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Tropism and innate host response of the 2009 pandemic H1N1 influenza virus compared with related swine influenza viruses and reassortants in ex vivo and *in vitro* cultures of the human respiratory tract and conjunctiva

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Keywords :2009 pandemic H1N1 influenza virus, conjunctiva, *ex vivo*, *in vitro*, innate host responses, respiratory tract, swine influenza virus, tropism

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Background

Pandemic influenza H1N1 (H1N1pdm) virus of swine-origin causes mild disease, but occasionally is associated with acute respiratory distress syndrome and death.[1,2] It is important to understand the pathogenesis of this new disease. Previously we showed a comparable virus tropism and host innate immune responses between H1N1pdm and seasonal H1N1 influenza virus in the human respiratory tract,[3] however H1N1pdm virus differed from seasonal H1N1 influenza virus in its ability to replicate in human conjunctiva, suggesting subtle differences in receptor-binding profile and highlighting the potential role of the conjunctiva as an additional route of infection. We now compare the tropism and host responses elicited by pandemic H1N1 with that of related swine influenza viruses and a pandemic-swine reassortant virus in *ex vivo* and *in vitro* cultures of the human respiratory tract and conjunctiva. We have used recombinant virus to investigate the role of the hemagglutinin (HA) and neuraminidase (NA) of H1N1pdm virus in its conjunctival tropism. These findings are relevant for understanding transmission and therapy.

Materials and methods

Fragments of human conjunctiva, bronchi, and lung tissues were cut into 2–3 mm fragments within 2 h of collection and infected with influenza A viruses at a titer of 10^6 $TCID_{50}$/ml. Viruses investigated included H1N1pdm (A/HK/415742/09), swine H1N2 virus (A/swine/HK/915/04), which shares a common derivation for seven genes with H1N1pdm, a natural swine reassortant H1N1 (A/swine/HK/201/10), which has acquired the NA gene from H1N1pdm and other swine influenza H1N1 viruses. Reverse genetics derived recombinant viruses with HA and NA gene segments of seasonal H1N1 and pandemic H1N1 swapped were also studied. Lung fragments were cultured at 37°C in culture plates; conjunctival and bronchial biopsies were cultured in air-liquid interface at 33 and 37°C respectively. Tissue fragments were infected for 1 h and incubated for 1, 24, and 48 h post infection. Infectious viral yield was assessed by titration in MDCK cells. The infected tissues were fixed with formalin and analyzed by immunohistochemistry for influenza antigen. Cytokines profiles induced by influenza virus infected respiratory epithelial cells *in vitro* were measured by quantitative RT-PCR and ELISA.

Results

We found comparable replication in seasonal and pandemic H1N1 viruses in human respiratory tract, while the swine influenza A/swine/HK/4361/99 (H1N1) virus and A/swine/HK/915/04 (H1N2) virus failed to infect and replicate in human lung *ex vivo* culture, but it replicated

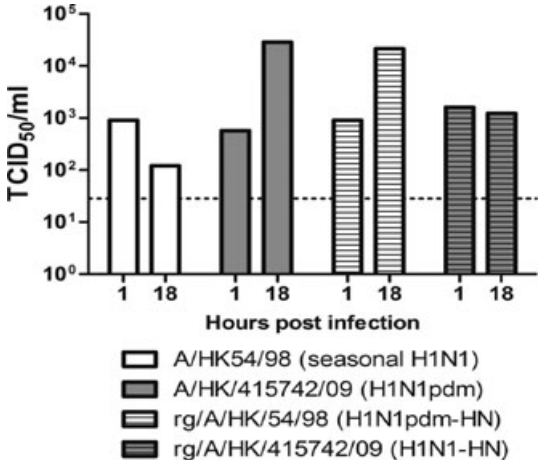

Figure 1. *Ex vivo* organ cultures of lung infected with 10^6 $TCID_{50}$/ml of influenza virus of (A) A/HK/54/98 (H1N1); (B) A/HK/415742/09 (H1N1pdm); (C) rg/A/HK/54/98 with HA/HA of A/HK/415742/09 and (D) rg/A/HK/415742/09 with HA and NA of A/HK/54/98. Viral replication in lung biopsies infected with influenza viruses was determined by $TCID_{50}$. The chart showed the means of duplicate assays at various times after infection.

productively in human bronchus *ex vivo*. Interestingly, the swine reassortant influenza H1N1 (A/swine/HK/201/10) virus (with the NA from H1N1pdm) infected and productivity replicated in lung *ex vivo* and *in vitro*. Pandemic H1N1pdm virus, but not seasonal H1N1 virus, was able to infect *ex vivo* cultures of human conjunctiva, suggesting subtle differences in receptor binding profile in H1N1pdm, seasonal viruses, and the swine related H1N2 viruses. Using reverse genetics derived recombinant viruses, we were able to demonstrate that the HA and NA segments of H1N1pdm, but not the polymerase genes, were required for the conjunctival tropism of H1N1pdm (Figure 1). In contrast with highly pathogenic influenza H5N1 virus, which induced high cytokine and chemokine decretion, the related swine viruses, A/swine/HK/915/04 (H1N2), as well as the swine pandemic reassortant virus, A/swine/HK/201/10 (H1N1) we studied were similar to H1N1pdm and seasonal influenza viruses in their intrinsic capacity for cytokine dysregulation.

Discussion

Collectively, our results suggest that pandemic H1N1pdm virus differs in modest but subtle ways from seasonal H1N1 virus in its intrinsic virulence for humans, findings that are in accord with the epidemiology of the pandemic to date. The HA and NA gene segments are key to the conjunctival tropism manifested by the H1N1pdm virus. The pandemic reassortant influenza H1N1 (A/swine/HK/201/10) virus isolated from swine with the NA from H1N1pdm shares with H1N1pdm the capacity for productive replication in lung *ex vivo* and *in vitro*. These findings are relevant for understanding transmission and therapy.

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Differential cell culture susceptibility and proliferative response to avian, human, and swine influenza viruses

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Abstract

Growth characteristics of different influenza viruses in various human and animal cell cultures were tested, and their influence on cell culture growth was evaluated. It was shown that pandemic influenza viruses replicated poorly in almost all cultures tested. Still, though they didn't induce cytopathic effect, the replication of the virus could be detected by *de novo* NP synthesis. These viruses, as well as those which replicated efficiently in cell cultures, induced

apoptosis. Moreover, low doses of viruses had a stimulating effect on cell proliferation in monolayer and suspension cell cultures.

Introduction

Isolation of influenza viruses from specimens is traditionally performed in two classical systems: embryonated chicken eggs and MDCK cell culture. Nevertheless, several publications are dedicated to the theme of alternative cell culture systems, which may be used for influenza virus isolation and cultivation.[1–3] This is in part because MDCK cells are of animal origin, which means that they cannot be used as a proper model for estimating interactions between a human virus and a human cell culture as a host.

A variety of human monolayer and suspension cell cultures have been tested on their capability to support influenza virus replication. Among them, some support influenza A virus growth as well as MDCK cells do,[4] others support replication of a virus, but do not enable the formation of mature viral particles,[5] whereas others show only a weak level of replication or are not permissive at all.[2] CaCo-2 cells, for example, represent a good substitute for MDCK cells, because it has been shown that the rate of viral isolation in CaCo-2 cells is as effective as in MDCK, and sometimes is even better.[6]

The success of viral replication is determined not only by the cell culture type, but also by the virus itself. Despite the accepted view that it is the type of receptor that defines the interaction between the virus and the host cell, there is evidence that it is not the only factor that predetermines the fate of the cell.[7]

The fate of the infected cell can also differ. A series of articles show that apoptosis is the most probable mechanism of cell killing by influenza viruses.[8,9] Influenza A viruses of different subtypes induce apoptosis to a different extent (e.g. H3 viruses provoke more strong apoptotic response than H1 viruses do [10]). Nevertheless, it has been demonstrated that CaCo-2 cells do not follow the apoptotic pathway and die through necrosis.[11] The SJPL cell line also dies through necrotic pathway and not apoptosis.[12]

The aim of our work was to compare growth characteristics of different flu viruses (e.g. avian, swine, and human) in various human and animal cell cultures and to evaluate their influence on cell culture growth. The parameters measured in the study were as follows: cytopathic changes of cell cultures following virus infection, hemagglutinin production, NP synthesis, the dose-dependent effect of infection on cell proliferation, and the ability of viruses to induce apoptosis.

Materials and methods

Influenza viruses

Influenza viruses used included: highly pathogenic avian H5N1 A/Kurgan/5/05, low pathogenic avian H5N1 A/Gull/Kostanai/7/07, swine H1N1 A/Swine/1976/31, human H1N1v A/California/07/09, human H1N1v A/Saint-Petersburg/5/09, human H1N1 A/Brisbane/59/07, and human H3N2 A/Brisbane/10/07.

