VN1203/PR8 (clade 1) vaccine collected at 4 days after challenge infection showed clear cross reactivity with Clade 2.1 virus. Results from microneutralization tests showed a similar trend of antibody reactions (data not shown).

Table 2a. Hemagglutination inhibition antibodies of sera of vaccinated mice challenged with VN1203 (Clade 1) virus.

Vaccine	Alum	1 week after 2 nd dose ^a			4 days after challenge ^b		
		HI titer against			HI titer against		
		VN1203	VN30408	Indo05	VN1203	VN30408	Indo05
PBS	-	<20	<20	<20	<20	<20	<20
	+	<20	<20	<20	<20	<20	<20
VN1203/PR8	-	80	80	<20	80/80/160	160/160/320	<20/<20/40
	+	160	160	<20	160/160/640	320/320/640	20/40/80
VN30408/PR8	-	20	80	<20	20/20/40	80/80/160	<20
	+	20	160	<20	40/40	320/640	<20
Indo05/PR8	-	<20	<20	160	<20	<20	160/160/320
	+	<20	<20	640	<20	<20	640/640

Table 2b. Hemagglutination inhibition antibodies of sera of vaccinated mice challenged with VN30408 (Clade 1) virus.

Vaccine	Alum	1 week after 2 nd dose ^a			4 days after challenge ^b		
		HI titer against			HI titer against		
		VN1203	VN30408	Indo05	VN1203	VN30408	Indo05
PBS	-	<20	<20	<20	<20	<20	<20
	+	<20	<20	<20	<20	<20	<20
VN1203/PR8	-	40	40	<20	80/160/160	80/80/160	<20/<20/80
	+	160	320	<20	80/320/640	160/320/640	40/40/80
VN30408/PR8	-	20	80	<20	20/20/40	80/160/160	<20
	+	40	320	<20	80/80	320/320	<20
Indo05/PR8	-	<20	<20	160	<20	<20	20/160/160
	+	<20	<20	640	<20/20	<20/20	320/640/640

Table 2c. Hemagglutination inhibition antibodies of sera of vaccinated mice challenged with VN30408 (Clade 1) virus.

Vaccine	Alum	1 week after 2 nd dose ^a			4 days after challenge ^b		
		HI titer against			HI titer against		
		VN1203	VN30408	Indo05	VN1203	VN30408	Indo05
PBS	-	<20	<20	<20	<20	<20	<20
	+	<20	<20	<20	<20	<20	<20
VN1203/PR8	-	40	40	<20	40/80/80	40/160/160	<20/<20/20
	+	160	320	<20	20/320/640	160/640/640	80/160/160
VN30408/PR8	-	20	80	<20	20/20/40	160/160/160	<20
	+	40	320	<20	20/40/40	80/320/320	<20
Indo05/PR8	-	<20	<20	160	<20	<20	160/160/160
	+	<20	<20	640	<20/20	<20	320/320/640

^aSerum samples were collected and pooled at 1 week after the second immunization.

^bSerum samples were collected from individual mice at 4 days after the challenge infection.

Discussion

The H5N1 influenza virus strain is considered to be a potential cause of the next influenza pandemic. Since 2003, viruses have evolved into different clades which are genetically and antigenically distinguished from each other. Thus, it is important to develop updated vaccines relevant to the strains currently circulating in the field and to assess protective efficacy of pre-pandemic vaccine strains developed against different clades. The high-growth reassortant viruses we produced using reverse genetics for clade 1 and clade 2 H5N1 influenza viruses in compliance with Good Laboratory Practice and WHO guidelines possessed desired characteristics for pre-pandemic vaccines. They were safe in chickens and mice, propagated in high titers and immunogenically similar to the parent viruses. Inactivated whole virion vaccines for clade 1 and clade 2 viruses protected mice from lethal infection with homologous and heterologous strains. Mice not only produced protective level of HI antibodies against the homologous strain after the second immunization, but also some cross-reactive HI antibody was detected in mice immunized with VN1203/PR8 vaccine after challenge infection with homologous and a heterologous viruses. Thus some vaccines from heterologous strain could be still useful as a primary vaccine in the face of a pandemic. Further studies are needed to optimize pre-pandemic vaccination while the search for vaccines with broad immunogenicity continues.

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A Novel Dry Powder Vaccine Formulation (GelVac™) for Influenza Vaccines

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A novel dry powder vaccine formulation (GelVac) suitable for nasal delivery as well as reconstitution for injection is being developed for influenza vaccines. It is based on a distinct ionic polysaccharide (GelSite polymer) that provides an *in-situ* gelling property for sustained antigen release and also an antigen stabilization effect. The powder vaccine has been stable at ambient room temperature for over 2 years (ongoing). When delivered intranasally, GelVac powder vaccine was highly immunogenic and protective in rats, an animal model suitable for powder delivery. A distinctive protective effect in the upper respiratory tract was observed compared to immunization by injection. A phase I clinical safety study with GelVac powder without an antigen demonstrated consistent nasal delivery, no lung deposition or sneezing, and good acceptance. This same powder or equivalent dried vaccine formulation could be readily reconstituted for injection. A much higher immune response based on serum HAI titer was obtained following one or two intramuscular immunizations in animal models. A strong immunoenhancing effect of the GelSite polymer (≥ 2 fold higher by HAI) was demonstrated at a very low polymer concentration. These results from preclinical and clinical studies clearly indicate the potential advantages of this innovative dry powder vaccine formulation for influenza vaccines – room temperature stability, prolonged shelf life, and cold chain free distribution that are critical for pandemic preparedness and epidemic control.

Introduction

Influenza, current influenza vaccines, and immunization routes. Influenza A viruses undergo constant antigenic changes, causing seasonal epidemics and potentially pandemics. In recent years, an H5N1 avian influenza virus caused human infections with high mortality and raised concerns that another pandemic may be imminent. Current influenza vaccines are all made of liquid formulations which have a limited shelf life, require refrigeration for storage and distribution, and most are administered by injection. Inactivated trivalent influenza vaccines containing split or subunit antigens are by far the most widely used, although a live vaccine is also available as a liquid nasal spray. The immunogenicity of these inactivated trivalent vaccines has been evaluated extensively. In humans, a hemagglutination inhibition (HAI) titer of ≥ 40 is generally considered the protective threshold [1,2]. To reach an HAI titer of ≥ 40 , two doses of these vaccines are often needed in unprimed or elderly populations. Vaccines are commonly administered by

intramuscular injection. However, the nasal cavity represents an easily accessible and effective route of vaccination. Nasal vaccination induces not only systemic, but also mucosal immunity compared to the intramuscular injection which induces primarily a systemic antibody response with minimal or no mucosal response. Mucosal immunity is mediated by secretory IgA produced in the secretions along the respiratory tract, especially the upper respiratory tract [3]. Since influenza infection infects the respiratory tract, nasal immunization has the advantage in providing protection at the site of infection.

Unmet needs. The challenge facing influenza pandemic preparedness and epidemic control worldwide is development of an effective and antigen-sparing vaccine that can be stored at ambient room temperature with a long shelf life, distributed without refrigeration, and able to be administered without a needle. That is, a vaccine that is not only effective, but one that also meets all logistic requirements.

GelVac dry powder formulation for influenza vaccines. GelVac is a novel dry powder formulation for inactivated influenza vaccines that can potentially meet these challenges. It can be used for nasal delivery as well as injection after reconstitution. It does not contain an immunostimulatory adjuvant and is based on a distinct ionic polysaccharide (polygalacturonic acid; GelSite® polymer). GelSite polymer is inert and does not stimulate immune cells. However, it is capable of *in-situ* gelling upon contact with nasal or interstitial fluids. The gelation provides a sustained antigen release and also prolonged residence time when delivered intranasally, thus increasing antigen exposure and immune response. The GelVac dry powder formulation possesses distinct advantages. They are:

- Room temperature stability
- Cold chain – free distribution
- Prolonged shelf life
- Preservative free
- Needle-free administration (nasal)
- Induction of both systemic and mucosal immunity when administered intranasally
- Antigen-sparing when administered by injection

Materials and Methods

Antigen. Inactivated whole virion antigens were prepared and purified by gradient centrifugation from viruses propagated in MDCK cells. Different virus strains (H1N1, H3N2, B, and H5N3) were used. A standard split antigen vaccine was also used as a reference administered by intramuscular (IM) injection. The total protein content of inactivated whole virion antigens was determined with the Lowry assay (Pierce Chemicals, CA). The HA content was calculated with the 37.5% of total proteins based on the densitometry measurement and published reports. *Formulation.* GelVac powder formulation consists of inactivated influenza antigen, GelSite polymer, and other USP excipients. GelSite polymer is manufactured under cGMP at a kilogram scale. Powder formulations were prepared by either freeze-drying or spray drying processes. Due to its distinct chemical and functional properties,

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the GelSite polymer was used only at a very low concentration, ~0.25% of the powder (w/w) or ~0.025% (w/v) as a liquid formulation (reconstituted). This concentration was found to be optimal after examining various formulation and immunogenic parameters. *Immunogenicity and protection.* Powder formulation experiments were carried out with Sprague-Dawley (SD) rats (5 per group) as powder delivery requires an animal larger than mice. The powder dose was individually weighed and delivered intranasally after animals were lightly anesthetized with isoflurane. Serum antibodies were measured by HAI assays using chicken red blood cells. A rat-adapted H1N1 virus was used for the challenge. Live viruses in the lung tissue and nasal washes were measured by the plaque assay in MDCK cells. Geometric mean titers and standard deviation were determined for each group. Means were compared using student t test. A p value < 0.05 was considered significant.

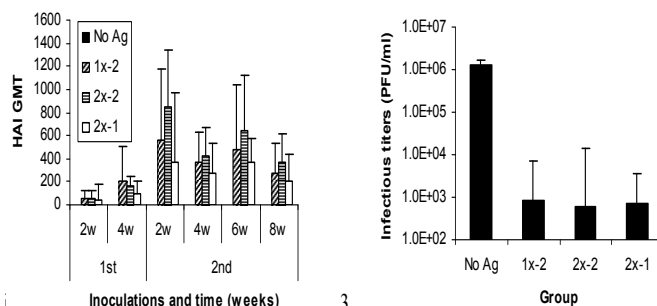
Results

Antigen stabilization and vaccine room temperature stability. The drying process can significantly influence antigen stability. Thus, a novel process condition for producing stable dry powder vaccine formulations has been identified. GelSite polymer was found to confer a stabilizing effect to the antigen during powder preparation. The powder vaccine has been stable at ambient room temperature for over 24 months based on HA activity and immunogenicity (ongoing).

GelVac powder for nasal delivery. GelVac nasal powders prepared with a desired particle size distribution are delivered into the nasal cavity with a nasal delivery device. Upon hydration by nasal fluids, the powder particles change into gel particles, which provide longer nasal residence times and sustained antigen release. Two GelVac powder formulations that differed by two fold in the antigen (A/Taiwan/1/86, H1N1) content (1x and 2x) were administered to rats intranasally (IN) via single (2x-1 group) or both (1x-2 and 2x-2 groups) nostrils (Figure 1). The results showed that the HAI titer reached 40 at week 2 and continued to rise above 100 at week 4 after just one immunization at a low dose of whole virion antigen (3.3 μg HA /rat) via one or both nostrils. A strong boosting effect (>2 fold increase in HAI titer) was observed after the second immunization ($p < 0.05$ for all groups at all time points except for the 2x-1 at week 8; Figure 1). The immune response was antigen dose-dependent. The titer obtained at 6.6 μg HA/rat (2x-2 group) was higher than that at 3.3 μg /rat (1x-2 group), although by < 2 fold ($p > 0.05$, Figure 1). Similarly, two-nostril inoculation (1x-2 group) induced a higher immune response than the single-nostril inoculation at the same antigen dose (2x-1 group), although also by < 2 fold ($p > 0.05$, Figure 1). At week 9 after the second immunization, rats were challenged with rat-adapted A/Taiwan/1/86 virus. The results showed that a $>1,000$ fold reduction in the lung virus titer was obtained in all three immunized groups and no significant differences in infectious titers were observed among them, suggesting that a similar protection level was reached by all groups (Figure 1). Several other immunogenicity and protection studies have also been conducted. The findings from these studies are summarized as follows:

- A strong immune response after one immunization followed by a strong boosting effect after the 2nd immunization
- The immune response by GelVac nasal powder with whole virion antigens is comparable to that of standard split antigen vaccine by injection
- The immunoenhancing effect of GelSite polymer
- A strong boosting effect by GelVac nasal powder was shown in animals primed by injection
- A distinct protection of the upper respiratory tract beside the lung
- GelVac nasal powder is effective with monovalent as well as trivalent antigens
- GelVac powder prepared by freeze drying and spray drying methods are equally effective

Figure 1. Immunogenicity of GelVac nasal powder with H1N1 (A/Taiwan/1/86) whole virion antigen in female SD Rats (N=5). Geomean HAI titers+SD were shown at different time points. Each animal was immunized with either 3.3 μg HA (1x) or 6.6 μg HA (2x) Via Single (2x-1) or two nostrils (1x-2 and 2x-2) followed by boosting on day 35. The animals were challenged with rat adapted A/Taiwan/1/86 (1×10^5 PFU/ Rat) at week 9 after the 2nd immunization.



GelVac powder for injection after reconstitution. GelVac powder is also suitable for injection after reconstitution with water. A strong immunoenhancing or antigen-sparing effect is obtained when the reconstituted GelVac powder is injected intramuscularly (IM). In this experiment, a GelVac powder incorporating H3N2 (A/Wyoming/03/2003) inactivated whole virion antigen was prepared in the presence or absence of GelSite polymer and administered to animals by IM injection after reconstitution with water (Figure 2). A strong immune response was achieved after just one immunization as measured by HAI titers (Figure 2). GelSite polymer, or the GelVac formulation, enhanced the immune responses by 6 – 12 times (week 4 after 1st inoculation; $p < 0.05$). This immunoenhancing effect was achieved at a very low polymer concentration (0.025%, w/v). This concentration is lower or comparable to that of aluminum adjuvants commonly used in the vaccines. Similar results have been obtained with monovalent H1N1 and trivalent (H1N1, H3N2, and B) antigens in mouse or rat model.

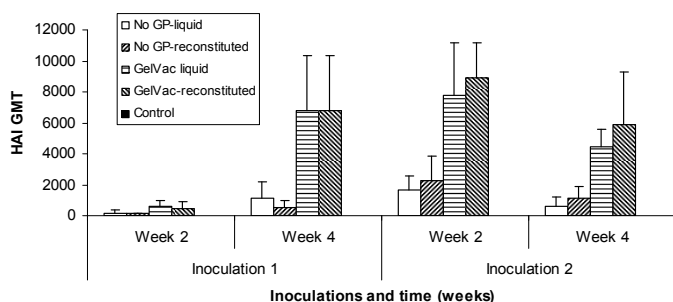
Discussion

The GelVac powder influenza vaccine formulation possesses

Options for the Control of Influenza VI

distinct advantages and is highly immunogenic and protective in the preclinical studies. It is suitable for nasal delivery as well as injection after reconstitution, thus allowing one base formulation for two different delivery routes. In order to develop this GelVac powder technology for influenza vaccines, we have 1) Completed the cGMP manufacturing of GelSite polymer, 2) Completed nasal and injectable toxicology studies of the polymer in two species, 3) Filed Master files for the polymer with CDER and CBER, 3) Completed an extensive series of preclinical immunogenicity and protection studies, 4) Completed a phase I safety study of nasal powder delivery platform (without antigen), and 5) Completed a pre-IND meeting with FDA in late 2006. The future steps include animal toxicology studies and IND filing prior to initiation of clinical studies.

Figure 2. Immunogenicity of GelVac powder with H3N2 (A/Wyoming/03/2003) whole virion antigen in female SD rats (N=5) after reconstitution and intramuscular injection in the presence or absence of GelSite polymer (GP). Geomean HAI titers+SD were shown at different time points. Each animal was immunized with 3.3 µG HA followed by boosting on day 35.