The viruses were propagated in 10-days embryonated chicken eggs, the allantoic fluid was collected, the aliquots were made and stored at −70°C for further use.

Cell cultures

Fourteen cell lines were used, all from Cell Culture Collection of the Research Institute of Influenza. Monolayer cell cultures were cultivated in alpha-MEM+2% of bovine fetal serum. Suspension cell cultures were cultivated in RPMI 1640 + 2% of bovine fetal serum. These cell cultures comprised two animal cell cultures: MDCK (canine kidney) and SP (in-house culture of porcine kidney); and twelve human cell cultures: eight monolayer cultures – A-549 (lung carcinoma), ECV-304 (endothelial line), L-41 (monocytic leukemia), FLECH (human embryo lung fibroblasts), RD (rhabdomyosarcoma), T-98G (glioblastoma), A-172 (glioblastoma), Girardi-Heart (auricle cells) – and 4 suspension cell cultures: Jurkat (hemablastoid T-lymphocytes), NC-37 (B-lymphoblastoid line), Raji (B-lymphoblastoid line) and U-937 (histiocytes).

Infection of monolayer cell cultures

To evaluate TCID50 for each virus on all cell cultures, 96-well plates were used. The cells were seeded 0·2 ml per well (concentration of 1–1·5 × 10 cells/ml). The confluent 24-h old monolayer was used for viral inoculation. The cells were washed twice with serum-free medium, then 0·05 ml of ten-fold viral dilutions from viral aliquots were added and left for 45 min for contact at 37°C. The cells were then washed to remove the non-attached particles, and the wells were filled with TPCK-trypsin (2 μg/ml)-containing medium without bovine fetal serum. The plates were observed daily for cytopathic effect, and the results were evaluated at 72 h after infection for cytopathic effect and by reaction of hemagglutination with suspension of chicken erythrocytes (0·75%).

Infection of suspension cell cultures

Infection of suspension cell cultures was done in centrifuge tubes. Cells (concentration 3–5 × 10^5) were inoculated with viral dilutions (MOI = 1–10). After 45 min of contact, cells were washed, resuspended in RPMI with trypsin and fetal serum, and seeded in 24-well plates (1 ml in each well). The results were fixed after 72 h, calculating the number of

cells grown and estimating the rate of apoptosis by Hoechst-33258 staining.[13]

Influence of viral infection on monolayer cell cultures proliferation

Cells were grown in 24-well plates with seeding concentration 1×10 cells/ml. One millilitre of cell suspension was placed in each well, inoculated with viral dilution (MOI = 1–1000) and left for 72 h. After, the cells were detached from plastic with versene and calculated in Fuks-Rosental camera to evaluate the number of cells.

Immunofluorescence with use of monoclonal antibodies

The monoclonal antibodies obtained in Research Institute of Influenza towards viral nucleoprotein NP were used following the standard protocol described in.[13]

Results

Influenza A virus reproduction in animal cell cultures

For all viruses tested, MDCK turned out to be more permissive than SP cell culture. Avian viruses, independently of their pathogenicity, replicated efficiently on both animal cultures tested. Human H1N1 and H3N2 viruses demonstrated weaker replication in SP cells. The most significant differences were seen for swine influenza and pandemic H1N1v viruses which replicated in MDCK cells at the rates comparable with other viruses, but showed poorer growth in SP cell line (see Table 1).

Influenza A virus replication roduction in human cell cultures

Human cell lines displayed clear differences in their susceptibility to viruses of various origins. Avian influenza viruses replicated in all cell lines except Girardi Heart, and the most intense replication rate was observed for ECV-304, L-41, and RD lines. A-549 and A-172 were poorly infected, as well as all suspension cell lines tested.

Seasonal human H1N1, as well as H3N2 viruses, replicated in all cell cultures tested, but the rate of infectivity was rather low in practically all cultures tested with the exception of RD and T-98G cell lines.

Strikingly, swine influenza virus and human pandemic H1N1v viruses didn't replicate well in any of human lines tested. A weak replication rate was observed in ECV-304, RD, and T-98G, but in general, human cell lines were

Table 1. Titres of influenza virus at 72 h post–infection in different cell cultures. The numbers represent the $\log_{10}$ TCID$_{50}$/0·1 ml calculated by Reed-Muench method as described in[17]

	Influenza viruses						
	Avian		Swine	Human			
Cell culture/Influenza virus (subtype)	A/Chicken/Kurgan/5/05 H5N1**	A/Gull/Kosta- nai/7/07 H5N1*	A/Swine/1976/31 H1N1sw	A/California/7/09 H1N1v	A/S.Petersb/5/05 H1N1v	A/Brisbane/59/07 H1N1	A/Brisbane/10/07 H3N2
Animal cell cultures							
MDCK	5·5	6·3	5	4	5·7	5	5·2
SP	5·5	5·5	2	1	2	4	4·7
Human cell cultures							
A-549	2	1	–	–	–	1	1
ECV-304	4·5	4	1	–	1	1	1
L-41	2·5	4	–	–	–	1	1
FLECH	2·5	1	–	–	–	1·5	1·8
RD	3	4	1	1	–	4	4
T-98G	1·5	3·5	–	1	–	4	3·6
A-172	1	1	1·5	–	–	1·5	1·5
Girardi-Heart	–	<0·5	1	–	–	1	1

The titers produced by swine and pandemic influenza viruses are shaded in grey.
*low-pathogenic avian influenza virus;
**highly-pathogenic avian influenza virus

poorly susceptible to pandemic H1N1v. Swine influenza virus differed because it infected weakly A-172 and Girardi Heart cell cultures, which was not the case for H1N1v viruses.

Induction of apoptosis

Our study has shown that all influenza viruses were able to induce apoptosis in the cell cultures tested. The degradation of chromatin found in the nucleus with Hoechst-33258 staining was seen before the first symptoms of cytopathic effect (CPE) in monolayer of cells. In cell cultures where the CPE was not visible, high doses of virus still induced apoptotic response.

The process of apoptosis is rather well studied in MDCK cells and some other cell types, so we've focused on three human monolayer cell cultures that are relatively poorly studied: A-549, ECV-304, and FLECH. These cell cultures are less susceptible to viral infection, and besides, it was interesting to find out whether the viruses that do not cause any CPE do infect these cultures. A-549 turned out to be most sensitive to apoptotic response, while FLECH turned out to demonstrate weak reaction. Time needed for apoptosis induction by different flu viruses also varied. The earliest apoptosis was noted for H5N1 and H3N2 viruses and H1N1 viruses induced apoptosis at about 20 h post-infection.

It is well-known that apoptosis can be induced only by a reproducing virus, and that UV-kills viruses that are not capable of it. We tested whether swine and pandemic H1N1v viruses (that do not show CPE in these cultures) do replicate in them and induce apoptosis with the help of monoclonal antibodies against viral NP. The obtained data show that they indeed do replicate in these cell cultures, as we observed NP fluorescence, and that they also induce apoptosis (see Table 2).

Stimulation of cell culture proliferation

We've shown earlier [13] that influenza A viruses can stimulate proliferation and apoptosis in T- and B-lymphoblastoid lines and histiocytes when infected with low MOI. Thus, we've tested the ability of swine and pandemic H1N1v viruses in this aspect. It was shown that these viruses were comparable with the effect seen for seasonal H1N1 virus. Moreover, swine influenza virus induced stronger apoptotic response in hemablastoid cell lines in comparison with pandemic H1N1v viruses, which also have a swine origin.

We also checked the ability of flu viruses to influence monolayer cell cultures growth. The data clearly indicated that only ECV-304 endothelial line and T-98G glioblastoma line displayed cell proliferation in response to low MOI. Apoptosis wasn't registered in these stimulated cultures, apparently because the MOI was very low. All the other monolayer cultures didn't respond to low MOI by stimulation of their proliferation.

Discussion

Interaction between an influenza virus particle and a host cell can follow several scenarios. CPE seen in infected cells is accompanied with high rates of viral particles production and leads to cell death. The death itself may be through apoptotic or necrotic pathways.[4,8] Also, infection process in low doses can stimulate cell proliferation – the effect seen for hemablastoid lines, histiocytes, peripheral blood cell lines,[14,15] and in glioblastoma and endothelial cell lines as it was described here. Considering the origin of ECV line,[16] these cells bear all the antigenic, biochemical, and physiological traits of umbilical cord and are actively used in pharmacological tests as well as glioblastoma cells; they also are of special interest for oncogenesis studies.

Table 2. Replication, apoptosis induction, and NP synthesis of influenza viruses in A-549, ECV-304 and FLECH cell cultures. The numbers represent the $\log_{10}$ $TCID_{50}$/0·2 ml calculated by Reed-Muench method as described in.[17] The (−) symbol means that no CPE could be observed in any dilution and no hemagglutination could be registered. The (+) symbol means that apoptosis was observed with Hoechst-33258 staining

	A-549			ECV-304			FLECH		
Virus/Cell culture	Apoptosis	lg TCID 50/0·2 ml	NP fluorescence	Apoptosis	lg TCID 50/0·2 ml	NP fluorescence	Apoptosis	lg TCID 50/0·2 ml	NP fluorescence
A/Brisbane/10/07 (H3N2)	(+)	1·5	Yes	(+)	2	Yes	(+)	2	Yes
A/Brisbane/59/07 (H1N1)	(+)	1·5	Yes	(+)	2	Yes	(+)	1·5	Yes
A/Chicken/Kurgan/5/05 (H5N1)	(+)	2	Yes	(+)	4·5	Yes	(+)	2·5	Yes
A/S.-Petersburg/5/09 (H1N1v)	(+)	(−)	Yes	(+)	1	Yes	(+)	(−)	Yes
A/Swine/1976/31 (H1N1 swine)	(+)	(−)	Yes	(+)	1	Yes	(+)	(−)	Yes

Though the productive replication and production of progeny viruses in human cell lines was generally low, it is evident that viral infection does occur in these cells, even for swine and H1N1v viruses. It can be demonstrated by the presence of NP *de novo* synthesis and by stimulation of virus-induced apoptosis.

In fact, we observe a contradiction: avian influenza viruses actively reproduce in human cell lines, but we do not see their vast spreading in human population, while H1N1v viruses that hardly replicate in all human cultures tested have caused the latest pandemic.

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Genetic characterization of H4, H5, and H13 type avian influenza viruses isolated from wild birds in Kazakhstan

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Introduction

Influenza viruses continue to cause problems globally in humans and their livestock, particularly poultry and pigs, as a consequence of antigenic drift and shift, resulting frequently and unpredictably in novel mutant and reassortant strains, some of which acquire the ability to cross species barriers and become pathogenic in their new hosts.

Long-term surveillance of influenza in migratory waterfowl in North America and Europe have established the importance of Anseriformes (waterfowl) and Charadriiformes (gull and shorebird) in the perpetuation of all known subtypes of influenza A viruses. The available evidence suggests that each of the 16 hemagglutinin (HA) and nine neuraminidase (NA) subtype combinations exist in harmony with their natural hosts, cause no overt disease, and are shed predominantly in the feces.[1,2]

In this study we determined the subtypes and prevalence of low-pathogenic influenza A viruses present on the territory of Kazakhstan in 2004–2006 and further analysed the HA and NA genes of these isolates in order to obtain a more detailed knowledge about the genetic variation of influenza A virus in their natural hosts.

Materials and methods

Virus isolation and characterization

Virus isolation (A/mute swan/Aktau/1460/2006(H5N1), A/common pochard/Aktau/1455/2006(H4N6), A/coot/Aktau/1454/2006(H4N6), A/great black-headed gull/Atyrau/743/2004(H13N6), A/great black-headed gull/Atyrau/744/2004(H13N6), A/great black-headed gull/Atyrau/767/2004(H13N6), A/great black-headed gull/Atyrau/773/2004(H13N6)) was performed in a laboratory of ecology viruses at the Institute of microbiology and virology, Almaty, Kazakhstan (expect for A/swan/Mangystau/3/2006(H5N1) (Institute for Biological Safety Problems, Gvardeiskiy, Zhambyl Oblast, Kazakhstan)). Samples that were identified as influenza A virus positive by matrix rRT-PCR were thawed, mixed with an equal volume of phosphate buffered saline containing antibiotics (penicillin 2000 U/ml, streptomycin 2 mg/ml, and gentamicin 50 μg/ml), incubated for 20 minutes at room temperature, and centrifuged at 1500 ***g*** for 15 minutes. The supernatant (0·2 ml/egg) was inoculated into the allantoic cavity of four 9-day old embryonated hens' eggs as described in European Union Council Directive 92/40/EEC.[3] Embryonic death within the first 24 hour of incubation was considered as non-specific, and these eggs were discarded. After incubation at 37°C for 3 days the allantoic fluid was harvested and tested by haemagglutination (HA) assay as describe in European Union Council Directive 92/40/EEC. In the cases where no influenza A virus was detected on the initial virus isolation attempt, the allantoic fluid was passaged twice in embryonated hens eggs. The number of virus passages in embryonated eggs was limited to the maximum two to limit laboratory manipulation. A sample was considered negative when the second passage HA test was negative. The subtypes of the virus isolates were determined by conventional haemagglutination inhibition (HI) test and neuraminidase inhibition (NI) test, as describe in European Union Council Directive 92/40/EEC.[3]

RNA extraction and PCR with specific primers

RNA was extracted from infective allantoic fluid using RNeasy Mini Kit (QIAGENE, GmbH, Germany) according to the manufacturer's instructions. The RNA was converted to full-length cDNA using reverse transcriptase. The RT mix comprised 2·5 μl of DMPC water, 5 μl of 5× First Strand buffer (Invitrogen), 0·5 μl of 10 mm dNTP mix (Amersham Biosciences), 2 μl of 50 mm UNi12 primer, 32 U of RNAguard (Amersham Biosciences), 200 U of

MMLV reverse transcriptase (Invitrogen) and 5 μl RNA solution in total volume of 25 μl. The reactions were incubated at 42°C for 60 minutes followed by inactivation of the enzyme at 95°C for 5 min.

PCR amplification with HA and NA gene specific primers was performed to amplify the product containing the full length NS gene. Twenty-five microliter PCR-mix contained 1× Platinum Taq buffer (Invitrogen), 200 μm dNTP, 2·5 mm $MgCl_2$, 240 nm each of Fw primer and Rw primer, 1 U Platinum Taq DNA Polymerase (Invitrogen) and 3 μl cDNA. Reactions were placed in a thermal cycler at 95°C for 2 min, then cycled 35 times between 95°C 20 seconds, annealing at 58°C for 60 seconds, and elongation at 72°C for 90 seconds and were finally kept at 8°C until later use.

Phylogenetic and sequence analysis

Sequences of the purified PCR products were determined using gene specific primers and BigDye Terminator version 3·1 chemistry (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Reactions were run on a ABI310^{TM} DNA analyzer (Applied Biosystems). Sequencing was performed at least twice in each direction. After sequencing, assembly of sequences, removal of low quality sequence data, nucleotide sequence translation into protein sequence, additional multiple sequence alignments, and processing were performed with the Bioedit software version 7·0·4·1 with an engine based on the Custal W algorithm. The phylogenetic analysis, based on complete gene nucleotide sequences were conducted using Molecular Evolutionary Genetics Analysis (*MEGA*, version 4·0) software using neighbor joining tree inference analysis with the Tamura-Nei γ-model, with 1000 bootstrap replications to assign confidence levels to branches.[4–7]

HA and NA sequences obtained from GenBank

The HA and NA gene was analyzed both with selected number of influenza isolates and in comparison with virus genes obtained from GenBank were used in phylogenetic studies [22].

Nucleotide sequence accession numbers

The nucleotide sequence data obtained in this study has been submitted to the GenBank database and is available under accession numbers FJ434373, FJ436942·1, FJ434369, FJ434370, GU982281- GU982284 for HA and FJ434374, FJ436943·1, FJ434371, FJ434372, GU982285- GU982288 for NA.

Results and discussion

Avian influenza prevalence

In our study H4, H5, and H13 influenza A virus subtypes were found to circulate at the same time, in the same geographic region in the Kazakhstan. This finding most likely indicates the existence of a large reservoir of different influenza A viruses in Kazakhstan.

Phylogenetic analysis

We analyzed the HA and NA gene sequences of the eight influenza A viruses isolated in Kazakhstan together with selected number of isolates, reported between year 1941 to 2008, and previously published in the GenBank.[8]

Phylogenetic analysis of the H4 HA gene showed that all viruses separated into the American and Eurasian lineages (Figure 1). An evolutionary tree suggests that North American isolates have diverged extensively from those circulating in other parts of the world. Geographic barriers which determine flyway outlay may prevent the gene pools from extensive mixing. The lack of correlation between date of isolation and evolutionary distance suggests that different H4 HA genes co circulate in a fashion similar to avian H3 HA genes and influenza C genes, implying the absence of selective pressure by antibody that would give a significant advantage to antigenic variants.