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Late Breaking Proceedings:

Oral Presentations

New Effective Nasal Immunizations of Influenza Vaccine by a Natural Mucosal Adjuvant From the Lungs and its Synthetic Compound

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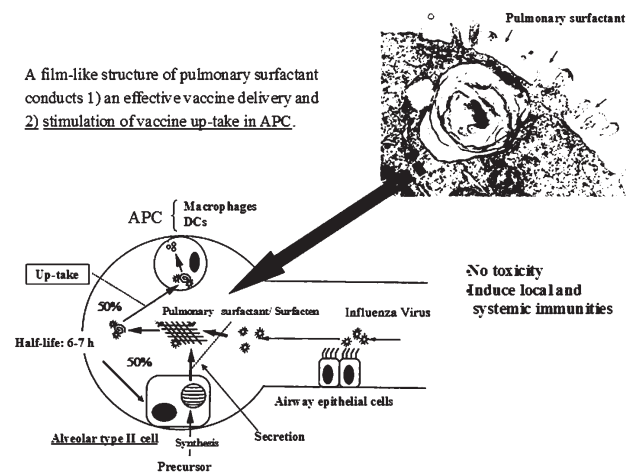
The intramuscular or subcutaneous administration of influenza vaccine induces IgG-mediated protection systemically but does not induce enough mucosal sIgA in the airway, the site of initial virus infection. To achieve both systemic and local protective immunity, intranasal vaccination has been studied. Mucosal adjuvants reported are Toll-like receptors-mediated DC activators, such as poly (I:C), CpG, LPS and bacterial lipids, and toxin based adjuvants, such as CT and *Escherichia coli* heat-labile toxin. However, these adjuvants stimulate not only antibody productions but also inflammatory cytokines through the innate immune system. We recently found a natural mucosal adjuvant in the lungs, pulmonary surfactant which efficiently delivers and increases in uptake of HA vaccine into the DCs, resulting in both local and systemic immunities. Intranasal administration of influenza HA vaccine combined with Surfacten, a modified bovine pulmonary surfactant free of antigenic SP-A and SP-B proteins, or a synthetic adjuvant mimicking human Surfacten induced mucosal sIgA in the airway and antigen-specific IgG in the serum without side effects in mice. The adjuvanticities of Surfacten and the synthetic human Surfacten were also observed in mini-pigs which have similar nasal lymphoid systems to human.

Introduction

The respiratory mucosal surface is the initial site of viral infections as well as a major site of immune defense by the interconnecting inductive nose-associated lymphoid tissue [1]. While several currently available HA vaccines are administered intramuscularly or subcutaneously, inducing an IgG-mediated protection in the systemic immune compartment, this immunization offers insufficient protection at the mucosal surface [2]. To induce mucosal immunity and systemic immunity, intranasal administration of HA vaccines combined with mucosal adjuvant have been studied. The powerful nasal adjuvants are toxin-based, such as CT and *Escherichia coli* heat-labile toxin, which induce antigen-specific serum IgG and mucosal sIgA [3,4], although they cause severe diarrhea and Bell's palsy [5]. Toll-like receptors-mediated DC activators, such as poly (I:C), CpG, LPS and bacterial lipids as mucosal adjuvants have also been studied. To develop safe and effective mucosal adjuvants conferring mucosal protective immunity in humans, we have searched natural compounds in the lungs and found that the pulmonary surfactant, a lamellated and/or film-like

structure of lipid-protein complex, has a potent mucosal adjuvant activity. Pulmonary surfactant has an innate host defense activity [6] and is rapidly turned over in alveolar type-II cells, macrophages and DCs [7] in the airway (Figure 1). In this study, we report the mucosal adjuvant activity of Surfacten, a modified pulmonary surfactant free of SP-A and SP-D, administered intranasally with the HA vaccine in mice. We also discuss our recent results of the synthetic human Surfacten mimicking human pulmonary surfactant and its adjuvant activity in mini-pigs.

Figure 1. Surfacten and its synthetic compound are promising adjuvants for nasal vaccination.



Materials and Methods

Virus and animals. The mouse-adapted IAV A/Aichi/2/68(H3N2) was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs, at 35°C for 48 h. The infectivity of the virus was measured by plaque assay. All experiments were performed in 6-weeks-old BALB/c female mice obtained from Japan SLC, Inc., Shizuoka, Japan.

HA vaccines and Surfacten. HA vaccine was prepared from IAV by the method of Davenport et al. [8]. Pulmonary surfactant was prepared from bovine bronchoalveolar lavage by the method of Hawgood et al. [9], and the commercially available modified bovine pulmonary surfactant, Surfacten (Mitsubishi Pharma Co., Osaka), a product free from SP-A and SP-D, was used.

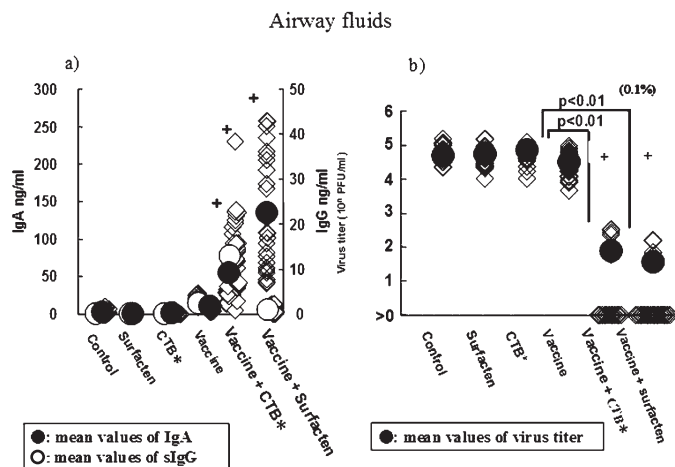
Immunization of mice and anti-HA vaccine-specific IgA and IgG levels. The mice were immunized intranasally on day 0, and boosted on day 28 by instilling 1 µl of the adjuvant-combined HA vaccine into each nostril. CTB* as a positive mucosal adjuvant was prepared by adding 0.2% of native CT to CTB in all experiments. Two weeks after the second immunization, serum, nasal wash and lung wash specimens were prepared and anti-HA vaccine-specific IgA and IgG levels were measured by ELISA as reported [10].

Infection and virus titer. The immunized mice were infected

intranasally with 3.5×10^4 PFU of IAV in 3 μ l of saline instilled into each nostril. On days 3 after infection, virus titers in the nasal and lung wash were measured by a plaque assay on Madin-Darby canine kidney cells [11].

Statistical analysis. All data were expressed as mean \pm SD. Differences between groups were examined for statistical significance using the unpaired Student's *t*-test. Results: To compare the mucosal and systemic antibody responses of mice treated with intranasal administration of HA-Surfacten or HA-CTB* as a positive control mucosal adjuvant, we analyzed the concentrations of anti-HA sIgA and IgG in the airway fluids 2 weeks after the boost inoculation. CTB* is a mucosal adjuvant, which enhances both mucosal and systemic antibody responses [3]. Intranasal instillation of HA vaccine, Surfacten and CTB* alone (0.2 μ g of each) did not affect the immune responses in the airway fluids while HA-Surfacten (0.2 μ g of each) or HA-CTB* (0.2 μ g of each) induced marked augmentation of mucosal anti-HA sIgA (Figure 2a). In contrast to HA-CTB*, which induced both sIgA and IgG in the airway fluids, Surfacten ($\leq 0.2 \mu$ g) combined with HA did not induce enough IgG in the airway fluids, but higher doses of Surfacten ($\geq 1.0 \mu$ g) significantly induced antigen-specific IgG in the airway fluids and in the serum (data not shown) [10]. The levels of IgG in the airway fluids and serum were almost equivalent to those induced by HA-CTB*. The results suggest that the induction of sIgA in the airway is more responsive to Surfacten than that of IgG. The achievement both systemic and local protective immunities by intranasal vaccination of HA-Surfacten or HA-synthetic human Surfacten was also observed in mini-pigs (manuscript in preparation).

Figure 2. Effects of surfacten on mucosal protective immunity. (a) The concentrations of anti-IAV sIgA (closed circle) and IgG (open circle) in the airway fluids were measured after the secondary inoculation for 2 weeks. Each circle represents the mean value in each group of 15 – 30 mice. (b) Two weeks after the second vaccination, the mice were infected with 7×10^4 PFU of IAV. Three days after infection, virus titers (PFU/ml) in the airway fluids were measured. CTB* was used as a positive control. + indicates a significant ($p < 0.01$) values compared with those in mice that received HA vaccine alone.



The mucosal adjuvant effects of Surfacten in the protection against influenza virus in mice was compared with the effects of CTB*. The mice received a primary intranasal inoculation with HA-Surfacten (0.2 μ g of each) or HA-CTB* (0.2 μ g of each), and a boost 4 weeks later. The mice were challenged with 7×10^4 PFU, a semi-lethal dose, of IAV 2 weeks after the second immunization. Over half of animals without vaccination died within 10 days. The virus titers in the airway fluids were measured 3 days after the challenge (Figure 2b). Although separate intranasal administration of HA vaccine, Surfacten and CTB* did not show significant protective mucosal immunity in the airway, HA-Surfacten or HA-CTB* markedly lowered the virus titers to $< 1/1000$ in the airway fluids. It is particularly noteworthy that the virus titers were below the detection level in 81% of mice inoculated intranasally with HA-Surfacten, versus 72% of mice inoculated with HA-CTB*.

Discussion

This study demonstrates that the intranasal inoculation of influenza virus HA antigen entrapped with Surfacten, a modified natural pulmonary surfactant, induced a prominent production of mucosal neutralizing sIgA in the airway, as well as systemic IgG responses in mice, its efficacy being almost equivalent to CTB* protein. In contrast, the s.c. inoculation of HA-Surfacten, augmented neither the mucosal nor the systemic IgA and IgG responses induced by treatment with the HA vaccine alone (data not shown) [10]. The pulmonary surfactant is rapidly turned over and recycled *in vivo* by alveolar cells, macrophages and DCs, with a half-life of 6-7 h [7,12], and SP-B and SP-C proteins stimulate the uptake of surfactant-like liposomes by these cells [13]. Surfacten has been clinically used for over 20 years as an effective medicine for acute respiratory distress syndrome, although Surfacten is prepared from bovine lungs and has a risk of BSE. To avoid the risk of BSE, we have synthesized Surfacten mimicking human pulmonary surfactant and recently succeeded. The adjuvanticity of synthetic human Surfacten was also observed not only in mice but also in mini-pigs having nasal lymphoid systems similar to those of human. Mini-pigs after booster inoculation of HA-synthetic human Surfacten intranasally showed local and systemic protective effects (manuscript in preparation). Hemagglutination-inhibition titers in pig serum were $\geq 1:40$, protective levels, and no inflammatory and adverse reactions were observed after vaccination. These data suggest that synthetic human Surfacten, mimicking the natural human pulmonary surfactant, is a safe and effective mucosal adjuvant, which enhances the protective mucosal and systemic immunities without incurring a risk of inflammation.

Acknowledgements

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Late Breaking Proceedings:

Poster Presentations

Protection From Influenza by Recombinant Nucleoprotein Requires CD40 Expression

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Introduction

Immune responses to influenza infection involve both B and T lymphocytes. Whereas B cells generate influenza-specific antibodies that neutralize virus, CD8 T cells respond with cytotoxicity toward infected epithelia and are important for viral clearance. Current influenza vaccines are designed to induce neutralizing antibodies to hemagglutinin (H) and antibodies to neuraminidase (N). The efficacy of these antibodies is primarily limited to preventing infection from challenge viruses with HN molecules identical to those of the vaccine, with little or no effect on disparate HN types [1, 2]. Due to the possibility of encountering viruses with unexpected HN, an alternative strategy is to induce immune responses against more conserved viral proteins. Intramuscular DNA vaccination with influenza nucleoprotein (NP) cDNA confers protection from viral challenge in BALB/c mice [3]. This protection strongly correlates with an increased frequency of cytotoxic T cell precursors in the vaccinated mice prior to challenge [4]. Because CD40 signaling is important for optimal CD8 T cell responses [5] as well as for B cell responses [6] to influenza, we asked whether this protein was necessary for protection in NP-vaccinated C57BL/6 mice. We found that protection of C57BL/6 mice from morbidity by vaccination with recombinant NP required the expression of CD40, and unexpectedly that this expression was specifically required on B lymphocytes.

Materials and Methods

Protein purification. The influenza PR8 NP gene was cloned into pTrcHis2C (Invitrogen), expressed in Top10F' *E. coli*, and affinity purified using the ProBond Purification System. Purified protein was dialyzed into PBS and sterile-filtered. SDS-PAGE analysis followed by Coomassie blue staining confirmed purity and anti-6Xhis tag western blot showed specificity.

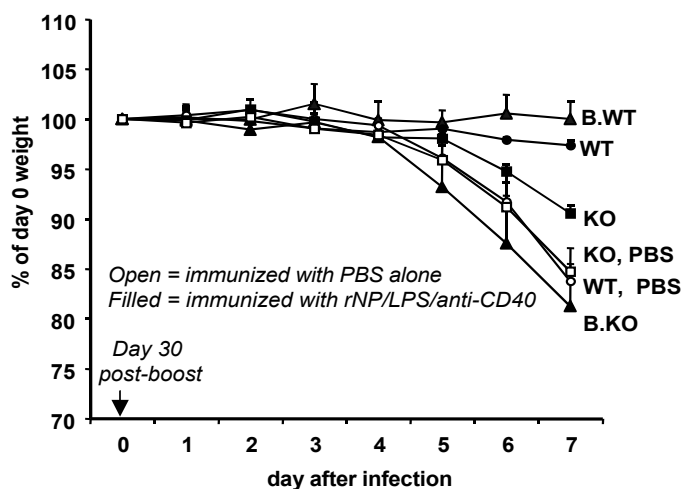
Immunization and challenge. μ MT mice were irradiated with 950-1000 Rad and reconstituted with 10^7 bone marrow (BM) cells as indicated. After 8 weeks, radiation chimeras, intact C57BL/6, or intact CD40^{-/-} mice were immunized intraperitoneally (i.p.) with combinations of 30 μ g rNP, 20 μ g LPS, and 50 μ g anti-CD40 (10C8). Immunized mice were challenged intranasally (i.n.) with 200-500 EIU of influenza A/PR8/34 in 100 μ l PBS 10-30 days post-boost. **Analysis.** Lung and spleen tissues were homogenized, RBC-lysed, and mesh-filtered. Recovered cells were treated with 2.4G2 FcR block, then stained with fluorochrome-conjugated anti-CD8, anti-CD62L, and D^b tetramers presenting either NP₃₆₆₋₃₇₄ or PA₂₂₄₋₂₃₃ (Trudeau Institute Molecular Biology Core Facility), and then analyzed with a FACSCalibur flow cytometer (Becton-

Dickinson). ELISA plates were coated with rNP and incubated with serially diluted serum samples. Bound antibody was detected with goat anti-mouse IgG^{HRP} (SBA).

Results

To determine the requirements for protection from influenza following NP vaccination, we first immunized C57BL/6 mice with recombinant nucleoprotein (rNP) followed by challenge with a sublethal dose of influenza A/PR8/34. Whereas mice immunized with PBS or with adjuvant alone (LPS \pm anti-CD40) lost ~15% of their initial body mass by one week after challenge, mice immunized with rNP/LPS \pm anti-CD40 lost \leq 5%. Similar results were seen when lethally irradiated mice were reconstituted with 100% C57BL/6 bone marrow (Figure 1, "WT"). Seven days after challenge, lung and spleen were analyzed by flow cytometry to measure antigen-specific CD8 T cell responses. At this timepoint, C57BL/6 mice vaccinated with LPS alone showed few if any detectable CD8 cells specific for the co-dominant NP₃₆₆ or PA₂₂₄ MHC class I-restricted epitopes (Figure 2, *open* symbols). However, mice previously immunized with rNP showed a variable but significant NP-specific CD8 response in the lung (Figure 2, *closed* symbols) as well as in the spleen (not shown). Therefore, systemic immunization with rNP can protect C57BL/6 mice from morbidity induced by sublethal influenza challenge, and this protection is associated with a more rapid NP-specific CD8 T cell response in the lung and spleen.

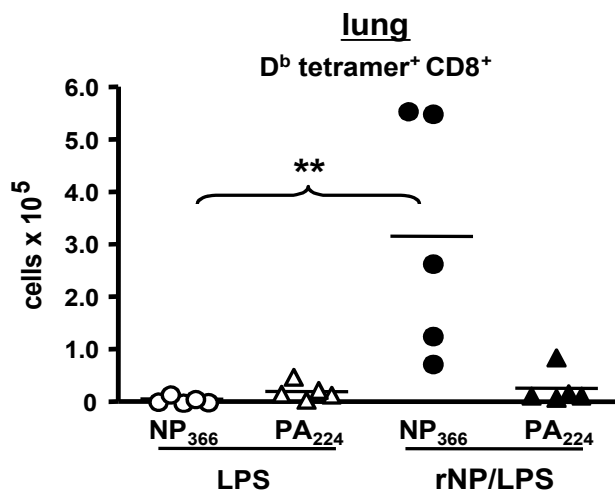
Figure 1. rNP-immune protection requires CD40 expression. Irradiated B cell-deficient (MT) mice were reconstituted with 100% C57BL/6 (WT), 100% CD40^{-/-} (KO) BM, 75% μ MT plus 25% C57BL/6 (B. MT), or with 75% μ MT plus 25% CD40^{-/-} BM (B.KO) Mice were immunized i.p. with rNP/LPS/anti-CD40 (filled symbols) or with PBS alone (open symbols) on days 0 and 10. On day 40, all mice were challenged i.n. with 500 EIU influenza PR8, and body weights were monitored daily.



Because optimal CD8 T cell responses to influenza require CD40 signaling [5], we asked whether this protein was necessary for protection following rNP vaccination. CD40^{-/-} mice or irradiated mice reconstituted with 100% CD40^{-/-} bone marrow

were each immunized with rNP/LPS. After PR8 challenge, the immunized CD40-deficient mice still lost 10-15% of their initial body weight by one week after infection (Figure 1, "KO" and not shown). Therefore, protection from morbidity by rNP vaccination requires the expression of CD40 on bone marrow-derived hematopoietic cells. CD40 is expressed on antigen-presenting cells such as dendritic cells, macrophages, and B lymphocytes, with B lymphocytes being the most numerous CD40⁺ cells. To determine which cells require CD40 to mediate protection in our system, we generated mixed chimeras, in which B cell-deficient μ MT mice were lethally irradiated, then reconstituted with a mixture of 75% μ MT bone marrow and 25% CD40^{-/-} bone marrow. After full reconstitution of the hematopoietic system, nearly all B cells were CD40-negative, whereas most non-B antigen-presenting cells, such as CD11c⁺ dendritic cells, expressed CD40 (confirmed by flow cytometry). When immunized with rNP and challenged with influenza PR8, these chimeric mice also lost 20% of their initial body weight (Figure 1, "B.KO"), in contrast to control chimeras reconstituted with 75% μ MT + 25% C57BL/6 bone marrow (Figure 1, "B.WT"). This observation suggests that CD40 expression is required specifically on B lymphocytes for rNP to protect C57BL/6 mice from morbidity induced by sub-lethal influenza challenge.

Figure 2. rNP -enhance CD8 T cell response after challenge infection. C57BL/6 mice were immunized i.p. on days 0 and 10 as indicated. On day 40, the mice were challenge with 500 EIU influenza PR8. Seven days post-infection (day 47) lungs were harvested for flow cytometry. **p 0.008 by Mann-Whitney U test.



Discussion

Collectively, our results demonstrate that rNP vaccination can protect C57BL/6 mice *via* a CD40-mediated mechanism. Because NP is an intraviral and intracellular protein, it has been inferred that protection after NP vaccination is likely mediated by NP-specific cytotoxic CD8 T cells exclusively [4]. In on-going experiments, we are analyzing the CD8 T cell response in our system more carefully and asking whether it too, is dependent on CD40 signaling. Although dendritic cells appear to be the most competent antigen-presenting cells for CD8 responses

involving influenza virus [7-9], we find that expression of CD40 on dendritic cells is insufficient for NP-immune protection, and instead CD40⁺ B cells are required. There are several possible and non-mutually exclusive explanations for this observation. One is that NP-specific B cells take-up rNP and cross-present its peptides to CD8 (or possibly cytotoxic CD4) T cells in a CD40-dependent manner. Secondly, CD40 signaling may help B cells generate high-affinity, isotype-switched antibodies against NP. In fact, we find that C57BL/6 mice immunized with rNP generate NP-specific serum IgG titers in excess of 10⁵, whereas the levels in rNP-immunized CD40^{-/-} mice are barely detectable. We are currently investigating whether these antibodies are necessary and sufficient for protection. It is unclear how such anti-NP antibodies, which would not be expected to neutralize or even bind to intact virions, could interfere with the influenza virus infection cycle. One possibility is that they form immune complexes with NP from lysed, infected epithelia early in infection. It has been previously shown that immune complexes are readily taken up by dendritic cells for efficient cross-presentation to CD8 T cells [10]. Either of these proposed CD40-dependent B cell responses may enhance cytotoxic T cell responses for early viral clearance and protection from morbidity. However, since a direct cause-and-effect relationship has not been shown for the NP-specific CD8 T cells induced in this system, we cannot rule-out the possibility of alternative mechanisms.

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Does Influenza Viral Population Change in a Patient Infected With Influenza?

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Introduction

New epidemic strains of influenza are always generated and seasonal influenza epidemics occur every year in the world because influenza viruses continually change genetically to evade host immunological attacks [1]. However, little is known about when and where influenza viruses change in the world. There are two ideas; a new strain comes into the human population in one place on the earth and spreads around the world from there or some new strains appear somewhere in the world at about the same time and spread from local areas all over the world. In both cases, the viral genetic variation must occur in a reservoir host infected with influenza virus. It is thought that an influenza virus isolated from an individual is genetically heterogeneous. The composition of the influenza viral population (quasispecies) at an early infection phase can differ from that at a later infection phase. Our objective for this study was to observe the changes of the influenza virus dominant population during a natural clinical course from a patient infected with influenza. Thus, we cloned and sequenced the RT-PCR products of the HA1 region of the HA gene in influenza virus isolates and compared the composition of the viral population between the early and late phase of infection in patients.

Materials and Methods

We collected nasal washes from patients with influenza-like illness at the early and late phase (from one to five days after onset of illness) of the clinical course at a pediatric clinic in Kadoma, Osaka, during the 2001-2004 influenza seasons. We inoculated specimens onto MDCK cells and harvested supernatants when CPE was observed. We identified isolates using the PAP staining technique with type and sub-type specific monoclonal antibodies to influenza viruses [2] or using the hemagglutination inhibition test with type specific polyclonal chicken sera (Denka-Seiken, Tokyo, Japan) [3]. Viral RNA from isolates was extracted with QIAamp Viral RNA Mini Kit (Qiagen Japan, Tokyo, Japan). We performed RT-PCR on the HA1 region of HA gene in H3N2 influenza virus by using Ready-to-Go RT-PCR Beads (GE Healthcare, UK) with specific primers (5'-CTATCATTGCTTTGAGCTAC-3' and 5'-GTTTCTCTGGTACATTCCGC-3'). The PCR products of 1,023 base pairs were cloned with TOPO TA-Cloning Kit for Sequencing (Invitrogen, California USA) or sent to a commercial laboratory (Takara-bio, Otsu, Japan) where each RT-PCR product was cloned and sequenced. We analyzed a 981 base pair nucleotide sequence of the HA1 gene with Genetyx software (Genetyx, Tokyo, Japan).

Results

Preliminary experiment. There is a chance to change the viral population by passage in MDCK cells. To confirm this possibility, compositions of viral populations among nasal washes, primary viral cultures in MDCK cells and secondary passage viral cultures in MDCK cells were compared. We undertook an investigation of two cases during the 2005-06 season. More than 45 clones of each PCR product of 6 specimens (two nasal washes, two primary viral cultures and two secondary viral cultures) were sequenced and analyzed. No changes of the dominant population among the three isolation procedures were observed in the two experiments (Table 1). Therefore, we used primary viral cultures as the first choice or secondary viral cultures if primary viral cultures were not available, to amplify the isolates instead of nasal washes to achieve our objective.

Table 1. Changes of the dominant population between nasal washes and viral cultures.

	Dominant population clone / total clones sequenced (%)		
	Nasal wash	Viral culture with MDCK cells	
		Primary	2 nd passage
Examination 1	36/45 (80.0)	44/54 (81.5)	42/48 (87.5)
Examination 2	48/52 (92.3)	38/51 (74.5)	44/47 (93.6)

Background of cases. Ten infant/adolescent cases were investigated. Backgrounds of all cases are described below: Case 1; a 3-month-old boy, sampling dates on 2 and 5 Feb. 2002, Case 2; a 3-year-old girl, sampling dates on 2 and 5 Feb. 2002, Case 3; a 5-month-old boy, sampling dates on 13 and 16 Feb. 2002, Case 4; a 1-year-old girl, sampling dates on 12 and 16 Feb. 2002, Case 5; a 1-month-old boy, sampling dates on 29 Mar. and 1 Apr. 2002, Case 6; a 6-year-old girl, sampling dates on 2 and 5 Feb. 2003, Case 7; a 4-year-old boy, sampling dates on 20 and 23 Jan. 2004, Case 8; a 2-year-old girl, sampling dates on 19 and 23 Feb. 2004, Case 9; a 4-year-old girl, sampling dates on 20 and 23 Feb. 2004, Case 10; a 4-year-old girl, sampling dates on 9 and 12 Feb. 2004. Case 1 and 2 were siblings. Additionally, a specimen from the father of Case 5, sampling dates on 1 Apr. 2002, was investigated, because he appeared to catch influenza from his baby. H3N2 influenza viruses were isolated from all of the specimens above. All cases completely recovered from the disease and there were no complications.

Viral population and nucleotide sequences of dominant population. Nucleotide sequenced clones from each isolate were classified into dominant population (a large number of identical clones), minor population (a small number of identical clones) and no cluster (a group of unique clones). The percentage of dominant populations in each isolate ranged from 54.5 to 90.0 percent (Table 2). At the early infection phase, all isolates consisted of one dominant population and some unique clones. At the late infection phase, all isolates had one dominant population, a few or no minor populations and no cluster clones. As far as we tested, the dominant population in isolates at early and late infection phases from individual

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patients demonstrated the same nucleotide sequences. However, the sequences of the dominant populations from all cases differed from one another except for case 1 and 2 (siblings) and case 5 and his father. As for the deduced amino acid sequences of the dominant populations, comparable results were obtained.

Table 2. The Percentage of the dominant population in each isolate from patients infected with influenza.

Case	Early phase		Late phase	
	Total number of sequenced clones	Dominant population clones (%)	Total number of sequenced clones	Dominant population clones (%)
1	58	45 (77.6)	46	29 (63.0)
2	51	40 (78.4)	60	44 (73.3)
3	57	39 (68.4)	52	35 (67.3)
4	45	35 (77.8)	50	34 (68.0)
5	45	30 (67.8)	55	30 (54.5)
6	20	17 (85.0)	20	14 (70.0)
7	20	14 (70.0)	20	14 (70.0)
8	20	18 (90.0)	20	16 (80.0)
9	20	13 (65.0)	20	13 (65.0)
10	20	17 (85.0)	20	15 (75.0)

Chronological analysis. The changes to the nucleotide and deduced amino acid sequences of the HA1 region of the HA gene of the dominant viral population occurred chronologically. Influenza isolates have been proceeding genetically like as on the natural course. Of the antigenic epitopes in HA of H3N2, the variations on site A and B were regularly occurred and accumulated (Table 3).

Table 3. Chronologically change of deduced amino acid in antigenic site of HA1 region of the dominant viral population.

Case	Sampling date	Site A		Site B						
		Position number	Position number	144	145	155	156	159	173	189
1	02-02	N	K	H	Q	Y	E	S		
2	02-02	N	K	H	Q	Y	E	S		
3	02-02	D	K	H	Q	Y	K	S		
4	03-02	D	K	H	Q	Y	K	S		
5	03-02	D	K	H	Q	Y	K	S		
6	02-03	N	K	T	H	Y	K	S		
7	01-04	N	K	T	H	Y	K	S		
8	02-04	D	K	T	H	F	K	N		
9	02-04	N	N	T	H	F	K	N		
10	02-04	N	K	T	H	F	K	N		

Discussion

The dominant population of influenza virus from an infected individual does not change significantly during the natural clinical course or during transmission, as was suggested from

those cases that were relatives, whereas isolated influenza viruses from each patient are not identical. Influenza viruses chronologically lined have been proceeding genetically. Our data show that influenza virus does not change as rapidly as one may predict. However, because influenza virus is believed to mutate in a human host, the data suggest that in this study we are likely to have missed the time and place of such change. Therefore, this study suggests that in order to significantly diverge genetically and to accumulate measurable variation, influenza viruses must go through numerous cycles of human-to-human transmission. Paradoxically speaking, influenza virus would, therefore, not evolve according to this paradigm if we were able to interrupt human-to-human transmission. In order to slow evolution of influenza, it is of utmost importance to prevent influenza infection and transmission, which from a public health standpoint indicates that prophylaxis may be more suitable than therapy for influenza control.

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A Vaccine Based on the M2 Ectodomain: Heterosubtypic Protection, Intranasal Delivery and Mechanism of Action

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Introduction

The continual antigenic variation of hemagglutinin (HA) and neuraminidase (NA) of human influenza viruses limits vaccine efficacy of currently licensed vaccines. A vaccine based on the ectodomain of matrix protein 2 (M2e) of influenza A viruses has received considerable attention because its high degree of sequence conservation allows the induction of intra- and heterosubtypic immunity [6]. The strong sequence conservation of M2e likely results from its genetic link with matrix protein 1 (M1), the most conserved influenza protein: the first 9 amino acid residues of M1 and M2 are identical and encoded by the same nucleotides. Amino acid residues 10-23 of M2 and 239-252 of M1 are also coded by the same nucleotides but in a different reading frame. Two M2e-consensus sequences can be discerned, a human-type (hM2e), and an avian-type (avM2e), differing at five positions. Of note, A/Brevig Mission/1/1918 virus and most isolates from H5N1-infected patients have an intermediate M2e sequence. The strong conservation of these two types and the limited changes allowed in the avM2e to hM2e adaptation are mainly due to constraints imposed by the overlapping M1 (matrix) gene. In serum from influenza A infected humans, ferrets and mice, M2-specific antibodies are detectable, sometimes only transiently, but remain much lower than those raised against HA and NA, even following multiple subsequent infections [1,3]. The apparent immunodominance of HA and NA over M2e can be explained by the difference in antigen size implying that there are more HA- or NA- than M2e-specific B cell precursors available in the vertebrate host. Currently licensed influenza vaccines aim at inducing neutralizing antibody responses, meaning the induction of anti-HA responses that prevent *in vitro* the interaction of HA with the sialic acid receptor present on cells. This type of antibodies is also present in convalescent sera and protects against a homologous challenge but not against drifted influenza strains. Our approach to develop a new influenza A vaccine has been entirely different: M2e was rendered immunogenic by creating a chimeric virus-like particle (VLP) comprised of M2e-epitopes densely arrayed on the surface of a hepatitis B core VLP. This M2e-VLP proved to be an effective vaccine in a lethal influenza A challenge model [6]. Protection was dependent on anti-M2e antibodies, was long lasting (at least 26 weeks in BALB/c mice), reduced virus replication in the mouse lungs and the M2e-VLP

vaccine also provided protection when administered intranasally in mice. We improved the initial format of the M2e-VLP vaccine by increasing particle stability and M2e immunogenicity and improved protection provided by intranasal administration. In the mouse model we demonstrate that pre-existing HBC antibodies do not interfere with M2e-VLP immunogenicity or protection. Finally we show that anti-M2e antibody-mediated protection is dependent on host immune cell functions.

Materials and Methods

Pathogen-free, female BALB/c mice were obtained from Harlan (The Netherlands). Fc receptor gamma (FcR γ) deficient mice with a BALB/c genetic background were purchased from Taconic (Germantown, USA). The animals were housed in a specific-pathogen free, temperature-controlled environment with 12 h light/dark cycles; food and water were delivered *ad libitum*. Mice were adapted for 1 week in the animal room and immunized at 8 weeks of age. All experiments were authorized by the institutional ethical committee on experimental animals. The M2e-vaccine used contains three tandem copies of M2e genetically fused at the amino-terminus of HBC. Vaccination and follow up details are described in [2] the legend of table 1. Recombinant detoxified heat-labile toxin LTR192G was kindly provided by Dr. John Clements (Tulane University Medical Center, New Orleans, LA) and used at 1 microgram/mouse per vaccination. For challenge experiments, PR8, WSN, X47 and A/swine/Belgium/1998 (the latter kindly provided by Dr. Kristien Van Reeth, Ghent University, Belgium) were mouse adapted and subsequently grown and purified from embryonated chicken eggs or MDCK cells.