Analysis of phylogenetic relationships among the HA5 HA genes reported in this study clearly shows that viruses belong to the western Pacific flyway, one of the major migratory flyways in this region that have subsequently spread throughout Eurasia.[9] These findings provide further

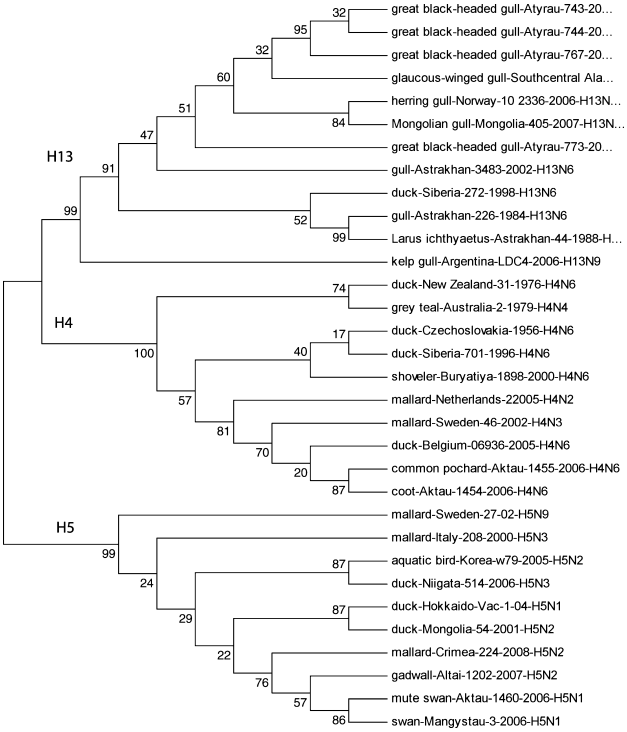

Figure 1. Phylogenetic relationships of the HA genes of representative influenza A viruses isolated in Kazakhstan. Analysis was based on nucleotides 22–1032 of the HA gene.

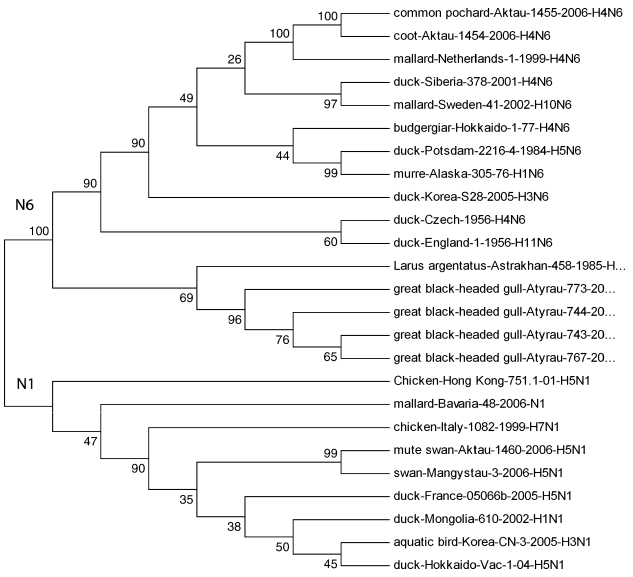

Figure 2. Phylogenetic relationships of the neuraminidase (NA) genes of representative influenza A viruses isolated in Kazakhstan. Analysis was based on nucleotides 7–1400 of the NA gene.

evidence of the dynamic influenza virus gene pool in this region. Along the western Pacific migratory flyway, the influenza virus gene pool in the domestic waterfowl of southern China has 'mixed' longitudinally with viruses isolated from Japan, Mongolia, and Siberia. However, it appears that there has also been 'mixing' latitudinally through overlapping migratory flyways, thereby facilitating interaction between the influenza virus gene pool in domestic waterfowl in the eastern and western extremities of the Eurasian continent. This helps to explain the latitudinal spread of the Qinghai-like (clade 2·2) H5N1 virus in the last 2 years, while H5N1 outbreaks in Korea and Japan may represent the longitudinally transmitting pathway.[10]

HA of subtype H13 so far has been found exclusively in shorebirds, such as gulls, and in a pilot whale (potentially a spillover from shorebirds), but not in other avian species that are natural hosts of influenza A virus, such as ducks and geese; therefore the study of the evolution of these viruses is very interesting. Phylogenetic analysis H13 HA gene revealed three significantly different evolutionary lines: an American line, a European line, and a line comprising the isolates from America and Eurasia.[11]

Further we analyzed NA genes of influenza viruses (Figure 2). The NA gene is important both because of its functional role in promoting the dissemination of the virus during infection, and because, like HA, it is a principal target of the immune system. It was shown that phylogeny of NA genes of influenza have the same properties as hemagglutinin. NA genes of kazakhstanian viruses belong to Eurasian lineage of virus evolution.

Obtained data are important for surveillance and diagnostics because some of the LPAI viruses examined in this study can infect and be shed by chickens and turkeys and may have epidemiology potential during further recombination with other influenza viruses.

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Evolution of H6N1 avian influenza viruses in chickens

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Introduction

Influenza virus is divided into different subtypes based on hemagglutinin (HA) and neuraminidase (NA) on the virus surface. Within each subtype, HA continues to mutate and produce immunologically distinct strains, as antigenic drift. The continuous mutation of influenza virus (IV) is important for annual epidemics and occasional pandemics of disease in humans. Antigenic drift requires vaccines to be updated to correspond with the dominant epidemic strains. In humans, IVs show both antigenic drift frequently. In contrast, IVs from birds are in evolutionary stasis,[1] and they show little amino acid changes.[2,3] The reason is that IVs in bird intestine are not subjected to strong immune selection.

Hemagglutinin (HA) gene of influenza A virus encodes the major surface antigen, which is the target for the protective neutralizing antibody response that is generated by infection or vaccination. In humans, influenza A viruses show antigenic drift with amino acid changes in the globular head of the HA so as to evade herd immunity of the population. On the contrary, avian influenza A viruses show evolutionary stasis in wild birds. H6 AIVs have occurred frequently in chicken farms in the world.[4] Although vaccination is not permitted, H6N1 AIVs have circulated in Taiwan for a time.[5] The seroprevalence in chicken flocks reaches about 50% in the field. H6N1 AIVs invades internal organs, such as kidney and lung.[6] Thus, viruses in chicken flocks are pressured into antibody selection. Here, we report that H6N1 AIVs in the field have showed evolutional changes instead of evolutional stasis.

Materials and methods

Virus isolation

In response to requests from poultry farmers for diagnostic investigations of illness in poultry flocks, the authors did necropsy at the pen-site. After careful examination, tracheae were taken and kept in cold for virus isolation in the laboratory. For avian influenza virus isolation, trachea was homogenized 1:10 in TPB with antibiotics. The homogenate was frozen and thawed three times and then centrifuged at 1157 g for 15 minutes. The supernatant was passed through a 0·45 μm filter. The homogenate was examined for the presence of virus by inoculation into five 9- to 11-day-old specific-pathogen-free (SPF) chicken eggs for two passages. Thirteen H6N1 AIVs were isolated in this laboratory during 2000 and 2008 from different parts of Taiwan.

Virus strains from the GenBank

Besides the viruses isolated in this laboratory, the HA sequences of 27 chicken H6N1 AIVs were from the GenBank. The accession numbers of hemagglutinin of AIV reference strains included in this study were as the following: G2/87, DQ376619; G23/87, DQ376620; 0824/97, DQ376621; na3/98, DQ376622; 165/99, DQ376626; 0705/99, DQ376624; ns2/99, DQ376623; SP1/00, DQ376628; 0329/01, DQ376633; 1205/01, DQ376630; 1212/01, DQ376631; 1215/01, DQ376632; 0208/02, DQ376641; 0408/02, DQ376642; PF1/02, DQ376635; PF2/02, DQ376636; PF3/02, DQ376637; A37/02, DQ376639; 0320/02, DQ376638; 0107/02, DQ376640; 0222/02, DQ376634; 0706/03, DQ376644; 1203/03, DQ376645; ch1006/04, DQ376649; 0114/04, DQ376647; A342/05, DQ376653 and 0204/05, DQ376652.

Virus growth and RNA extraction

The viruses isolated were propagated in the allantoic cavities of 10-day-old embryonated SPF eggs for 72 hour. The virus RNA was extracted using QIAamp Viral RNA Miniprep Kit (Qiagen).

Molecular cloning and sequencing

Viral RNAs were reverse-transcribed, and full-length HA cDNAs were amplified by PCR using P5(+): atg att gca atc att gta ata gc and p4(−): tta tat aca tat cct gca ttg cat. PCR products were cloned in yT&A (Yeastern, Taipei) and sequenced.

Sequence and structure analysis

DNA sequences were compiled and edited using Lasergene sequence analysis software package (DNASTAR). Sequence alignment analysis was performed with the MegAlign program using the Clustal W multiple alignment algorithm. The 3D-structure of HA were modified using UCSF Chimera (University of California).

Monoclonal antibodies (mAbs)

Six-week-old BALB/c mice were injected emulsion intraperitoneally with 100 μg of purified and concentrated A/chicken/Taiwan/2838V/00 (H6N1) virion with complete Freund's adjuvant. Every two weeks, the mice were boosted supplementary five times with 50 μg of virion in incomplete Freund's adjuvant. When the mice were boosted, blood was collected from tail vein and tested by the western blot assay to check the antibody titers. The mice were then injected intraperitoneally with 50 μg of virion at week 8. Five days after the last injection, the splenocytes in the mice were fused with myeloma cells (SP2/0-Ag14). One week before fusion, the myeloma cell line was expended in DMEM medium (HyClone Laboratories, Logan, UT) with 10% fetal bovine serum at 37°C to ensure they were in the exponential growth phase. The spleen cells from immunized mice were washed, harvested, and mixed with the previously prepared myeloma cells and fused by gradually adding 50% polyethylene glycol-1500. The resulting pellet was plated into 96 well tissue culture plates. Only the fused cells grew in medium with hypoxanthine- aminopterin-thymidine (HAT). With fresh medium replacement over 2 weeks, the hybridomas were ready for screening. High-titer monoclonal antibody (mAb) preparations were obtained from the ascetic fluid of mice injected with the selected hybridoma clones. The antibody from mouse ascetic fluids was purified by precipitation with ammonium sulfate, then aliquoted and frozen at −70°C, avoiding repeated freezing and thawing. Eventually, six mAbs were obtained and named CH11-D10, EB2-B3, EB2-E5, EB2-F9, FF9-F5, and FF9-F7, respectively.

Hemagglutination inhibition test

The HI test was performed following a standard method. All the viruses were diluted twofold and reacted with 1% chicken erythrocytes in the v-bottomed microtiter plate by the hemagglutination test. After agglutination, four hemagglutinating units of A/chicken/Taiwan/2838V/00 (H6N1) and ascetic fluids from the immunized mice of the six mAbs were prepared for HI test. HI titers of 2^4 or more were regarded as positive.

Results

Clinical characteristics

The cases submitted for diagnosis from chicken farms had respiratory signs, increase in mortality, or drop in egg production (e.g. egg production dropped from 10% to 40%). The extent of drop in egg production depended on the chicken ages. For example, the age of case 3511 was 27 weeks, a stage of increasing egg production. However, after H6N1 AIV infection, the egg production decreased 1% instead of increasing and then stayed at 65% for a week. The infected chickens showed signs of decreasing activity, anorexia from 150 g per bird to 90 g per bird, and respiratory signs. Case 2829 showed infection in the second floor first and then transmitted to third and fourth floor, indicating that the virus transmitted by air or human movement. However, most cases showed air borne transmission from one flock to another in spite of enforcing restrictions of persons entering the poultry pens and changing clothes and booths. In most cases, males' mortality was higher than that of female pen mates.

Amino acid changes in HA protein

By comparing the sequences of HA of those H6N1 viruses, we found that amino acid changes in HA1 were higher than those in HA2, showing that antigenic changes on the globular head of HA molecule rather than randomly on the whole HA protein, indicating that H6N1 viruses in Taiwan had been selected in the presence of antibody pressure. The aa residues and changes that showed yearly trends were the followings: A-5S, I19S, V30I, N35S, E39K, L45M, E54D, Q66K, A94V, or T, S127N, S128R, K133N, Y138D, N139T, S140I, G141D, L149V, I152V, G155E, T188N, G226S, A285V, K304E, D386N, I533M, and M535I. However, their significance on antigenic variation was previously unknown.

Hemagglutination inhibition of monoclonal antibodies to viruses

By hemagglutinination inhibition (HI) assays, except mAb CH11-D10, all other monoclonal antibodies elicited from 2838V/00 showed different HI titers with the different H6N1 viruses (Table 1). However, those 5 mAbs showed negative HI to 2829 and 2831, the early H6N1 strains. This indicated that the epitopes recognized by those mAbs were undergoing antigenic drift.

Table 1. Hemagglutination inhibition titers of different monoclonal antibodies to different H6N1 AIV strains

Virus strain	HI titers of monoclonal antibody					
	CH11-D10	EB2-B3	EB2-E5	EB2-F9	FF9-F5	FF9-F7
2829/00	0	3	3	3	0	1
2831/00	0	3	3	2	0	1
2838V/00	3	8	7	7	6	6
2896/01	3	8	6	7	6	6
3072/03	3	8	6	7	6	6
3115/03	2	8	6	6	5	6
3127/03	3	8	7	7	6	6
3152/03	3	8	8	7	6	7
3153/03	3	7	7	6	5	5
3511/08	4	9	7	7	6	6

Discussion

Although some H6N1 AIVs have been isolated from wild birds, no related virus was detected in domestic poultry in Taiwan. The sequences of H6N1 AIVs from wild ducks are different from those originated from chickens.[5] The transmission of AIVs from wild birds to domestic poultry is low because most poultry farms in Taiwan are located away from migratory flyways.

Many H6N1 AIVs have circulated in chicken farms in Taiwan since 1972. About 50% of chicken flocks have anti-H6 AIV antibody in Taiwan.[6] The present results indicated that the viruses mutated more frequently on the globular head than HA2 because they face antibody pressure from chickens. This phenomenon is similar to the viruses in humans. Human IVs show antigenic drift by antibody selection in the human population. These mutations are clustered at the five epitopes, A, B, C, D, E, of globular head of HA protein (H3 reference). On the contrary, avian viruses in wild birds show evolutionary stasis because the viruses grow in the intestine and induce no antibody to the hosts. Thus, the mutations are randomly distribution through HA1 and HA2.

Unlike AIVs in wild birds, the H6N1 AIVs in chickens have showed mutation in the presence of antibody selection pressure in chickens.

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Influenza virus surveillance in migratory ducks and sentinel ducks at Poyang Lake, China

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Keywords gene pool, migratory ducks, sentinel ducks, virus interactions.

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Introduction

Aquatic birds are recognized as the natural reservoirs of the influenza A virus as all known subtypes (H1-H16, N1-N9) have been found in them. Phylogenetic analyses of influenza viruses found in other animals revealed that all were directly or indirectly derived from viruses resident in aquatic birds.[1] However, the prevalence, movement, and evolutionary dynamics of influenza viruses in these avian hosts have not been well defined.

Southern China was hypothesized to be an 'epicenter' for the generation of human pandemic influenza viruses as all major influenza pandemic viruses in the 20th century emerged from this region.[2] The ecological background that facilitates the occurrence of these pandemic influenza strains has not been fully explored.

In the past two decades, four lineages, belonging to H5N1, H9N2, and H6N1 viruses, have become established and long-term endemic in different types of poultry in this region.[3–5] Some of these viruses were disseminated to many countries in Eurasia and Africa and have continued to cause sporadic human infection, posing a persistent pandemic threat to the world.[6] In the mean time, the endemic influenza lineages have undergone extensive genetic reassortment events giving rise to many variants, dramatically increasing the genetic diversity of the influenza virus in this region.

Table 1. Prevalence of AIV from migratory and sentinel ducks in Poyang Lake during 2002–2007

Year	Migratory duck			Sentinel duck		
	Sample No	Isolates No	Isolation rate (%)	Sample No	Isolates No	Isolation rate (%)
2002	153	1	0·65[a]	739	91	12·31
2003	1080	13	1·20	5265	100	1·90
2004	2951	14	0·47	8026	439	5·47
2005	5925	38	0·64	8308	489	5·89
2006	1140	15	1·32	8385	350	4·17
2007	259	9	3·47	5752	212	3·69
Total	11 508	90	0·78[b]	36 475	1681	4·61

[a]Isolates No/Samples No.
[b]Total isolates No/Total samples No.

Questions remain as to how and where these viruses emerged, and what were the sources of the gene segments incorporated within the novel reassortant variants of the H5N1, H9N2, and H6N1 virus lineages. To address these questions, surveillance of influenza in migratory and domestic (sentinel) ducks has been conducted since 2002 at Poyang Lake, the biggest fresh-water lake and the major migratory bird aggregation site in southern China. The aim of this study is to identify the prevalence, seasonality, and movement of virus between migratory and domestic ducks.

Materials and methods

Sampling

Migratory ducks were captured during over-wintering, from November to March. Cloacal swabs and blood samples were collected from each individual bird. All birds were released after sampling. To observe the interaction between migratory ducks and domestic birds, we also sampled domestic ducks from two duck farms (designated as sentinel ducks) surrounded by rice fields and inaccessible to other types of poultry, but accessible to migratory birds. That is, the sentinel ducks share the same water body with migratory ducks and have the chance to spread viruses to each other.

For sentinel ducks, sampling was conducted fortnightly, all year-round, on the two farms from August 2002 onwards. Cloacal swabs and fresh fecal droppings were taken. About 200 birds were randomly sampled fortnightly from these farmed ducks. All swabs were soaked in vials containing 1·5 ml transport medium with antibiotics and kept on ice-packs during sampling and immediately stored in −80°C freezers for further use.