Results

We have further exploited the mouse model to obtain an optimized M2e-VLP prototype vaccine for human use. We first focused on new constructs to enhance anti-M2e serum antibody responses, with or without adjuvants that are suitable for human use, and aimed to identify the antibody subtypes that correlate best with protection. From this study we concluded that M2e-VLPs with three tandem repeats of M2e-fused at the N-terminus of HBC induced approximately 10-fold higher anti-M2e serum IgG responses in mice as measured by a peptide-based ELISA [2]. Adsorption of the particles on alhydrogel improved protection in mice compared to unadjuvanted M2e-vaccine and protection correlated with a balanced M2e-specific IgG1/IgG2a response. Although there are precedents for the use of HBC-VLP based vaccines in clinical trials [5] there is some concern that pre-existing immunity in chronic HBV patients could affect the immunogenicity of this particular carrier system. To address this, we compared the immune response and protective efficacy in BALB/c mice, which were primed with either PBS or 10 microgram carrier HBC particles, adsorbed on alhydrogel and injected via the intraperitoneal route. Subsequently mice received either 10 microgram of empty HBC or M2e-HBC vaccine again adjuvanted with alhydrogel. Serum samples isolated three weeks after each

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immunization were analyzed for anti-M2e peptide responses in ELISA. Three weeks after the last immunization mice were challenged with 0.4 LD₅₀ of mouse adapted X47 virus and lung virus titers were determined. As summarized in Table 1, priming of mice with HBc was associated with a slight increase of the immunogenicity of subsequently administered M2e-HBc. In addition, lung virus titers at day 6 after challenge were lowest in the HBc-primed group. These results suggest that pre-existing antibodies have no negative impact on the M2e-HBc vaccine but rather may slightly enhance the immune response.

Table 1. Pre-existing anti-HBc antibodies do not impair M2e-VLP vaccine efficacy.

1 st immunization	2 nd immunization	3 rd immunization	Anti-M2e IgG2a ^b	Lung virus titer ^c
HBc	HBc	HBc	< 100	3.6 x 10 ⁷
PBS	M2e-HBc	M2e-HBc	24.300	5.3 x 10 ⁵
HBc	M2e-HBc	M2e-HBc	218.700	1.6 x 10 ⁵

^a Groups of 10 mice (female, BALB/c, 8 weeks old) were primed by intraperitoneal injection of PBS or 10 microgram HBc adjuvanted with alhydrogel. 3 and 6 weeks later mice were immunized intraperitoneally with 10 microgram of HBc or M2e-HBc particles containing three tandem repeat copies of M2e. ^b Serum samples were prepared 2 weeks after the third immunization and the M2e-specific serum IgG2a endpoint titer in serum pooled from each group was determined in an M2e peptide ELISA. ^c Three weeks after the last immunization, mice were challenged with 0.4 LD₅₀ of the X47 virus. Virus titers were determined by titration on MDCK cells of lung extracts prepared from mice sacrificed at 6 days post challenge and are expressed as TCID₅₀/ml of lung extract.

The universality of protection was assessed by challenging M2e-VLP immunized BALB/c mice with different mouse adapted strains including PR8, WSN and Swine/Belgium/1998 (all H1N1). Although the latter virus carries an avian type M2e, all M2e-immunized mice survived a 4 LD₅₀ challenge that proved lethal to control animals (data not shown). We have tested and improved intranasal M2e-VLP vaccine delivery [2,6]. Admixing recombinant detoxified heat-labile toxin LTR192G with M2e-VLP vaccine significantly reduced weight loss compared to the unadjuvanted group. In a follow-up study we have focused on the use of the rationally designed mucosal adjuvant CTA1-DD, which is composed of the enzymatically active A1 domain of Cholera toxin fused to two tandem copies of the immunoglobulin-binding domain of *Staphylococcus aureus* protein A. The latter part of this potent non-toxic mucosal adjuvant acts as a targeting domain for immune cells and improves mucosal vaccine responses [4]. M2e-VLPs coadministered with CTA1-DD in the mouse nasal cavity induced strong systemic anti-M2e IgG1, IgG2a and IgA responses and protected mice from lethal challenge. IgG antibodies against M2e are needed for protection by an M2e-based vaccine as was shown by passive serum transfer experiments [6]. Antisera raised by M2e-VLP vaccination strongly bind to influenza virus infected cells, which most likely are also the *in vivo* target cells. Furthermore, we have noticed a consistent correlation between the presence of both M2e-specific serum IgG1 and IgG2a and strong protection by M2e-VLP vaccination. Anti-M2e antibodies do not block virus replication *in vitro* and passive transfer of the antibodies failed to provide protection in FcRγ knock out

animals, indicating that an antibody-dependent cell mediated effector mechanism is responsible for protection *in vivo* (data not shown).

Discussion

Others and we have demonstrated that a vaccine based on the conserved M2e can provide strong protection against challenge with influenza A strains in mice and ferrets. Both parenteral and intranasal administration are effective delivery routes of this novel vaccine and protection is clearly dependent on antibodies although a minor contribution from M2e-specific T cells can not be excluded. Vaccination with M2e-VLPs protected against a lethal challenge with a virus strain containing an avian type M2-protein (A/Swine/Belgium/1998), in accordance with the finding that hM2e-immune serum specifically binds to a large panel of avM2e-sequences. In preparation of a Phase 1 clinical trial with M2e-HBc we have obtained a stabilized M2e-VLP that induced stronger anti-M2e responses concomitantly with lower anti-HBc carrier IgG responses, resulting in better protection in the mouse model compared to our original construct [2]. When mice were primed by vaccination with HBc particles to mimic the presence of pre-existing anti-HBc antibodies in HBc seropositive individuals, we noticed a slight enhancement of anti-M2e antibodies induced by subsequent immunization with M2e-VLPs compared to unprimed mice. The latter observation could be explained by immune complex formation, which is known to stimulate antigen presentation by dendritic cells [7]. Influenza enters the human host through the mucosa of the upper respiratory tract. Influenza-specific adaptive immunity, initially induced locally starting from this viral entry region is expected to be advantageous to prevent viral infection and spread. In our hands, the best protection observed with intranasally administered M2e-VLPs was obtained with CTA1-DD adjuvanted particles. The rational design of CTA1-DD explains its lack of toxicity but clinical testing is needed to evaluate its efficacy in humans. Having identified a role for Fc Receptors in immune protection, we are now focusing on the immune cells involved in protection and on identifying the most effective antibody isotypes for this universal vaccine. Clinical trials will reveal the future potential of an M2e-based "universal" influenza A vaccine.

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Latex Particles Coated With Sialic Acid-Containing Glycoprotein Are Agglutinated By Influenza Viruses

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Introduction

Influenza virus is a cause of respiratory illness which is a major public health problem worldwide. Although the term "influenza pandemics" has greater impact, over the long term the smaller influenza epidemics that occur each winter between "global pandemics" are responsible for greater overall mortality. To give immunization to large human population is the best protection against influenza outbreaks. Each year a new influenza vaccine is produced based on WHO recommendations. Therefore, prediction of the viral strains which will spread in the next season is critical for recommendations. Antigenic differences between isolates can be detected using ferret antisera and RBCs for analysis of cross reactivity in HI tests. However, the titers of HA tests depend on the species of RBCs used for the tests. In this study, we made the artificial particles which are able to be preserved for a long time, and examined whether they were able to be used instead of RBCs for HA and HI test.

Materials and Methods

Viruses. Influenza viruses used in this study were isolated from clinical specimens using MDCK cells or from avian feces using embryonated hen's eggs of our institute according to standard procedure. These isolates were then propagated using the same method as for isolation and stored at -80°C until use. We also used the inactivated strains for reference antigens provided by the National Institute of Infectious Diseases, Tokyo, Japan and A/turkey/Ontario/7732/66(H5N9) and A/duck/Hongkong/342/78(H5N2) avian influenza live strains stocked and propagated in our institute.

Buffers and reagents. LSTc and BSA (98-99%, fraction V) were obtained from Sigma-Aldrich Co, St. Louis, MO, USA. LSTa was obtained from Dextra laboratories, Ltd, Whiteknights Road, Reading, UK. MES, NHS, EDAC were also obtained from Sigma-Aldrich Co. CML particles were obtained from Polysciences, Inc, Warrington, PA, UK.

Sialylated glycoconjugates. The synthesis of the sialic acid containing glycoconjugates was according to the literature [1]. Briefly, 10.4 mg of LSTc and 10.7 mg of bovine serum albumin (98-99%, fraction V) were dissolved in 1.5 ml of 0.2M borate buffer pH8.7 [2]. The solution was stirred for 1h at 55°C , then 26 mg of NaBH_3CN (WAKO Chemical Co, Japan) was added, and the mixture was stirred for 24 hours at 55°C . The solution was dialyzed against distilled water and lyophilized to give 13.2mg of a white powder. The same procedure was applied for the preparation of linking with LSTa and BSA.

Coupling glycoprotein to microparticles. Covalent coupling procedure of glycoprotein to micro particles was guided by the maker's instruction book. In detail, 160 μl solution of 2.5% CML particles with 1.0 μm diameter, 40 μl of 500mM MES buffer, 92 μl of 50mg/ml NHS, 16 μl of 10mg/ml EDAC and 92 μl of distilled water were pipetted into a microcentrifuge tube, and incubated at room temperature on a mixing wheel for 30 minutes. The tube was centrifuged and supernatant was discarded, then particles were resuspended with 1ml of 50mM MES buffer, pH6.1. After repetition of centrifugation and removal of supernatant, the pellet was resuspended with 20 μl of 500mM MES buffer, 80 μl of glycoprotein stock (1mg/ml of 0.3M sodium phosphate buffer pH 8.7) and 100 μl of DW. The mixture was incubated at room temperature on a mixing wheel for 1 hour. The tube was centrifuged and microparticles were washed 2 times with 50mM MES to remove free glycoprotein. The glycoprotein coupled microparticles were suspended in the 0.1M glycine-NaOH buffer (pH 8.1) containing 0.14M sodium chloride and 0.1% BSA to make a final concentration of 0.05%.

HA, HI assay and PA, PI assay. All of the HA, HI assay and PA, PI assays were performed using 96-well microtiter plates with V-shape bottoms. The HA titration and the HI test were guided by the recommended protocol of WHO collaborating centre for influenza. The PA and PI assay were performed in the same way as the HA and HI. Erythrocyte suspension obtained from human (blood type O) or chicken were stored in Alsever's reagent at 4°C until use. 0.7% human erythrocytes suspension or 0.5% chicken erythrocytes suspension was used for the HA and the HI assay. The reactions of the HA and the PA titration were done at 4°C for 16 hours. Ferret post-infection antisera panels against each reference antigen were also provided by the National Institute of Infectious Diseases, Tokyo, Japan. Ferret antisera were reconstituted and treated with RDE at 37°C over night. The next day, the sera were heated at 56°C for 60 minutes and treated with packed RBC to remove of nonspecific agglutinins.

Neuraminidase treatment of microparticles. -2-(3,6,8,9)-Neuraminidase from *Arthrobacter ureafaciens* and reaction buffer were obtained from Sigma. Total 0.1ml of reaction mixture containing 4% (V/V) of washed LSTc-BSA-CML in reaction buffer with 2.5mIU of neuraminidase was incubated at 37°C for 3 hours. Neuraminidase treated particles were washed with 50mM MES buffer and pelleted by centrifugation. The microparticles were suspended in the 0.1M glycine-NaOH buffer (pH8.1) containing 0.14M sodium chloride and 0.1% BSA to make a final concentration of 0.05%.

Reading of the plates. Confirmation of the PA results was according to the previous report [3]. Briefly, the results were obtained by inclining the plates for 30 seconds. At the end of this time, if a layer or button of latex on the well bottom did not flow and no line was seen, it was judged as positive. If a discreet button of latex flowed and a line was seen, it was judged as negative.

Results

Comparison between HA and PA titers. The latex particles linking with LSTc-BSA showed comparable agglutination titers to HA titers when using H1 subtype, most of H3 subtype and B type influenza viruses. In contrast, the PA result of two H3 subtype isolates named A/Osaka/01/2004 and A/Osaka/18/2005 was very low (at<8). (Table 1) As the negative control, BSA-linked latex were not agglutinated by any influenza viruses. The latex particles linking with LSTc-BSA after the treatment by $\alpha 2$ -(3,6,8,9) neuraminidase at 37°C for 3 hours were not agglutinated by existence of any reference antigen. (data not shown).

Table 1. Comparison between the HA titers and the particles coated with LSTc-BSA agglutination titers used filed isolates.

Strain	HA titer*1	PA titer*2
A/Moscow/13/98(H1N1)	16	16
A/New Caledonia/20/99(H1N1)	64	64
A/Panama/2007/99(H3N2)	64	32
A/Wyoming/03/2003(H3N2)	64	64
B/Shandong/7/97(Victoria)	128	128
B/Johannesburg/5/99(Yamagata)	64	128
A/Osaka/374/2001(H1)	16	16
A/Osaka/585/2001(H1)	32	32
A/Osaka/868/2001(H1)	64	32
A/Kadoma/35/2002(H1)	128	32
A/Kadoma/51/2002(H1)	64	64
A/Kadoma/52/2002(H1)	64	64
A/Kadoma/54/2002(H1)	64	64
A/Osaka/01/2004(H3)	16	<8
A/Osaka/30/2004(H3)	16	8
A/Osaka/32/2004(H3)	64	16
A/Osaka/01/2005(H3)	16	8
A/Osaka/06/2005(H3)	128	64
A/Osaka/11/2005(H3)	128	128
A/Osaka/18/2005(H3)	64	<8
B/Osaka/01/2004(Yamagata)	32	32
B/Osaka/02/2004(Yamagata)	16	16
B/Osaka/03/2004(Yamagata)	32	64
B/Osaka/09/2005(Yamagata)	32	128
B/Osaka/10/2005(Yamagata)	64	64
B/Osaka/11/2005(Yamagata)	64	32
B/Osaka/13/2005(Yamagata)	32	64

*1 0.7% human typeO RBCs were used for hemagglutination assay

*2 0.05% particles were used for particle agglutination assay

Comparison between HI and PI titers. In case of A/Moscow/13/98 antiserum, the PI titers were ≤ 10 in all clinical isolates. In case of A/New Caledonia /20/99 antiserum, the PI titers were lower than HI titers when we tested the clinical isolates of H1 subtype. On the other hand, for type B influenza viruses, both HI and PI tests showed no cross-reactivity against B/Shandong/7/97 antiserum and very high titer against B/Johannesburg /5/99 antiserum. For H3 subtype isolates, the PI titers tend to be higher than HI titers against two distinct antiserum.

Comparison between HA and PA titers using avian influenza viruses. All of the avian influenza viruses agglutinated chicken erythrocytes as well as human erythrocytes. PA results using LSTa-BSA-CML show similar results to HA except for A/turkey/Ontario/7732/66 and A/duck/HongKong/342/78. (Table 2) Although their PA results for all avian viruses except the two mentioned above have shown slightly lower agglutination

titer, using LSTc-BSA-CML, the titer remained within a 2-fold difference compared with LSTa-BSA-CML results. Only the A/turkey/Ontario/7732/66 virus showed very high PA titer, when used with LSTa-BSA-CML which has just Neu5Aca2-3Gal sequence. This result suggests A/turkey/Ontario/7732/66 virus recognizes strongly Neu5Aca2-3Gal sequence on the cell surface glycoprotein. On the other hand, avian influenza viruses can bind LSTc-BSA-latex which has only Neu5Aca2-6Gal sequence.

Table 2. Comparison between the HA titers and the PA titers using the same avian influenza strains.

Viral strain	Hemagglutination titres*1		Particle agglutination titres*2	
	Chicken RBC	Human-O RBC	LSTa-BSA-CML	LSTc-BSA-CML
A/teal/Osaka/1/2002(H10N1)	256	256	256	128
A/teal/Osaka/2/2002(H10N1)	>256	256	256	128
A/teal/Osaka/3/2002(H10N1)	>256	>256	>256	>256
A/teal/Osaka/4/2002(H10N7)	>256	256	128	128
A/teal/Osaka/5/2002(H10N7)	>256	>256	256	256
A/wigeon/Osaka/1/2002(H7N7)	128	128	64	32
A/turkey/Ontario/7732/66(H5N9)	256	256	≥ 1024	32
A/duck/Hongkong/342/78(H5N2)	64	64	128	16

Maximum dilution of virus capable of agglutinating red blood cells or particles coated with sialic acid-containing glycoprotein.

Discussion

In this study, we produced latex particles(called LSTc-BSA CML) that were agglutinated by influenza viruses and are stable over long periods. The particles could be used successfully in agglutination inhibition assay and for the most part the PA Titers were comparable to the HA titers. There are many sialyloligosaccharides of various structures and lengths on the RBCs surfaces. The difference in the titers between HI and PI in clinical isolates might be due to structural differences or density differences of the sugar chain on the surface between the RBCs and the particles. Regarding as the two H3 strains, A/Osaka/01/2004 and A/Osaka/18/2005, the results of HA were not parallel to PA. We tested agglutination titers of these two strain with chicken erythrocytes or latex particles linking with LSTa-BSA that has Neu5Aca2-3Gal linkage on sialyloligosaccharides on their surface. Those two strains could bind chicken RBCs. The PA titer of A/Osaka/18/2005 was 32 PA units for LSTa-BSA-latex. So these two viruses may recognize the Neu5Aca2-3Gal linkage rather than the NeuAca2-6Gal sequence which is on the LSTc. Our results suggest that the latex particles LSTc-BSA CML can bind to influenza viruses and their binding is inhibited specifically by antigen specific antibody. The comparable sensitivity and specificity of PI to HI and its stability on storage are factors in favor of using LSTc-BSA CML to determine antigenicity of influenza viruses in a survey of epidemic strains. We also investigated whether influenza viruses isolated from avian feces bind NeuAca2-6linkage on sialyloligosaccharides of LSTc-BSA-CML as well as NeuAca2-3linkage which is known as a component of natural receptor. All five H10 subtype strains and a H7N7 strain showed similar agglutination pattern by LSTa-BSA-CML (which has only NeuAca2-3Gal sequences) and

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LSTc-BSA-CML (which has only NeuAc α 2-6Gal sequences). Our data suggest that the receptor specificity of these avian influenza viruses did not differ. So the restriction of their ability to infect and replicate efficiently in mammalian cells may be placed on another point such as receptor affinity or extracellular inhibitors in the respiratory tract. These particles may be useful for investigation about receptor and viral ligand interactions.

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The Application of Environmental Modeling to the Selection of Avian Influenza Study Locations and Sentinel Flocks

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The Bioagent Transport and Environmental Modeling System (BioTEMS™) is a robust modeling system capable of integrating biological factors, such as agent survival and host and vector population dynamics, with abiotic factors such as temperature, elevation, and soil type to predict where pathogens introduced or endemic to an area are likely to survive or amplify. This model has applications to surveillance for many pathogens, including influenza. There has, for the past several years, been heightened monitoring of the spread of the H5N1 strains of influenza in Asia, Europe and Africa. There is, however, the known potential for viral genes from Eurasia to reach North America as clearly demonstrated by the isolation in the Eastern US of AI virus that carries matrix genes closely related to those of Eurasian AI viruses. Isolates since 1986 include: A/Red knot/Delaware/2552/87 H9N5; A/Ruddy Turnstone/Delaware/34/93 H2N1; A/Ruddy Turnstone/Delaware/2589/87 H11N4; A/Laughing gull/New Jersey/798/86 H2N7; A/Sanderling/New Jersey/766/86 H2N7; A/Herring gull/Delaware/471/86 H13N7 (Widjaja, et.al. 2004). Eurasian virus and virus genes can reach the Americas through a number of routes, the most likely of which is through Alaska. However, less likely routes are also possible and monitoring of locations along additional flyways, e.g. the eastern flyway of North America, has merit. Monitoring efforts become more feasible if resources can be focused on sites likely to yield representative isolates of circulating virus strains. Models can be used as a tool for identifying optimal sites where limited resources can be applied most effectively to the effort of sampling wild birds, the environment, or placement of sentinel flocks. BioTEMS™ is a proprietary, unique biology-based system for evaluating the threat posed by the introduction and spread of pathogens. BioTEMS technology uses abiotic and biotic variables to rapidly identify bioagent dispersal, environmental spread and amplification. A method to quickly and economically identify optimal surveillance sites for AI virus was developed using an Avian Influenza Surveillance Index (AISI) and geographic information systems (GIS). The AISI is composed of habitat variables and waterfowl species diversity information. AISI values for selected sites were developed from direct observation of habitat and birds. Habitat (estuary, fresh water or terrestrial) was scored on a scale of 1-4 in accordance with the method used in the US Interagency Strategic Plan, and the waterfowl species diversity and density was scored in accord with the Shannon-Weiner Index of Species Diversity. The AISI for each site is linked to the geographic coordinates to produce information layers

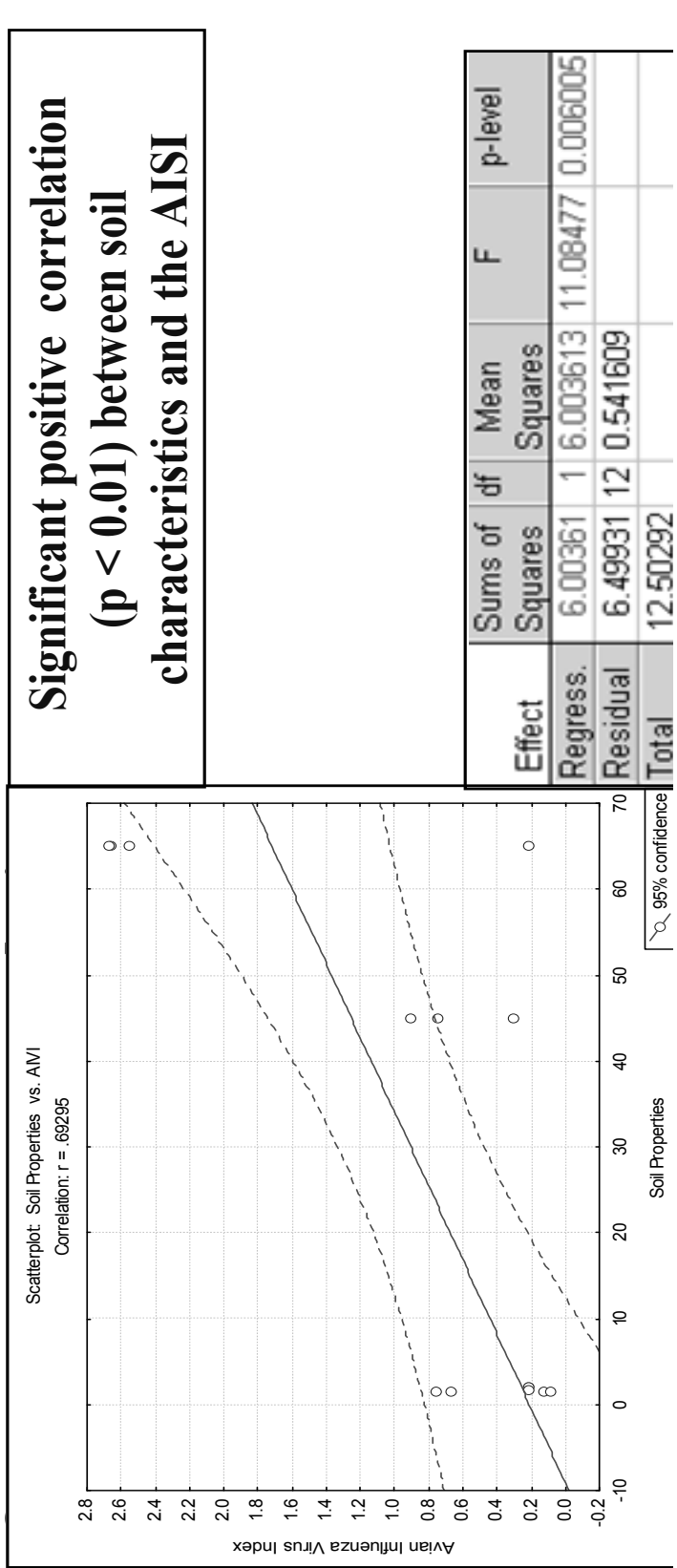
using geographic information system (GIS) software (ArcMap, ESRI, Inc.). Preliminary results indicate that the BioTEMS AISI can be used to optimize surveillance efforts for sentinel flocks and identify sampling sites for the isolation of virus from wild waterfowl and the environment. The AISI values provide an objective score for potential surveillance sites. In evaluating the initial AISI values for Edgewood, an apparent correlation of the AISI value with soil characteristics was noted. The ability of soil properties to predict the AISI for a new set of sites at Aberdeen, MD, was evaluated by using GIS to develop a soil property index from soil characteristics provided by the US Geological Survey (USGS) for the same sites to which AISI values were assigned. Site-to-site variation was observed for the AISI calculated for selected locations at Edgewood and Aberdeen, MD (Figure 1) and the soil property index developed from USGS data sites was found to correlate with the observed AISI (Figure 2).

Figure 1. AISI (AIVI) values for selected sites at Aberdeen, MD.



In summary, the BioTEMS model produces a quantitative index that can be evaluated for utility against observed data. The BioTEMS Avian Influenza Surveillance Application has been demonstrated to correlate with a USGS-based soil property index. Further environmental sampling and deployment of sentinel flocks is now warranted to test and validate the model over a wide geographic range.

Figure 2. Correlation of Soil Property Index with AISI.



**Significant positive correlation
($p < 0.01$) between soil
characteristics and the AISI**

Effect	Sums of Squares	df	Mean Squares	F	p-level
Regress.	6.00361	1	6.003613	11.08477	0.006005
Residual	6.49931	12	0.541609		
Total	12.50292				

Diclofenac Augments Intracellular Influenza Virus Replication

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Influenza virus infection in children treated with nonsteroidal anti-inflammatory antipyretics, such as aspirin and diclofenac sodium (DFS), sometimes cause severe neurological complications including brain edema and is diagnosed as Reye's syndrome or influenza-associated encephalopathy. However, the etiology is little known to date. In the present study, we analyzed the effects of DFS on influenza virus replication *in vivo* and *in vitro*. We found that a clinical dose of DFS (2 mg/kg) significantly increased the mortality of newborn mice infected with influenza virus A/Puerto Rico/8/34 H1N1. Under these conditions, the lungs of mice treated with DFS exhibited higher virus genome numbers, viral protein expressions, virus titers, cytokine expressions (IL-6 and TNF- α) and active caspase-3 in comparison with the data of mice without DFS treatment. With the *in vitro* system, we also found higher virus-genome numbers and viral protein expressions in infected MDCK and A549 cells treated with DFS at doses of up to 105 μ M in comparison to the data without treatment. Furthermore, we found that DFS markedly reduced virus release from the infected cells, resulting in the reduction of virus titers in the extracellular milieu. These results suggest that accumulation of influenza virus in the cells by DFS induces disorder of cell functions and apoptosis.

Introduction

IAV is one of the most common infectious pathogens in humans [1] and causes considerable morbidity and mortality in infants and aged individuals. There is a rare but often fatal encephalopathy due to infection by IAV of children in IAE [2,3] and RS [4,5] treated with aspirin and DFS, nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs are widely used for the alleviation of pain, fever and inflammation, but they have several side effects, such as gastrointestinal damage and platelet dysfunction, and can cause convulsions when co-administered with quinolone-derivative antibacterial drugs [6-8]. Although the mechanisms of these side effects have been studied, little is known about those of side effects of NSAIDs on the central nervous system and brain edema after IAV infection. In the present report, we analyzed the effects of DFS on IAV replication *in vitro* and *in vivo*.

Materials and Methods

Cells and virus. Madin-Darby canine kidney (MDCK) and human lung A549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. A/Puerto Rico/8/34 H1N1 virus (PR8) was used for infection.

Mice and virus infection. Ketamine-anaesthetized 9-day-old C57BL/6 mice were inoculated intranasally with various doses of PR8 in 6 μ l of saline. One hour after inoculation, mice received subcutaneous injections of either DFS (2 mg/kg) or vehicle (saline), and the treatments were repeated every 12 hours for the next 10 days. Uninfected control groups also received DFS or vehicle. Mice were monitored every 12 hours for weight and survival. **Virus titration.** Two groups of mice infected with 30 PFU of PR8 were treated with DFS or vehicle and then sacrificed indicated days after infection. Each lung was removed, weighed and homogenized in saline. The homogenates were centrifuged to remove cell debris and the supernatants were stored at -80°C until assay. Samples were 10-fold serially diluted and titrated by plaque assay in MDCK cells as described previously [9].

RT-PCR and quantification of viral RNAs. Total RNA from the lungs of infected mice and infected cells was prepared using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions and reverse transcribed with M-MLV reverse transcriptase (Promega) as described [10]. The cDNAs for M1 viral (v) RNA and complementary and/or messenger (c/m) RNA were synthesized using sense and antisense primers 5'-agtcttctaaccgaggtcgaa and 5'-cttgaaccgttgcatctgac, respectively. The β -actin was used as internal control of cellular RNA and cDNA for β -actin mRNA was synthesized using an antisense primer 5'-tgtaacgcaactaagtcataagtc. Quantitative PCR reactions were performed using the SYBR Green PCR kit (Qiagen) with an ABI 7300 system. The primer sequences used for detection of viral M1 RNA were 5'-tctatcatcccgcaggcccc and 5'-aaagcgtctacgctgcagtcc. The primer sequences used for detection of β -actin mRNA were 5'-ggctacagcttcaccaccac and 5'-tagctcttccaggaggga.

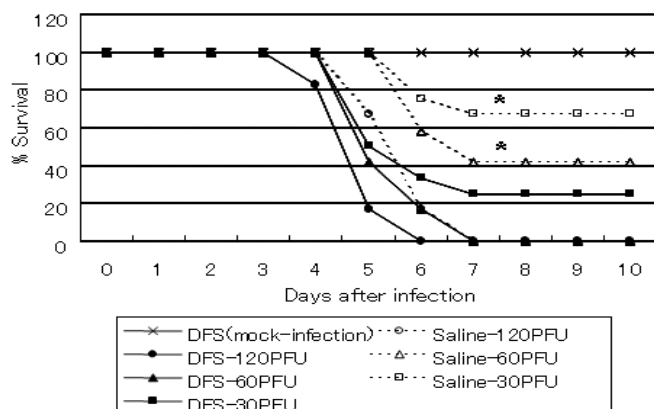
Statistical analysis. Statistical significance was determined from the means using the unpaired *t*-test for comparisons between the infected or stimulated groups and the control groups using the StatView 4.0 software. All values are expressed as mean \pm SD. A *p* value <0.05 was considered statistically significant.

Results

A clinical dose of DFS increases mortality of IAV-infected newborn/suckling mice. We analyzed the effect of DFS on the newborn/suckling mice after IAV infection. DFS at a clinical dose (2 mg/kg) was injected subcutaneously shortly after virus infection and the injection was repeated every 12 hours for 10 days. Although DFS itself did not affect growth and weight of mice, it significantly increased the mortality of IAV-infected newborn/suckling mice (Figure 1).

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Figure 1. DFS increases mortality of IAV-infected newborn mice. Mice were infected intranasally with 120, 60 or 30 PFU of PR8. DFS or vehicle was administered subcutaneously 1 h after virus inoculation and every 12 hours for 10 days. Mortality was recorded every 12 hours. Results are expressed as percentage of survival. n=12 per group, *P<0.05 (vs saline).



DFS increases virus replication in the infected lungs. To analyze the mechanisms of an increased mortality of infected mice treated with DFS, we measured virus titers in the lungs of infected mice. Two groups of mice treated with DFS and vehicle were infected with 30 PFU of PR8 and virus titers in each group (8 mice) were analyzed at indicated days after infection. Figure 2 shows that virus titers in the lungs of DFS-treated mice significantly increased after infection for 3-6 days. In addition, we detected an increase in the viral M1 protein in the lungs of DFS-treated mice (data not shown). The effect of DFS on the replication of viral genes was also analyzed. We found that M1 vRNA significantly increased in the infected lungs of DFS-treated mice after infection for 3 days. Since cytokines activate host antiviral defense system upon infection, we measured the levels of proinflammatory cytokines, IL-6 and TNF- α , in the lungs of mice before and after IAV infection. We found that the level of IL-6 and TNF- α were increased in the infected lungs treated with DFS after infection for 3 days (data not shown). As it has been demonstrated that an early increase in the levels of IL-6 and TNF- α in the lung homogenates is associated with the severity of lung pathology in the IAV-infected mice, we examined the effect of DFS on the levels of active caspase-3 in the lungs of mice. We found that the levels of active caspase-3 were increased in the infected lungs treated with DFS. These results suggest that DFS augments the viral multiplication in the infected organs and cytokine expressions, leading to exacerbation of virus infection.

Figure 2. DFS increases virus titer in the lungs of infected mice. DFS and vehicle were administered to two groups of mice infected with 30 PFU of PR8 virus and viral titers in the lungs were determined by plaque assay. *P<0.05 (vs saline).