Serum treatment, virus isolation, and identification

Blood samples from migratory ducks were treated according to methods previously described.[7] Serological survey and virus subtyping in migratory and sentinel ducks used hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests as previously described.[3] For isolates that were not identified by reference antisera, subtypes were determined by RT-PCR using subtype specific HA and NA diagnostic primers.

Results

Prevalence and seasonal patterns of influenza virus in migratory and sentinel ducks

During 2002–2007 a total of 11 508 cloacal swabs from migratory ducks and 36 475 cloacal or fecal swabs from sentinel ducks were collected at Poyang Lake. From these specimens, 90 influenza isolates were obtained from migra-

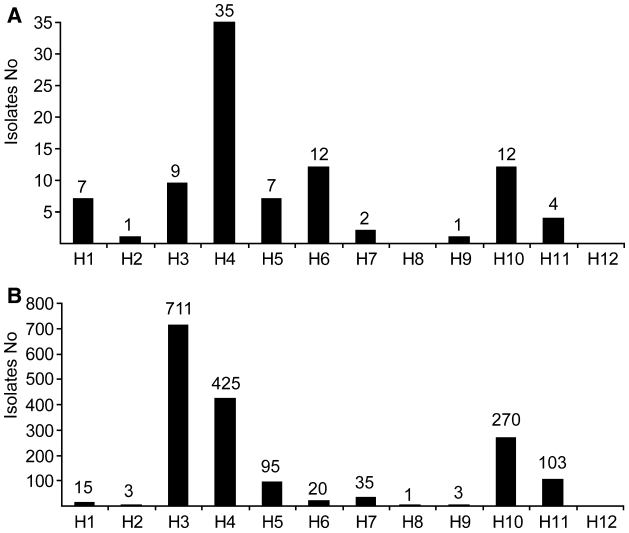

Figure 1. HA subtype distribution from migratory ducks (A), and sentinel ducks (B) at Poyang Lake during 2002–2007.

tory ducks and 1681 from sentinel ducks; isolation rates of 0·78% and 4·61%, respectively (Table 1).

It was noted in sentinel ducks that virus occurrence formed a seasonal peak from November to February, which completely overlapped the over-wintering months of migratory ducks. This suggests that virus movement or transmission between migratory and sentinel ducks occurred during this period at Poyang Lake.

Distribution of influenza virus HA subtypes in migratory and sentinel ducks

Eleven HA subtypes, from H1 to H11, of influenza viruses were detected from migratory and sentinel ducks (Figure 1). The most frequent HA subtypes from migratory ducks were H4, H6, H10, and H3, followed by H1 and H5, while H11, H7, H2, and H9 were only sporadically detected. There were seven H5 subtype isolates detected from migratory ducks, including one LP H5 virus and six HP H5N1 viruses. These H5N1 viruses were isolated on two sampling occasions in early 2005 and are phylogenetically related to the endemic H5N1 virus in domestic poultry in southern China.[8] No H8 subtype virus was detected from migratory ducks during the survey period.

For sentinel ducks, H3, H4, H10, and H11 were the most common HA subtypes, followed by H5, H7, and H6, while H1, H2, H9, and H8 were rarely detected. All H5 viruses isolated from sentinel ducks belonged to the LP H5 viruses. Only one H8 subtype virus was detected from sentinel ducks during the survey period.

NA subtype distribution and antigenic combinations in migratory ducks

Six NA subtypes were detected in migratory ducks, N1, N2, N3, N5, N6, and N8. Among these isolates, N6 was the most common subtype found followed by N1, N2, and N3, while N5 and N8 were only sporadically detected.

There were 27 HA and NA antigenic combinations found in migratory ducks. H4N6 was the most common combination and was found in 28 (>30%) of isolates. H5N1, H6N1, H6N2, and H10N6 were found in five to six isolates, while 11 antigenic combinations (e.g. H4N3, H10N3) had two to four isolates, and another eleven combinations (e.g. H1N6, H2N8) had only one isolate.

Serological surveys in migratory ducks

Thirty positive samples (HI titer ≥20) were identified from 715 blood samples collected during November and December in 2004. Among these, 15 samples were positive to H6, 8 were positive to H9, 5 were positive to H1, and 2 were positive to H4. One serum sample was positive to both H4 and H6, which suggested co-infection of influenza virus in migratory ducks might occur in natural conditions.

Discussion

Poyang Lake, which is located in the northeastern part of Jiangxi province, is the largest freshwater lake in China and is part of the Eastern Asia-Australia migration route.[9] Every year, hundreds of thousands of migratory ducks congregate at Poyang Lake during the migration season.[9]

Recent farming practice involves raising domestic waterfowl in dense populations in the Poyang Lake region. Farm-raised domestic waterfowl are allowed to feed in and share the same water body with migratory birds, thereby facilitating direct interactions between domestic waterfowl and free-ranging migratory birds. This makes Poyang Lake an ideal site to observe the dynamics of influenza virus interactions between migratory and sentinel ducks in southern China.

In our longitudinal surveillance during 2002–2007, the overall virus isolation rate from migratory ducks was less than 1%, which suggests a low prevalence of viral infection during the birds' southern migration. Similar results have been observed in Taiwan, which is also an important stop-over site for migratory birds along the Eastern Asia-Australia migration route during 10 years of surveillance.[10] The overlap in seasonal patterns of virus infection between migratory and sentinel ducks found in our study suggests that virus movement or transmission between migratory and sentinel ducks occurred during the period of time migratory birds were at Poyang Lake.

The HA subtypes harbored in migratory and sentinel ducks were similar in our study. For migratory ducks, H4, H6, H10 were the predominant subtypes, while H3, H4, and H10 were the major subtypes in sentinel ducks. HPAI H5N1 was only detected from migratory ducks in early 2005 on two sampling occasions. From phylogenetic analyses the H5N1 viruses isolated from migratory ducks were closely related to the viruses endemic in domestic poultry in southern China.[8] Therefore, it appears that H5N1 viruses endemic in domestic poultry could be transmitted to migratory ducks via close contact in southern China. Only LP H5 viruses were detected from sentinel ducks at Poyang Lake during this period. Whether H5N1 virus infection was absent from sentinel ducks at Poyang Lake needs further investigation.

Serological surveys provided further evidence for the prevalence of AIV in migratory ducks at Poyang Lake. The serological results in 2004 did not match well with the epidemiological results during 2002–2007, which suggests that influenza virus infection in migratory birds could be influenced by multiple factors, such as host immune status, population size, spatial and temporal variations, and migration routes.

Southern China has the biggest domestic duck population in the world. Our study demonstrates that dynamic interactions between migratory ducks and sentinel ducks

occurred frequently throughout the surveillance period. Thus, sentinel ducks could be treated as intermediate hosts between the ''real gene pool'' from migratory ducks and domestic poultry in the whole influenza virus ecosystem. A sentinel duck sampling system may be a feasible method to represent the viruses in the natural gene pool and a baseline for virus or gene interactions between migratory and domestic ducks.[11] Further investigations and surveillance are required to better understand the role of the domestic duck population in facilitating virus interactions and the generation of genetic diversity.

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Continuing evolution of H9N2 influenza viruses endemic in poultry in southern China

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Introduction

Two distinct lineages of H9N2 influenza viruses represented by A/Chicken/Beijing/1/94 (Ck/Bei-like) and A/Quail /Hong Kong/G1/97 (G1-like) have become established and endemic in poultry in southern China.[1] These established H9N2 lineages continue evolving to generate many different reassortant variants (or genotypes) [1,2] and are causing sporadic cases of human infection.[3,4]

Studies of H9N2 viruses isolated from pigs in Hong Kong and Shandong Province have also raised the possibility of reassortment with human-like viruses from pigs.[5,6] In addition, H9N2 viruses isolated beyond the late 1990s had preferential binding with α-2,6-NeuAcGal human-like receptors.[7]

These observations suggest that the H9N2 influenza viruses still have pandemic potential. Unlike highly pathogenic H5N1 influenza viruses that have been rarely detected in the live-poultry markets in Hong Kong since 2002, H9N2 viruses are still frequently isolated in our surveillance program. Therefore, we try to understand the continuing evolution of H9N2 viruses through genetic characterization and phylogenetic analyses of the viruses isolated in Hong Kong live-poultry markets from 2005 to 2009.

Materials and methods

A total of 47 421 terrestrial poultry were sampled at different live-poultry markets in the Hong Kong SAR between

January 2005 and December 2009. Of those samples, 29 691 were from chickens and the others were from minor poultry species including chukar, pheasant, guinea fowl, silky chicken, and pigeon. Fecal droppings, cloacal and tracheal swabs, drinking water, and environmental samples from cages were collected into transport medium. Viruses were isolated in 9- to 11-day old embryonated eggs as described previously.[8]

Virus isolates from positive sampling occasions were selected for sequence analysis. RNA extraction, cDNA synthesis, and PCR were carried out as described previously.[8] DNA sequencing was performed using BigDye Terminator v3·1 cycle sequencing kit on an ABI 3730 DNA analyzer (Applied Biosystems) following manufacturer's instructions.