DFS augments virus replication and inhibits virus release from infected cells. To confirm the augmentation by DFS of virus replication *in vitro*, we assessed the effect of DFS on virus replication in MDCK and A549 cells. These cells were pre-treated with various concentrations of DFS (30-105 μ M) for 14 hours, infected with 0.1 moi of PR8 virus, and then cultured for 7 hours in the absence of DFS. Intracellular viral M1 protein levels were determined by Western blotting and densitometric analysis revealed an increase in the expression of M1 in the cells treated with 30-105 μ M DFS. The quantitative RT-PCR was also performed using primers specific for the viral M1 gene. The results showed the increase in the expression of both M1 vRNA and c/mRNA in the cells treated with DFS. Under the conditions, virus titer in the medium was also increased along with an increase in the intracellular M1 protein expression. These cells pre-treated with various concentrations of DFS (30-105 μ M) for 14 hours and infected with 0.1 moi of PR8 virus were also analyzed in the presence of DFS for 8 hours. Under these conditions, virus yields in the medium were significantly and dose-dependently inhibited by DFS, although intracellular levels of virus genes and M1 protein were similar to those of the cells cultured in the absence of DFS after virus infection. In these experiments, virus titer in the medium was markedly reduced to 10% of levels reached with control cells without treatment of DFS after infection. These results indicate that pre-treatment (?) of cells with DFS augments intracellular virus replication and suppresses virus release in the presence of DFS in culture medium after infection.

Discussion

In the present study, we found that mice treated with DFS exhibited higher virus genome numbers, viral protein expressions, virus titers, cytokine expressions (IL-6 and TNF- α) and active caspase-3 in the lungs, compared to those of mice without DFS treatment, resulting in an enhancement of mortality by DFS. With the *in vitro* system, we found a suppressive effect of DFS on virus titers in the extracellular milieu. The suppression of virus titers in the medium was detected only in the cells treated with DFS before and after virus infection. Since DFS has a short elimination half-life (about

4 hours) *in vivo*, the suppression of virus release by DFS may not be evident *in vivo*. Although the mechanisms of neurological complications by DFS in IAE and RS have not been clarified, our results suggest that DFS increases virus-genome numbers and viral protein expressions in infected cells and induce higher inflammatory cytokine expressions and active caspase-3 than those of the cells without DFS treatment, resulting in an increase in apoptotic cells and tissue/cell damage.

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Influenza Awareness in The Asia-Pacific Region: The Role Of The Asia-Pacific Advisory Committee On Influenza (APACI)

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The APACI was established in early 2002 to promote influenza awareness and highlight the poorly understood impact of the disease in Asia. The Committee includes board members and associate members who are leading influenza and infectious disease experts from around Asia as well as guest advisors from international organisations such as the World Health Organization (WHO). The APACI is a joint initiative supported by five pharmaceutical companies (Sanofi Pasteur, Novartis Vaccines, GlaxoSmithKline, Roche and Solvay Pharmaceuticals), and works in cooperation with the WHO to complement its work on global influenza control. APACI activities include holding bi-annual meetings in different Asian countries; promoting influenza awareness to healthcare professionals in the region through a regular newsletter (*Influenza – Asian Focus*) and peer-reviewed publications; providing educational resources to support influenza awareness activities, including a website (www.apaci-flu.org); assisting with country-specific recommendations for influenza prevention and control and the development of country-specific public awareness programmes.

Mission Statement

To promote influenza awareness in the Asia-Pacific region, with the intent to improve the prevention and control of influenza. Objectives: 1) To promote influenza awareness to healthcare professionals in the region; 2) To provide educational resources to support influenza awareness activities; 3) To assist the process of establishing or reviewing country-specific recommendations for influenza prevention and control; 4) To advocate the timely access to and supply of influenza vaccines and antiviral medications; 5) To assist the development of country-specific public awareness programmes; 6) To identify and develop activities that complement the WHO Global Agenda.

How are we achieving our objectives?

- Promoting influenza awareness to healthcare professionals in the region. This is being achieved through the identification of key opinion leaders, with expertise either in influenza epidemiology, laboratory diagnostics or public health from each country for board membership. By keeping board members abreast with developments in the field of influenza and its control, especially within countries in the region, it is hoped that they will be able to provide more effective leadership in their own country. Communication within the region is facilitated both by publications, presentations at international meetings

and through press conferences. APACI's primary publication is the newsletter entitled *Influenza - Asian Focus*, which is produced twice each year. *Influenza - Asian Focus* carries articles written by board members and APACI advisors with the objective of providing timely information on influenza-related activities in Asia. Ten issues have been published to date. These articles are being developed into peer-reviewed publications. An article has been submitted to *European Respiratory Disease* focusing on influenza vaccine usage in Asia-Pacific, while a manuscript on influenza seasonality in the Asia-Pacific region is in progress. Material contributed by the board is also presented at regional and international meetings. Recent congress activity has included the 3rd Asian Congress on Pediatric Infectious diseases, in the Philippines, March 2006; The Lancet Asia Medical Forum, in Singapore, May 2006 and the Annual Singapore Paediatric Congress (ASPC), in Singapore, August 2006. Since 2005, regular press conferences have been held following APACI board meetings: Hong Kong, October 2005; New Delhi, India, February 2006; Beijing, China, September 2006 and Hanoi, Vietnam, March 2007.

- Providing educational resources to support influenza awareness activities. An APACI website (www.apaci-flu.org) has been established which houses past issues of *Influenza - Asian Focus*, and a comprehensive speaker slide kit. Education slides have been selected from presentations at APACI board meetings, and grouped into topics to support the influenza awareness educational activities of our board members and others. The website also contains APACI consensus statements and other educational material.

- Assisting the process of establishing or reviewing country-specific recommendations for influenza prevention and control. Through presentations by board members on influenza surveillance, and control strategies including vaccine and antiviral usage, international recommendations within a regional and country-specific context are discussed and reviewed. This strategy has been particularly useful for the development or modification of country specific vaccination recommendations based on surveillance data.

- Assisting with the development of country-specific public awareness programmes. An outcome of the identification of key opinion leaders from countries in the region, and their exposure to the successful influenza awareness strategies developed in Australia and New Zealand through the establishment of public-private partnerships, has been the spawning of mini-APACIs in the region. Board members have led the formation of the Influenza Foundation (Thailand) and the Influenza Foundation of India.

- Identifying and developing activities that complement the WHO Global Agenda on Influenza Surveillance and Control. WHO representatives attend APACI meetings in an advisory capacity and provide an important linkage between the WHO and individual countries. However, the primary focus of the board's activities is on seasonal influenza and ensuring board members are exposed to the differing influenza vaccine strategies and antiviral strategies being developed internationally. *Country Achievements*. Since the inception of the APACI, a number of national influenza awareness initiatives have been successfully implemented across member countries. Working closely with

Ministry of Health officials and other relevant professional bodies, APACI members have played active roles in helping their respective countries improve influenza surveillance, increase vaccine usage and develop targeted vaccination recommendations.

The Philippines. The Technical Working Group on Influenza (TWGI) was created at the end of 2003 to formulate national guidelines for the prevention and control of influenza, strengthen influenza surveillance, and provide information on influenza prevention and control to healthcare workers and the public. Surveillance data from 1998 to 2003 showed increased influenza activity during the June–November period – suggesting that the most effective timing for vaccination is from February to June. In view of this data and to achieve optimal protection, the Philippine Department of Health has recommended the use of the Southern Hemisphere formulation of vaccine, which is available in February each year. **Thailand:** Influenza Foundation (Thailand) (IFT) was founded in August 2004, under the leadership of Professor Prasert Thongcharoen, to improve knowledge of influenza epidemiology in Thailand, promote pandemic preparedness activity in Thailand and disseminate scientific information to the medical community and lay public. Members include representatives from academic and government sectors. Since its inception, the IFT has conducted numerous seminars and workshops around the country, covering topics ranging from influenza vaccination guidelines to pandemic preparedness planning. The IFT's website, www.ift2004.org, details its objectives and activities. **India.** The Influenza Foundation of India (IFI) was established by the Indian Council of Medical Research (ICMR) in late 2005 in response to calls from influenza experts. The IFI aims to increase knowledge of influenza and its epidemiology in India, produce unbiased scientific and educational materials for the medical community and the general public, promote influenza pandemic preparedness activities, and provide a link between private practice and the government. The IFI has established a panel of expert advisors and working groups focusing on virology, paediatric influenza, at-risk groups, research, pandemic preparedness and public health, and industry. To date, the IFI has met on three occasions and produced the inaugural issue of its official newsletter, *Influenza – Indian Focus*.

Recent meeting highlights. The APACI meets twice a year, once at the start of the Southern Hemisphere influenza season and once at the start of the Northern Hemisphere season. When possible, meetings are tied in with congresses or other related activities. The 9th APACI meeting was held in New Delhi, India, to coincide with the second meeting of the IFI in February 2006. One of the meeting highlights was a desktop exercise held to assess the capacity to react to a pandemic in member countries, partly modelled on a recent simulation exercise conducted in Europe. Members used this opportunity to discuss the strengths and weaknesses in their respective national plans and to identify areas where further work was needed. Other presentations at the meeting included updates on the expanding influenza surveillance system in India and other initiatives to fight avian

influenza. The 10th APACI meeting was held in Beijing, China, in September 2006. The meeting focused on pandemic planning initiatives and influenza surveillance data. Members presented on the status of pandemic planning in their respective countries, as well as the latest surveillance and seasonality data. Members whose countries were affected by avian influenza outbreaks at that time discussed the preventative measures that their governments and health sectors were undertaking. An overview of the Chinese influenza surveillance system, including its history and current structure, was also presented. The 11th APACI meeting was hosted by the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi, Vietnam, in March 2007. Local experts and international guest speakers joined APACI members to discuss the role of vaccines and antivirals in seasonal influenza, national recommendations for influenza vaccination and initiatives for influenza control in member countries, and avian influenza updates. The meeting was followed by a press conference. The next APACI meeting will be held in Taipei, Taiwan, in September 2007. **Current Members:** Since the founding of the APACI, board membership has grown into a true representation of the entire Asia-Pacific region, from South-East Asia to Northern Asia. Twelve countries are currently represented on the board, with the aim of each country having one main member and two associate members.

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Multiplex PCR for Typing Influenza and Human Respiratory Syncytial Viruses

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The frequency of co-infection of influenza virus and human respiratory syncytial virus (hRSV) is not well documented in the literature and is essentially unknown in developing countries especially India. In this report we describe the development and application of a multiplex PCR (mPCR) capable of detecting influenza A & B, and hRSV A & B in a single tube. mPCR was standardized with Influenza A & B and hRSV A & B in one tube. Standardized mPCR was applied on clinical samples. Sensitivity and specificity of the mPCR assay was determined to be 0.1TCID₅₀ for influenza A and B and 1TCID₅₀ for hRSV A and B respectively. Mixed infection with hRSV B with influenza A was detected in 2 (2%) samples.

Introduction

Most of the acute respiratory tract infections (ARTI) are caused by influenza viruses A and B as well as the human respiratory syncytial virus (hRSV), which are associated with the most severe complications (1-3). Virus culture is considered to be the "gold standard" for respiratory viruses but has the limitations of being time consuming, laborious and need of an expert for result interpretation. Rapid diagnostic tests have variable sensitivity (40 to 100%) and specificity (86 to 99%) (4-7). In spite of employing both rapid antigen tests along with virus culture, a number of clinically suspected cases of respiratory infections remain negative (8). Polymerase chain reaction (PCR) assays allow detection of small amounts of viral nucleic acid in clinical samples. Multiplex PCRs (mPCR) are designed to amplify more than one respiratory viral target in the same PCR test along with all the advantages of single PCR (9-11). Co infection of Influenza with hRSV is the most common dual respiratory infection detected in clinical samples (12). For patients with viral respiratory infections in whom several viruses have been tested for and in whom co infection has been detected, one of the virus present is often hRSV (13). The frequency of co infection with influenza virus and hRSV or with different subtypes of both viruses is not well known, although it is likely to be low, on the order of 3 to 4% of total infections with either pathogen (14-15). To date there is no published mPCR assay that has been reported from India that can successfully detect and subtype both Influenza and hRSV in one tube and assess the contribution of both influenza and hRSV to the overall burden of respiratory illness in the diagnostic settings. Also, India being a developing country has monetary constraints; commercially available rapid diagnostic tests and virus culture methods for detection of respiratory viruses are relatively expensive to be routinely implemented in a diagnostic setting. Therefore, our aim was to design an in house multiplex PCR (mPCR) that can

detect and subtype both Influenza and hRSV and to determine the usefulness and cost effectiveness of our mPCR over virus culture as an alternative laboratory method by comparing the sensitivity and specificity of both assays used.

Materials and Methods

Standard strains. Standard strains of viruses viz. hRSV (University of Alabama, Birmingham), influenza A and B (NIV, Pune) were grown in Hep-2 and MDCK cells with viral growth medium, respectively. Virus stocks consisted of a mixture of mechanically disrupted cells and supernatant and were stored at -70°C immediately after harvest and until use. **Titration of viruses.** Titrations of all standard strains of viruses were done to determine tissue culture infectious dose (TCID₅₀) using Reed and Muench method (16).

Primer designing. Four sets of primers were designed from highly conserved matrix and nucleocapsid gene of Influenza and hRSV, respectively according to nucleotide sequences available from GenBank. An online melting temperature (T_m) calculator (<http://www.operon.com/oligos/toolkit.php>) was employed to design primers with compatible T_m. This program also displays the complement to the oligonucleotide, plots the sequence against itself and can compare its sequence against another oligonucleotide. Basic Local Alignment Search Tool from the National Center for Biotechnology Information was used for checking specificity of the primer (<http://www.ncbi.nlm.nih.gov/BLAST.html>). Final selection of the primers was based on the following criteria: G/C content of 20–80%, the last five bases on the 3' end of the primers did not contain more than two C and/or G bases, the optimal length for single-stranded primers is about 20–25 bases and between primer pairs T_m should not differ by more than 1–2°C.

Clinical specimens. One hundred Nasopharyngeal aspirates (NPAs) were collected from children <5 years of age visiting All India Institute of Medical Sciences, New Delhi Pediatrics OPD from April 2005 to March 2006 with clinical diagnosis of acute lower respiratory infections (ALRI) according to WHO criteria (17). NPAs were collected in viral transport medium viz. VTM (Hank's balanced salt solution with 0.5% Gelatin). The samples were transported to Virology Laboratory on ice and stored at -70°C for further studies.

Nucleic acid extraction and cDNA synthesis. RNA extraction was done from 500µl clinical sample using RNeasy kit (Qiagen, Germany) as per manufacturers' instructions. RNA was eluted in 50µl RNase free water and 1µl (40U/µl) of RNasin enzyme (Promega Corp., USA) was added to prevent RNA degradation. The viral RNA was reverse transcribed to cDNA using Avian Myeloblastosis Virus Reverse Transcriptase enzyme (AMV RT), and random hexamers primer (Promega Corp., USA). RT and PCR were done in separate tubes. Briefly, for each 25 µl reaction, 10µL RNA and 500 ng of random hexamer primer along with 200µM of each deoxynucleoside triphosphate (dNTP) (Promega Corp., USA) and 20 units of AMV RT were used. The samples were incubated for 90 min at 37°C before inactivating the enzyme by heating for 5 min at 95°C. For DNA amplification, 5 µl

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of first-strand cDNA reaction was amplified in a total volume of 25 μ l containing 0.2 μ M of each primer for influenza A & B virus and 0.5 μ M for hRSV A & B along with 200 μ M each of the four dNTPs (Promega, Madison, WI), 6 units of the *Taq* polymerase (Bangalore Genei, India) and 1.5mM $MgCl_2$. The reactions were allowed to proceed in an thermocycler gene Amp PCR System 9700 (ABI,USA) under the following conditions: 1 cycle at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. Expected band sizes were: Influenza A as 105bp, Influenza B as 503bp, hRSV A as 260bp and hRSV B as 328bp respectively. PCR products were visualized under a digital gel documentation system (Bio-Rad, UK). To prevent possible PCR contamination and false-positive results negative controls and template-free reactions were included. Co-infections: The ability of the mPCR to detect the presence of all four viral templates in the same starting material was assessed by the preparation material spiked with various combinations of viral templates. *Immunofluorescence*. NPAs received in 2-3 ml of VTM were vortexed on arrival to release cells attached to the fibres of the swab. An aliquot of 500 μ l was taken off for RNA extraction after which the specimens were centrifuged at 2000 g for 10 min at 4°C. The resulting cell deposits were air-dried on Teflon coated glass multi-well slides (Cel-Line/Erie Scientific Co., USA) and fixed in chilled acetone prior to testing. Centrifugation enhanced cultures (CEC) were done for both Influenza and hRSV using published protocol [18]. Presence of influenza A & B and hRSV was detected by using monoclonal antibodies (Chemicon International, Inc.; Temecula, CA, USA). Evan's blue was used as counter stain at a final concentration of 0.01%. Slides were visualized under fluorescent microscope (Nikon Eclipse 80i, Japan). The positive samples yielded apple green fluorescence while the negative controls appeared red and showed typical cellular morphology with no specific fluorescence.

Results

Specificity and sensitivity of multiplex RT-PCR. The specificity of the four multiplex primer sets was tested by amplification of RNA from measles virus, herpes simplex virus type 1 and enteroviruses. No specific amplification was obtained, nor was any interassay cross amplification observed when the four multiplex primer sets were used in combination with any of these viruses (data not shown). Assay sensitivity was determined by TCID₅₀. The stock titer of viruses were as follows: Influenza A, 10^{6.1} TCID₅₀/ml, Influenza B 10^{5.1} TCID₅₀/ml, hRSV A, 10^{6.3} TCID₅₀/ml and hRSV B, 10^{6.7} TCID₅₀/ml. Amplified products detected by agarose gel electrophoresis were observed at various dilutions for each virus type and corresponded to a calculated minimal amount of detectable virus RNA of 0.1TCID₅₀ for Influenza A & B and 1TCID₅₀ for hRSV A & B.

Multiplex RT-PCR of four respiratory virus types. The influenza A primer set amplified a 105bp region and influenza B a 503bp matrix region of influenza viruses respectively. The hRSV A primer set amplified of 260bp region and hRSV B a 328bp nucleocapsid region of hRSV respectively*. Single-tube,

multiplex PCR was developed through initial optimization of each individual component of the process. First, RNA from all four prototype viruses were amplified separately to ensure buffer, $MgCl_2$ and temperature conditions were appropriate. An annealing temperature of 52°C for 1 minute was determined to be optimal. Second, primer concentrations in each reaction mixture were adjusted such that amplification of equal amounts of RNA provided similar band intensities (Figure 1). The optimized conditions were then applied to clinical samples viz. nasopharyngeal aspirates.

RNA detection in respiratory specimens. A total of 100 nasopharyngeal aspirates from patients presenting with symptoms of ALRI were tested by mPCR and virus culture both. In total, 13 out of 100 samples were positive for both influenza and hRSV by mPCR and virus culture (CEC) followed by IIF. Both the methods successfully detected hRSV B in 7 samples, Influenza A in 4, hRSV A and Influenza B in 1 sample each. hRSV B constituted majority of the positives as detected by both assays. Our in house mPCR detected mixed infection with hRSV B with Influenza A in 2 (2%) of the total 100 samples (Table 1). The sensitivity and specificity of both our in house mPCR and virus culture were 100%. However, the turn around time for mPCR was less than 24 hours whereas for virus culture followed by IIF, the turn around time was 2-4 days.

Discussion

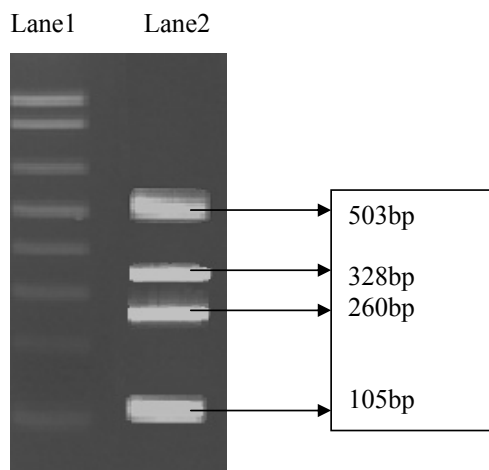
The benefits of the mPCR are its speed, sensitivity, specificity, low volume of specimen required for testing, ability to detect viruses inactivated during collection, and, most importantly, ability to assay for more than one respiratory virus in a single specimen. Previous studies from India (19) identified respiratory viruses in 22% of the specimens collected from acute respiratory infection (ARI) patients by IIF in which influenza A accounted for 3%, influenza B 1%, hRSV 5% of the respiratory viruses. Dual infections with more than one virus were identified in 1% of the cases. Recently, a study from an urban hospital, New Delhi, India revealed the presence of hRSV in 17%, Influenza A & B in 14.5% and dual infections with different viruses in 4% of the total cases on clinical samples by CEC (Chemicon, Inc, USA) followed by IIF (18). In another study, severity of ARI and incidence rates for viruses were determined in NPAs by Direct IF and found to be 9.5% for influenza A & B and hRSV and dual infections accounted for 42% of the cases (20). However these studies were based on rapid antigen based assays and virus culture which are considered less sensitive than PCRs and are dependent on stringent transport and storage conditions. The present study aimed to develop a rapid detection method that permitted identification of four respiratory viruses from a single specimen in one tube. The method developed in this study is the first from India to include a four-primer multiplex set detection and sub-typing of influenza and hRSV within a single tube. Our in house mPCR was capable of detecting all four major respiratory virus types in a single pooled extract, without requiring further steps for identification. Our mPCR was clearly capable of detecting the co infections when they were present.

Virus culture and IIF confirmed the presence of co infections. We also determined the cost effectiveness of our in-house mPCR in comparison to virus culture (CEC) followed by IIF. The cost of reagents alone for mPCR and virus culture followed by IIF assay was USD 10.34 and USD 14.07 respectively. Clearly, mPCR was more cost effective method for detection of viruses which is important when considering setting up a diagnostic assay for routine use in developing countries. Sensitive, rapid testing for respiratory viruses is crucial in the clinical setting to reduce the potential for nosocomial transmission to high-risk patients, to limit unnecessary antibiotic use, and to direct therapy following a specific diagnosis (11-21). We have demonstrated that mPCR can be used for the detection and sub typing of influenza A and B and hRSV viruses in clinical respiratory samples. The assay described here is both highly sensitive and specific for each individual pathogen and is capable of detecting co infections in clinical samples. It should prove to be useful in studies of viral respiratory illness in both surveillance and diagnostic settings. (*Sequences Of Primers Available On Request.)

Table 1. Distribution of different respiratory viruses as detected by our in house mPCR and virus culture.

Positive for viruses by mPCR	No. of positives/samples (%)
Respiratory viruses	13/100 (13)
Influenza A	4/13 (30.7)
Influenza B	1/13 (7.7)
hRSV A	1/13 (7.7)
hRSV B	7/13 (53.7)
Mixed infections (Influenza A and hRSV B)	2/13 (15.4)

Figure 1. Standardized mPCR for detection of influenza A, influenza B, hRSV A and hRSV B, respectively.



Lane1: 100bp marker
Lane2: Influenza A (105bp), Influenza B (503bp), hRSVA (260bp), hRSV B (328bp)

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Genetic Characteristics of the First Human Case of Highly Pathogenic Avian Influenza A (H5N1) in Sub-Saharan Africa

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Outbreaks of an highly pathogenic avian influenza A (H5N1) among poultry in February 2006 in Nigeria was a warning signal that the virus could cross the species barrier and cause many human fatalities and also increase pandemic threat. We describe the features of the first human case of influenza A (H5N1) detected in sub-Saharan Africa. An H5-specific reverse-transcriptase PCR assay (RT-PCR) and RT-PCR for NA gene were used for the detection of virus directly in clinical specimens. Samples from a 22-year old female with influenza-like illness from Lagos state of Nigeria were confirmed to be positive for H5N1 virus and the isolated virus (A/Nigeria/641/6/2007) was shown to be identical with a highly pathogenic H5N1 virus (A/chicken/Nigeria/641/2006) previously isolated from chickens in Nigeria during the 2006 outbreak in poultry. The complete genome sequencing of the virus has been carried out and details will be described. Owing to the pandemic potential of the virus, the strategic position of Nigeria and peculiar public health situation on the African continent, urgent and active measures must be taken to stop further spread of the virus.

Introduction

Outbreak of highly pathogenic Avian Influenza virus H5N1 in poultry in Africa was first reported by Nigeria in February 2006 (Figure 1). The country has an estimated poultry population of 159 million out of which 60% is backyard and 30% commercial. Nigeria also recorded the first human case of H5N1 infection in sub-Saharan Africa in February 2007. We report here the genetic characteristics of the human isolate.

Materials and Methods

Virus Isolation. Nasopharyngeal swabs and lung aspirates were taken from the deceased in Lagos and transported in VTM to Abuja and stored at -80°C in the National Clinical Research & Training Centre (NCRTC)/Institute of Human Virology (IHV Nigeria) Laboratory, Asokoro, Abuja, until tested. Sequencing And Phylogenetic Analysis: Viral RNAs were extracted from the

samples and standard reverse transcriptase-PCR and sequencing were performed in Nigeria and at the WHO Influenza Reference Centre, London, United Kingdom. The nucleotide sequences were compared using the multiple sequence alignment programme for DNA and proteins with the Clustal W alignment algorithm. Phylogenetic comparisons of the aligned sequences for each gene segment were generated by the neighbour joining (NJ) method with PAUP 4.0b10 software (Figure 2).

Conclusions

Sequences of the 8 genes we obtained for A/Nigeria/6/2007 (H5N1) isolated from samples from the fatal case of the 22-year-old Nigerian female patient have been analyzed. In line with agreed WHO policy, the sequence data had been uploaded to the restricted H5 sequence compartment of the LANL database and the two sequences for HA and NA to the public compartment of the LANL database. All 8 genes were closely related to those of clade 2, subclade 2 (WHO nomenclature), "Qinghai Lake" H5N1 viruses. The available sequences of Nigerian avian isolates fall into two principal subgroups in the phylogenetic trees: one represented by, for example, A/chicken/Nigeria/10478/2006, the other represented by, for example, A/chicken/Nigeria/641/2006 (closely related to viruses isolated in the Ivory Coast and Sudan). Five of the genes, encoding the polymerase complex, comprising PB1, PB2, PA and NP proteins, and M1 and M2 proteins, fall within the former subgroup, whereas sequences of the HA and NS genes are closer to those of chicken/Nigeria/641/2006. Amino acids characteristic of the respective subgroups include: alanine 654 in PB1, leucine 243 and valine 483 in PB2, glycine 272 in PA, isoleucine 270 in NP, and serine 58 in NS1. Although closely related, the NA gene does not fall into either subgroup. The genome of A/Nigeria/6/2007 is in fact more closely related to that of the avian isolate A/chicken/Nigeria/104762/2006 in 6 genes (except for NP and NA). These data illustrate how the viruses are related by genetic reassortment. The NA sequence of A/Nigeria/6/07 does not possess either a H275Y or N295S mutation which have been associated with reduced susceptibility to oseltamivir. The M2 protein also lacks any of the well-defined mutations associated with amantadine resistance. The results of an HI test including A/Nigeria/6/07 showed that it is antigenically closely related to the clade 2, subclade 2 candidate vaccine reference viruses, A/turkey/Turkey/1/2005, A/BHG/Qinghai/1A/2005 and A/whooper swan/Mongolia/244/2005 and the recent human isolate A/Egypt/914725/2006.

Future Directions. Build diagnostic laboratory capacity in all States of the Federation. Conduct nationwide baseline study for the spread of H5N1 among Nigerian population. Increase surveillance network. Establish African influenza sequence database. Establish research network for antivirals and vaccine development.

Figure 1. Map of Nigeria showing location of the first poultry and human outbreaks (Adapted from Ducatez, MF *et al*/Nature 2006 vol 442 pg 37)

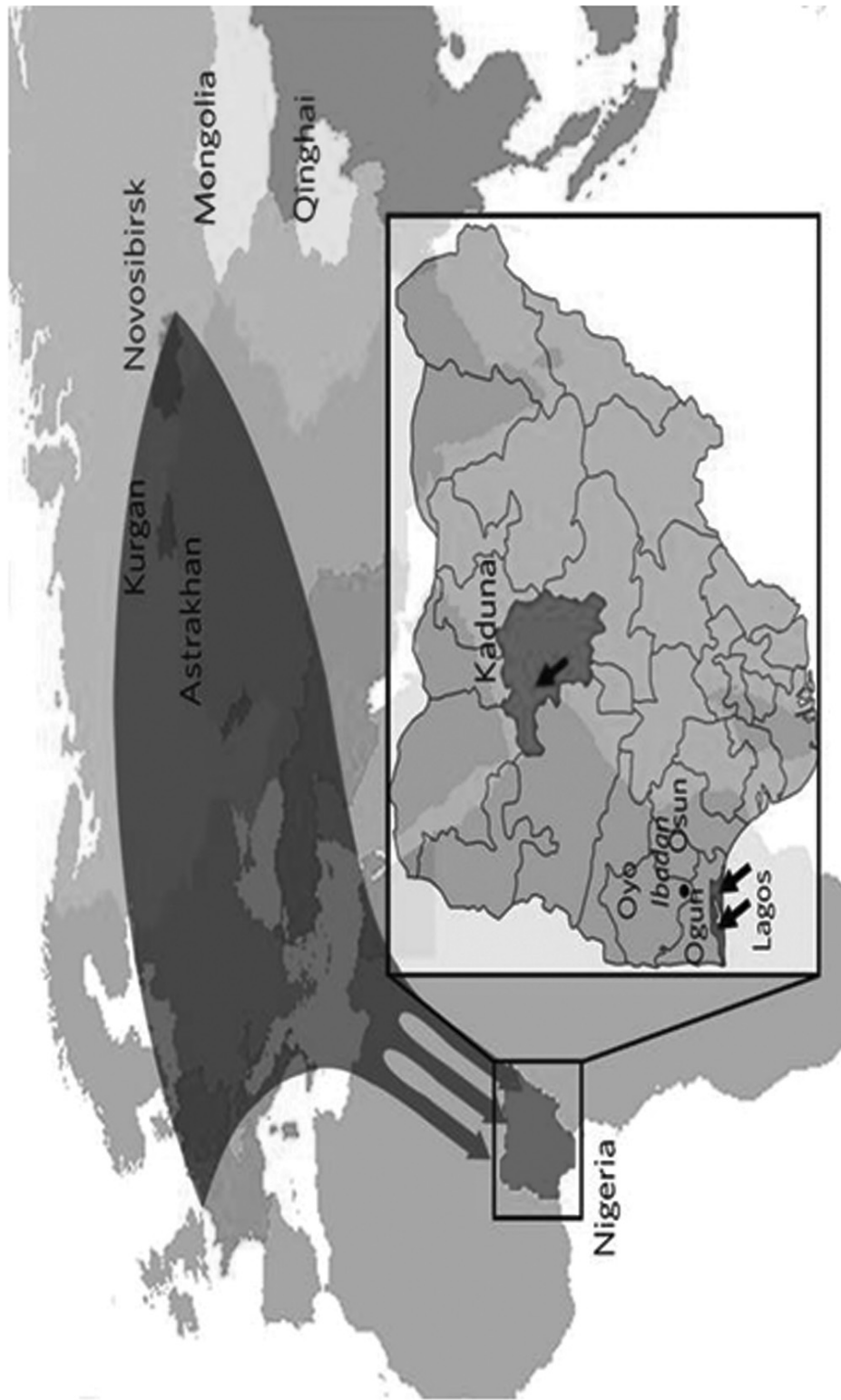
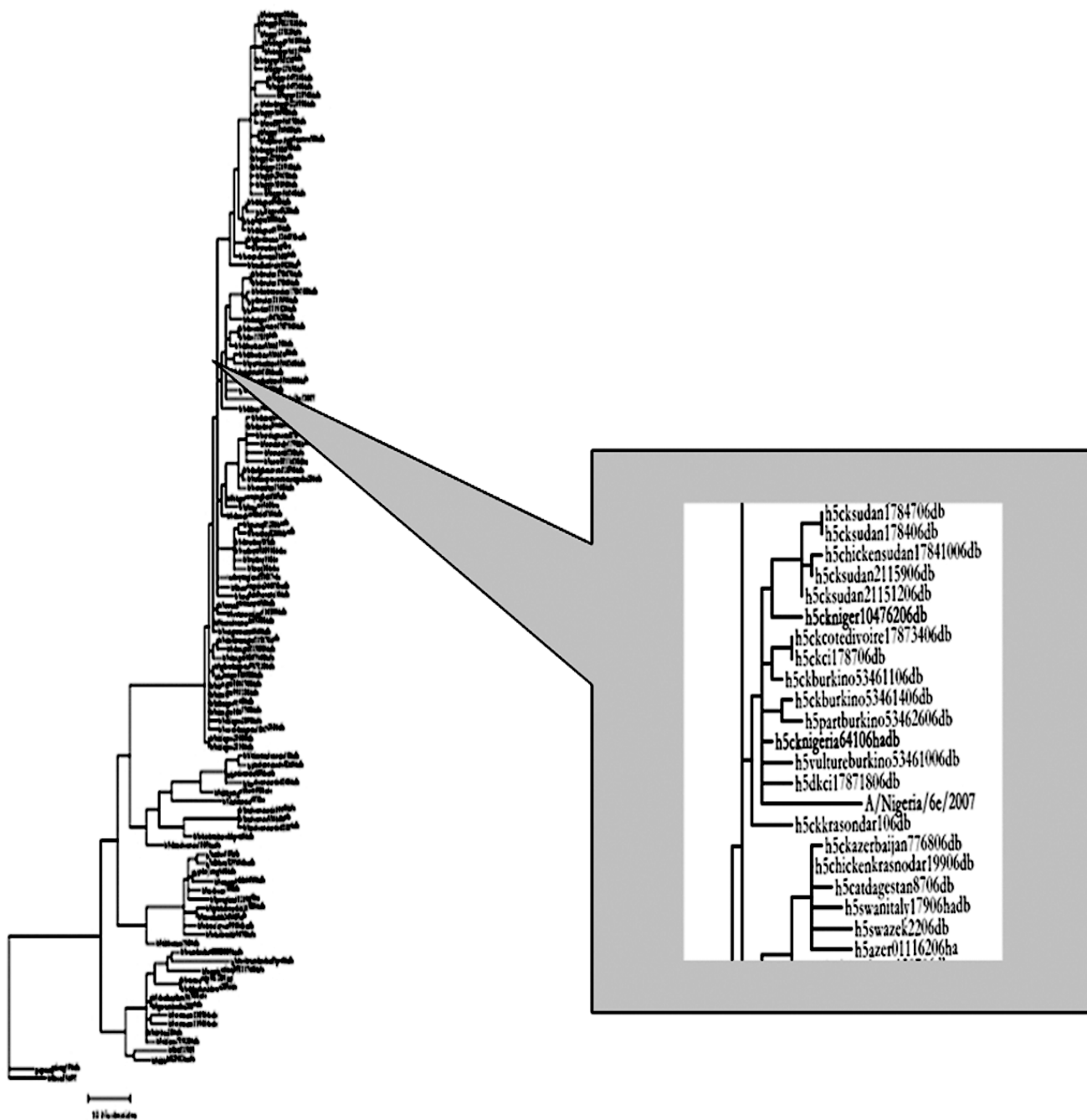


Figure 2. Phylogenetic comparison of H5 HA genes.