All sequences were assembled and edited with Lasergene 7·0 (DNASTAR, Madison, WI) software. Sequence alignment and residue analysis were performed with the BioEdit sequence alignment editor, version 7·0.[9] All eight gene segments of sequenced viruses were characterized and analyzed phylogenetically together with virus sequence data available in public databases. Maximum-likelihood trees were constructed using Garli 0·96.[10] Estimates of the phylogenies were calculated by performing 1000 neighbor-joining bootstrap replicates using PAUP*4·0.[11]

Results

Prevalence of H9N2 viruses in chickens and minor poultry species

Systematic surveillance of live-poultry in Hong Kong from 2005 to 2009 resulted in 1088 H9N2 isolates from 47 421 samples (overall isolation rate, 2·3%) (Table 1). There were 966 strains isolated from 42 253 chicken samples (isolation rate, 2·3%). Of these viruses, four were isolated from 1556 tracheal swabs (isolation rate, 0·3%), while 541 isolates were isolated from 29 030 cloacal or fecal swabs (isolation rate, 1·9%). An additional 421 isolates were collected from 6926 drinking water samples (isolation rate, 6·1%).

There were 122 strains of H9N2 viruses isolated from 5168 minor poultry samples (isolation rate, 2·4%) (Table 1). Of these viruses, only one was isolated from 1209 tracheal swabs (isolation rate, 0·1%), whereas 72 strains of viruses were isolated from 3610 cloacal or fecal swabs (isolation rate, 2·0%). The isolation rate in drinking water in minor poultry was again higher when compared with other sampling methods with 49 strains isolated from 297 drinking water samples (isolation rate, 16%).

Taken together, these findings suggest that the H9N2 viruses mainly replicated in the intestinal tract of chickens and minor poultry species. Also, the high isolation rate in drinking water samples could be a sensitive indicator for monitoring the prevalence of H9N2 viruses in the field.

Phylogenetic analysis of surface genes

To better understand the evolutionary pathway of H9N2 viruses in southern China, 50 representative viruses, isolated from Hong Kong live-poultry markets from 2005 to 2009, were sequenced and genetically characterized. Phylogenetic analysis of the H9 HA gene revealed that Ck/Bei-like viruses were predominant and one chicken isolate had a G1-like HA gene (Figure 1). This is the first time the G1-like H9 HA gene has been detected in chickens from live-poultry markets in Hong Kong. The Ck/Bei-like lineage is further divided into two subgroups as previously described.[1] Subgroup 1 is represented by Qa/ST/243/00 and subgroup 2 is represented by Dk/HK/Y280/97. All H9N2 viruses in this study belonged to subgroup 2 of the Ck/Bei-like lineage except for the virus with the G1-like HA gene.

Phylogenetic analysis of the NA gene also showed a similar evolutionary pattern to the HA gene with all viruses clustered within the Ck/Bei-like lineage. These results revealed that Ck/Bei-like viruses are predominant in both chickens and minor poultry.

Phylogenetic analysis of internal viral genes

All of the PB1, PA, NP, NS and M genes clustered with those of H9N2 lineage viruses previously prevailing in ter-

Table 1. Prevalence of H9N2 viruses from chickens and minor poultry species in Hong Kong live-poultry markets from 2005 to 2009

	Chicken			Minor Poultry		
Year	Total samples	Positive samples	Isolation rate (%)	Total samples	Positive samples	Isolation rate (%)
2005	11 019	235	2·1	1540	2	0·1
2006	10 689	223	2·1	1054	4	0·4
2007	9062	387	4·3	828	16	1·9
2008	6889	105	1·5	1017	99	9·7
2009	4794	16	0·33	729	1	0·1
Total	42 253	966	2·3	5168	122	2·4

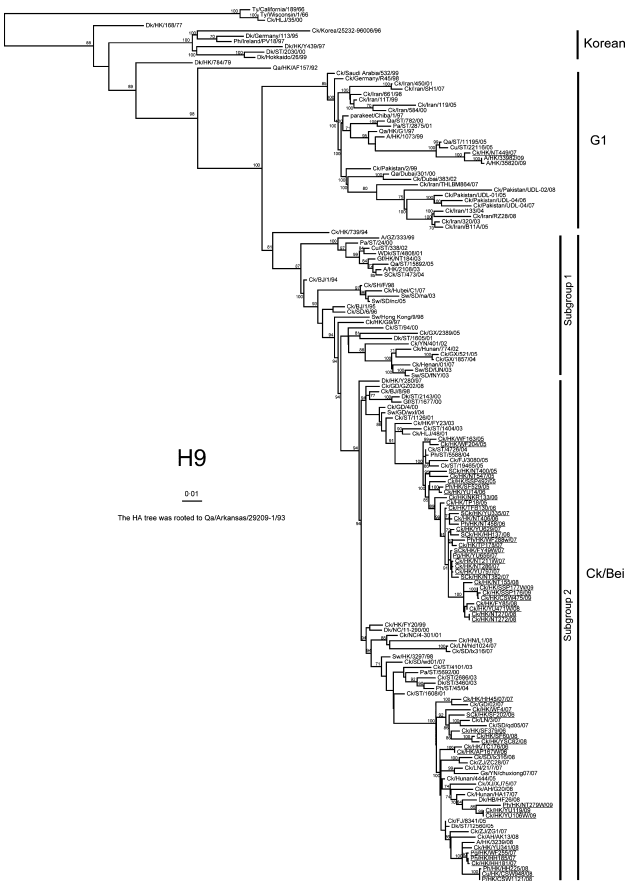

Figure 1. Phylogenetic relationship of HA genes of representative influenza viruses isolated in southern China. Viruses characterized in this study are underlined.

restrial poultry in southern China. Phylogenetic analysis of the PB2 gene revealed three different lineages; G1-like (n = 1), Ck/SH/F/98-like (n = 15), and Unknown avian (n = 34). The SH/F/98-like lineage (or F/98-like) was previously reported in eastern China [12] and was used previously for vaccine production in an intensive vaccination program.[13] This PB2 gene lineage was also distinguishable from the Ck/Bei-like lineage and its presence in the viral genome may be due to reassortment between the vaccine strain and field isolates, followed by selective establishment in terrestrial poultry.

Genotyping

Gene constellation analyses of the 50 viruses revealed six genotypes. Thirty-four of the viruses analyzed belonged to two genotypes, B14 and B15, which were also the prevailing reassortants found in other provinces in southern China since 2005.[2] The remaining sixteen viruses belonged to four novel genotypes that have not been identified before in this region.

Discussion

Characterization of H9N2 influenza viruses isolated from live poultry in Hong Kong markets from a 5 year surveillance program revealed that Ck/Bei-like viruses were predominant in southern China and were continuing to evolve. Two recognized and four novel genotypes were identified in this study.

One characterized virus, Ck/HK/NT499/07, had a G1-like HA gene (the first time this has been detected in Hong Kong poultry markets) that showed a close relationship with two human H9N2 strains isolated in 2009. G1-like viruses were usually detected and caused outbreaks in chickens of Middle Eastern and European countries,[14–16] and minor poultry, mainly quail, in southern China.[1] Whether the G1-like virus was transmitted from China to Middle Eastern and European countries, as the highly pathogenic H5N1 virus did in the last five years, or vice versa, is still unknown. Since the Ck/HK/NT499/07 strain clustered with other G1-like strains isolated previously in minor poultry in southern China, the G1-like viruses in chicken may be due to interspecies transmission from minor poultry species.

Genetic studies demonstrated that reassortants with genotypes B14 and B15 persistently occurred in either chickens or other minor poultry species from 2005 to 2009. Other genotypes that were prevalent in southern China might be being gradually replaced and four novel genotypes were identified in this study. These novel genotypes were generated through reassortment of viruses with different lineages. A newly emerged F/98-like lineage originating from eastern China is responsible for generation of some of the novel genotypes found in this study.[12] The Ck/Bei-like lineage is gradually being replaced by F/98-like lineages which are becoming dominant in northern and eastern China.[12,17] Animal experiments have also demonstrated that F/98-like viruses are more effective in replication and transmission in chickens compared with Ck/Bei-like viruses.[18]

Since the F/98-like lineage of the PB2 gene has been introduced into southern China, this newly emerged lineage may have a higher tendency to replace the RNP genes in the circulating Ck/Bei-like viruses and subsequently become the endemic virus in terrestrial poultry.