Re-Evaluation of Usefulness of Throat Swab Specimens for the Influenza Rapid Diagnostic Test

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To re-evaluate the throat swabs as the specimen for influenza rapid antigen-detection kit, throat swabs were collected from 439 patients with influenza-like symptoms, and were subjected to both virus isolation using cell culture system and a rapid diagnostic kit. The sensitivity and specificity of the kit test were 87.8% and 90.2% for type A influenza, and 80.4% and 95.0% for type B, respectively, which were almost comparable levels with those other same kind of studies using nasal swabs. Furthermore, there is no significant difference in results among various age groups.

Introduction

The rapid antigen-detection kit is a useful tool for diagnosis of influenza at the bedside. As to the specimen for the test, nasal and throat swabs are frequently used, but it has sometimes been considered that the former is superior to the latter to be recommended unconditionally [1]. We have suggested, however, that such difference in sensitivity between these two were only slight one [2] probably based on the skill of physicians in collecting throat swab specimens. We had an opportunity to know that even throat swab specimens could bring good results in the tests, comparable enough to those thought-to-be the best levels by nasal swab specimens.

Materials and Methods

Throat swab specimens collected by two physicians from 439 patients who attended a clinic with influenza-like symptoms in the 2004-2005 season, were subjected to both virus isolation using cell culture system and a rapid diagnostic kit, QuickVue influenza A+B Test (QUIDEL, USA); Japanese Brand name: QuickVue Rapid SP influ (DS Pharma, Japan) Results of the kit test were analyzed in terms of the sensitivity and specificity by comparing with those of virus isolation using MDCK cell culture system as the golden standard for the diagnosis.

Results

The sensitivity and specificity of the kit for all 439 specimens were 87.8 (72/82) and 90.2% (322/357) for type A influenza, and 80.4%(176/219) and 95.0%(209/220) for type B, respectively (Table 1). The sensitivity and specificity were calculated separately for various age groups set at every 10 years from 1 to 88, as well as less than 6 and over 7 years old, and there found no significant difference among them (Table 2).

Table 1. Sensitivity and specificity of the rapid kit test using throat swabs as the specimen.

	Virus isolation			Total
	Type A influenza	Type B influenza	Negative	
A+	72	7	27	106
B+	2	175	9	186
A+B+		1		1
Negative	8	36	102	146
Kit test Total	82	219	138	439
Sensitivity	87.8%	80.4%		
Specificity	90.2%	95.0%		

Table 2. The sensitivity and specificity determined at age groups.

Age	n	Type A Influenza		Type B Influenza	
		Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
1-6	82	90.0 (18/20) < 76.9 - 100% >	87.1 (54/62) < 78.8 - 95.4% >	72.2 (26/36) < 57.6 - 86.9% >	95.7 (44/46) < 89.8 - 100% >
7-88	357	87.1 (54/62) < 78.8 - 95.4% >	90.8 (268/295) < 87.6 - 94.1% >	82.0 (150/183) < 76.4 - 87.5% >	94.8 (165/174) < 91.5 - 98.1% >

Discussion

Results of our study confirmed that the sensitivity and specificity of the influenza virus rapid antigen-detection kit using throat swabs were almost comparable levels with those reported so far in the same kind of studies using nasal swab specimens [3] at any age groups. The throat swab would still be competent specimen useful enough in the clinical setting for diagnosing influenza using the rapid antigen-detection kit, regardless of the age of the patient, as far as it could be collected properly with the solid skill.

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Rescue of A/Chicken/Guangdong/04 (H5N1) Influenza Virus by Reverse Genetics and its Pathogenic Characterization in Mice

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The H5N1 avian influenza virus has recently demonstrated the ability to cross the species barrier between birds and mammals. However, the genetic mechanism of this ability is not well known. We successfully rescued an influenza A virus (A/Chicken/Guangdong/04) (H5N1), which is highly pathogenic to both SPF chickens and BALB/c mice, by plasmid-based reverse genetics. The rescued virus, R-A/Chicken/Guangdong/04 (R-CG), and the wild-type A/Chicken/Guangdong/04 (W-CG) were found to share similar biological properties, such as in titers of 50% egg infectious dose, 50% tissue culture infectious dose and intravenous pathogenicity index. R-CG, like W-CG, is highly pathogenic in mice following natural route infection. Both R-CG and W-CG viruses can be isolated from many organs of mice such as brain, lung, kidney and spleen. As a result, the constructed R-CG reverse genetic system can be used as a tool in the investigation of the molecular mechanism of infection of the mammalian host by avian influenza virus.

Introduction

Phylogenetic analysis of influenza viruses has revealed species-specific lineages of viral genes, as well as genes that have crossed species barriers. Avian influenza viruses are occasionally transmitted to other host animals. Some of the viruses may establish in these new hosts and cause epidemics and epizootics. During the 2003 and 2004 avian influenza A virus (H5N1) outbreak in South-East Asia, reports of fatal H5N1 virus infection in domestic cats and zoo felids, such as tigers and leopards, were described [1, 2]. These incidents as well as subsequent laboratory findings suggest that avian H5N1 influenza viruses are becoming progressively more pathogenic for mammals [3]. Rapid progress on the reverse genetics of influenza viruses, which enables the entire generation of virus from cloned cDNA, has been recorded [4]. An eight-plasmid DNA transfection system for the rescue of infectious influenza, possessing two transcription units, has been developed [5]. The orientation of the two transcription units allows the synthesis of negative-sense viral RNA and positive-sense mRNA from one viral cDNA template. The interaction of all molecules derived from the cellular and viral transcription and translation machinery results in the generation of infectious influenza A virus. Reverse genetics provides a powerful tool for dissecting the virus life cycle, the virus assembly, the role of viral proteins in pathogenicity, the interplay of viral proteins with components of the host cell immune response and the development of

influenza vaccines [6]. Using the eight-plasmid system, we successfully rescued A/Chicken/Guangdong/04 (H5N1) and found it to be highly pathogenic in BALB/c mice as the wild type virus.

Materials and Methods

Virus growth and animals. A/Chicken/Guangdong/04 (H5N1) propagated in allantoic cavity of 10-day-old specific pathogen free (SPF) embryonated eggs. Aliquots of viruses were stored at -70°C. Six-week-old White Leghorn SPF chickens and 6- to 8-week-old female BALB/c mice were housed in isolator cages. All work with the H5N1 virus was performed in biosafety level 3 laboratory facilities, and experiments with animals were handled in HEPA-filtered isolators.

Plasmid construction. RNA isolation, RT-PCR and sequencing. Viral RNA was isolated from allantoic fluid using the Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse viral RNA transcription and subsequent PCR were performed using primers specific for each viral gene, as described previously [7].

Construction of eight recombinant plasmids. The cloning vector pHW2000 (kindly provided by Dr. Webster, St. Jude Children's Research Hospital, Memphis, TN) was gel purified after cleaved by *BsmBI*. Following the digestion with corresponding endonucleases *BsmBI*, *BsaI* or *AarI*, the eight gene fragments were cloned into the vector pHW2000, using T4 DNA ligase and transformed into Top 10 competent cells. The recombinant plasmids were sequenced by commercial company.

Virus rescue and sequencing. The co-cultured MDCK and 293T cells were used for the transfection experiments as described previously [8]. After 72 h of incubation, titre of the cell supernatant was determined by infection of embryonated chicken's eggs. The allantoic fluid was tested by haemagglutination assay (HA). The rescued virus was designated as R-A/Chicken/Guangdong/04 (R-CG). The complete genes from the second-passage allantoic fluids containing the rescued R-CG viruses were amplified by PCR and purified PCR products were sequenced. Biological properties: comparison between R-CG and its wild-type virus, W-CG. Determination of 50% egg infectious dose (EID₅₀) and 50% tissue culture infectious dose (TCID₅₀). According to the WHO manual [9], EID₅₀ of viruses were determined by log₁₀ serial dilutions and the titration of virus in eggs was carried out; TCID₅₀ of viruses were determined by 0.5 log₁₀ serial dilution in PBS, and the dilution of virus was co-cultured with MDCK cells. After 72 h, the supernatant of cell culture was tested by HA assay. Titration endpoints were calculated by the method of Reed and Muench [10].

Chicken pathogenesis assessment. To determine the virus pathogenicity in an avian species, 10 chickens were inoculated intravenously with 0.1 ml 10⁻¹ dilution of viruses. Clinical signs and pathogenesis observed daily for 10 days. The intravenous pathogenicity indexes (IPVI) of were calculated according to the method established by the Office International des Epizooties [11].

Mouse pathogenesis assessment. Groups of six 6-week-old

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female BALB/c mice were lightly anaesthetized with CO₂ and inoculated with 106. ⁰EID₅₀ of viruses in 50µl PBS by intranasal route. The control mice received PBS, instead of virus. The mice of three groups were monitored daily for morbidity (measured by weight loss) and death for 10 days after inoculation. Tissue samples (lungs, kidneys, spleen and brain) were collected from euthanized mice on days 3, 4, 5 and 7. Tissue homogenates were prepared in cold PBS and titrated for virus infectivity in eggs from initial dilutions of 1: 10 (lung) or 1: 2 (brain, kidney and spleen).

Results

Construction of plasmids. The eight plasmids containing the full-length cDNA of viral genes were designated pCG-PB2, pCG-PB1, pCG-PA, pCG-HA, pCG-NP, pCG-NA, pCG-M and pCG-NS. The sequencing results showed that eight segments of W-CG were inserted into pHW2000 at the appropriate sites. Although there were a few nucleotide differences between the sequences of the PCR products of W-RG and that of the segments inserted into the recombinant plasmids, the amino acid sequences displayed 100% homology between them.

Rescue of A/Chicken/Guangdong/04 and sequencing. The first passage of the supernatant in embryonated eggs resulted in a haemagglutination activity with a titre of 1: 32. A higher titre of virus (HAU, 1: 256) was obtained after two passages in embryonated eggs. After two passages in embryonated eggs, R-CG demonstrated identical sequences as that of corresponding recombinant plasmids.

Biological properties of W-CG and R-CG EID₅₀, TCID₅₀ and IVPI. The EID₅₀, TCID₅₀ and IVPI of W-CG are 10-9.85/ml, 10-8.73/ml and 2.91; R-CG are 10-9.67 /ml, 10-8.75/ml, and 2.88, correspondingly. Seven of 10 chickens intravenously inoculated with W-CG virus died within 24 h after inoculation. Six chickens died within 24 h post-infection (p.i.) in the group inoculated with R-CG. The remaining chickens in both groups died within 48 h p.i.. According to the OIE criteria, both W-CG and R-CG are high pathogenicity in chickens.

R-CG and W-CG pathogenicity in BALB/c mice. Mice showed abnormal behaviors including coughing, unwillingness to move, hunched posture and ruffled fur. Weight loss was observed in infected mice. All mice in the control group showed no signs of illness and gained weight throughout the course of infection (Figure 1). W-CG-infected mice began to succumb to infection 3 days p.i., while the R-CG-infected mice began to succumb day 4 p.i.. All of the W-CG and R-CG infected mice died within 8 days p.i. (Fig. 1). Infection of mice with each of the viruses resulted in high titers of virus in the lungs on day 3 (W-CG) or day 4 p.i. (R-CG). Virus was present in extrapulmonary tissues, including the brain, spleen and kidneys of W-CG-infected mice on 3, 5 and 7 days p.i.; however, mice infected with R-CG virus had undetectable titers in these tissues on day 3 p.i. (Table 1).

Figure 1. Pathogenicity in BALB/c mice. A, Weight change of negative control and BALB/c mice after infection with wild type (W-CG) and rescued (R-CG); B, Survival percent of BALB/c mice after infection wild-type (W-CG) and rescued (R-CG).

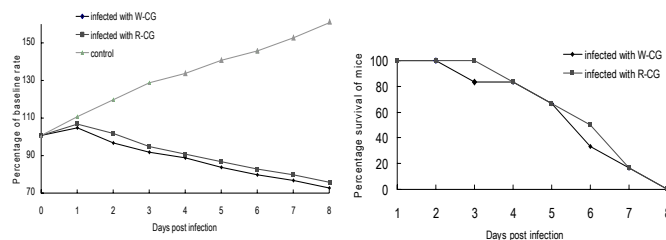


Table 1. Virus isolation in different organs of mice Infected by wild-type (W-CG) and rescued (R-CG).

Groups	Days after Virus infection	Virus titer (log ₁₀ EID ₅₀ /ml)			
		Lung	Brain	Spleen	Kidney
W-CG	3	5.8	-	-	+
	5	5.6	2.3	+	1.5
	7	5.1	3.1	+	2.2
R-CG	4	6.0	-	-	-
	5	5.4	2.2	1.3	1.4
	7	5.0	3.3	+	1.9
Control	3	-	-	-	-
	5	-	-	-	-
	7	-	-	-	-

+Virus was detected in the undiluted sample; -virus was not detected in the undiluted sample

Discussion

In recent years, reports have been issued about the avian influenza crossing the species barrier and directly infecting mammals, such as human beings, tigers, leopards and cats. The viral genetic characteristics that have allowed the transmission of avian influenza viruses to humans remain unknown. The BALB/c mouse has been a useful mammalian model system for the pathogenesis study of H5N1 and H9N2 avian influenza viruses. The H5N1 avian influenza viruses isolated from ducks in mainland China have acquired a progressively greater capacity to infect mammals was reported [12]. Some of these viruses can replicate efficiently in many organs without prior adaptation and cause mortality of the mouse. Plasmid-based reverse genetics has been used to elucidate the molecular basis that determines viral pathogenicity by generating reassortants between virulent and avirulent viruses [13]. In this study, we rescued an avian influenza virus A/chicken/Guangdong/2003 (H5N1), which had high pathogenicity in chickens, ducks (data not shown) and mice. The rescued virus genome had amino acid sequence homology with that of its wild type. EID₅₀, TCID₅₀ and IPVI of the rescued

virus were similar to that of the wild type. The R-CG pathogenesis was characterized in BALB/c mice and compared with the wild type W-CG. Both R-CG and W-CG viruses replicated efficiently in lung, but they could be detected from non-respiratory organs such as brain, kidney and spleen of the infected mice on 5 days post infection. All of the infected mice died by 8 days post infection. All of these data indicated that the rescued virus R-CG shares similar genomic and biological characteristics with that of the wild-type W-CG. As a result, the constructed H5N1 reverse genetic system can be used as a tool in the investigation of the molecular mechanism of infection of the mammalian host by an avian influenza virus.

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