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Swine influenza in Vietnam: preliminary results of epidemiological studies

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Introduction

In Vietnam, the modelling of the pandemic H1N1 progression estimates that 460 000 (260 000–740 000) pigs might be exposed to the virus on the basis of 410 000 cases among swine owners (220 000–670 000).[1] A poor level of biosecurity, high animal densities, and a mix of species could increase the risk of influenza virus flow, persistence, and emergence on swine and poultry farms. This study was set up in the Red River Delta, where a third of the national pig husbandry is produced.[2] The aims are to give preliminary information of the epidemiological state of swine influenza and in order to further assess the risk of infection of SwIV, through cross-species transmissions from poultry to pigs. This paper will present the preliminary results on SwIV and the risk factors of pig seropositivity in Vietnam.

Materials and methods

A cross-sectional study was conducted in two provinces of the Red River Delta in April 2009. Pig farms were randomly selected from nine communes representative of at risk area of avian H5N1. In each farm, pig and poultry were sampled and collected to virological and serological analyses. Interviews were conducted in all farms by trained interviewees. Questionnaires included closed and open questions on

livestock husbandry/management and household characteristics, such as herd size and structure, health history and vaccination, pig housing, watering and feeding system, reproduction, purchasing of animals, biosecurity measures, pig contact with poultry, and environmental factors.

The virological detection assay was performed on pools of nasal swab specimens from pigs. We investigated whether real-time RT-PCR assay could detect gene M on pools of nasal swab specimens before attempting virus isolation from individual nasal swab specimens. The poultry and pig sera were tested against influenza type A with an Enzyme-like immunosorbant assay (ELISA) competition test IDVET©. This commercial kit is designed to specifically detect antibodies directed against the NP protein antigen of influenza type A viruses. The positive serum samples were examined in hemagglutination inhibition (HI) to determine antibody titers and subtypes. The HI test was tailored for H1, H3, and H9 subtypes in pigs and H6 and H9 subtypes in poultry. Seroneutralization tests by pseudo particles were used to test the presence of antibodies directed against H5 subtype.

We analysed the data for relationships between Influenza A serological status (the outcome variable) and possible risk factors using R version 2·11·1 (R Development Core team). The statistical unit was the individual. Initially, the quantitative variables were encoded into categorical variables according to the quartiles or median. Descriptive statistics (e.g., means or medians, proportions, standard deviations) were calculated for all herd-level and commune level predictors to assist in the subsequent modeling process. We also performed the independence test among all variables to determine if variables were dependant. Then, univariate analysis of potential risk factors for the pigs being positive for SwIV and estimation of odds ratios were performed using generalised linear mixed models with binary outcome and logit link function for each herd-level and commune-level variable to determine which variables were individually associated with influenza A seropositivity at a significance level of $P < 0{\cdot}30$. Herd and commune of residence were included as a random effect to account for the correlation of observations at the herd level.

The third stage of the analyses included the four herd-level variables found to be significantly ($P < 0{\cdot}30$) associated with Influenza A seropositivity. An automatic process using all possible associations between the selected variables was computed into a mixed logistic regression models, with random effects. When two variables were collinear, as determined before, only one variable was likely to enter the multivariable model, and therefore, the selection of which collinear variable to enter the model was guided by biological plausibility and statistical significance.

Results

All of the 146 pools of nasal swabs were RT-PCR negative. The maximal possible prevalence considering perfect diagnostic tests would be of 2·03% at a confidence level of 95%, in an infinite population within these regions (WinEpiscope 2·0).

Six hundred-and-nine pig sera were tested in 76 non-vaccinating farms. The herd seroprevalence of swine influenza in the commune previously infected by the avian H5N1 in the Red River Delta raised by 17·1% [8·7; 25·6] in April 2009. But among 13 seropositive farms, only four had at least two seropositive pigs. The within-herd seroprevalence is very low, and no seropositivity was detected in the majority of farms. Estimates had large confidence intervals due to small sample sizes. The individual seroprevalence raised 3·62% [1·98; 5·27]. The subtyping of seropositive sera is still in process.

Descriptive statistical analyses on five major risk factors of SwIV: farm size, breeding vs. fattening, purchasing, percentage of family income, and poultry production, were conducted. Based on this analysis, three types of farming systems were identified and included in mixed models (Table 1). Percentage of family income by pig production and poultry production were not differentiating factors for this typology. Whereas types 1 and 2 seem to be specialized in fattening, the type 3 produces and might sell piglets on the farm site.

The exploration of the different variance components indicated that the random effect variances were mainly associated with the herd, while the commune did not seem to have any effect. Therefore we included in all models only the herd as a random effect. The random effect term for herd was modelled, assuming a normal distribution with a

Table 1. Typology of farming system

Type 1: Large fattening farms	Largest scale production, with more than 40 pigs per year Specialized in fattening, and purchase more than 20 pigs per year
Type 2: Small fattening farms	Small scale of production, with less than 20 pigs per year Specialized in fattening, and purchase less than 20 pigs per year
Type 3: Medium breeding-fattening farms	Medium scale of production, with less than 40 pigs per year Breeding and fattening piglets, with rare purchase

Table 2. Seroprevalence of SwIV and univariate analysis with typology as fixed effect and herd as random effect

	Seroprevalence (%)	IC95%	OR	*P*-value
Type 1	1·93	0·53–4·87	1	–
Type 2	4·76	1·77–10·08	3·11	0·39
Type 3	6·47	3·00–11·94	5·26	0·18

common variance [$\sim N(0,\sigma 2herd)$].[3] The univariate analyses were conducted on 22 variables and typology variables, with herd as random effect. Some coefficient or confidence intervals were inconsistent because of small effectives, especially for the percentage of self-product culture or the pig free-grazing because of the lack of positive results in the dataset. The only one significant (P value < 0·1) parameter was the percentage of pig sales in the familial annual income. Surprisingly, common risk factors of swine influenza infection, such as farm size, animal movements, and sanitary parameters got low odds ratio individually (without being significant); the typology provides the hypothesis of complex interactions effects that increase the risk of infection. As shown in Table 2, the farming system type 3 got a higher seroprevalence of 6·47% [3·00–11·94] and a higher risk indicator, with OR = 5·26 (P-value = 0·18) in comparison with type 1. This finding was not significant. In the multivariate mixed model, the percentage of familial income provided by pig production was the only one significant variable, with OR = 0·22 [0·04–1·25].

Discussion

The focus on diseased animals in the winter-time is usually required in order to increase the likelihood to isolate the virus, although the isolation rate on healthy or clinical samples never exceed 6%.[4] The season and the lack of disease reports might explain the difficulties to detect influenza viruses. Additionally, the pooling method tends to decrease the isolation rate because of a dilution effect, potential presence of PCR assay inhibitors, or uneven distribution of virus in the sample.[5]

Our seroprevalence results must be confirmed and the subtypes identified, especially because we found only one positive animal in a few farms that could be attributed to false positive results of the ELISA test (performances are not known). These preliminary results are in favor of a virus circulation at low level in the spring, but must be completed by further surveys in the winter and before the New Year (Têt celebration) when pig production, trade, and movement increase at their maximum.

No clear prior information on the expected prevalence of swine influenza in Vietnam, tests sensitivity, and specificity could be obtained from literature or reliable sources. Bayesian methods will be carried out in the future in order to compute prevalence and/or to estimate the probabilities of freedom.

The risk factors analysis was limited by the lack of positive results. Further studies are necessary to identify the at-risk season and type of farming systems at risk of swine influenza infection. However, this investigation of risk factors leads to the hypothesis that medium size breeding-fattening farms had a higher risk than large or small size fattening farms. Further investigation are needed to precise this typology. The risk of SwIV infection increases with a combination of three major factors. Poultry production does not seem to play any role on swine infection. The generalized linear mixed model afforded to take into account all the non investigated parameters at the herd level. Although we investigated the most common risk factors of swine influenza infection covering different kind of fields, the herd random effect might explain risk variations. Mixed models have become a frequently used tool in epidemiology. Due to software limitations, random effects are often assumed to be normally distributed. Since random effects are not observed, the accuracy of this assumption is difficult to check.[6]

Further studies, such as case-control or cohort studies could help to identify more precisely risk factors of swine influenza seropositivity, as these study designs are more adapted than cross-sectional studies.

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Surveillance of influenza viruses in swine in Hong Kong abattoir: methods and feasibility

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Introduction

The concept that swine are a mixing-vessel for the reassortment of influenza viruses and for the emergence of pandemic influenza viruses has been re-enforced by the emergence of the recent pandemic.[1] The pandemic H1N1 virus of 2009 (H1N1pdm) is believed to have emerged through the reassortment of North American triple reassortant and Eurasian avian-like swine influenza viruses.[2] Since the immediate precursor of this pandemic virus has not yet been identified, it is not possible to be definite whether the reassortment leading to the pandemic occurred in swine, but swine influenza viruses are the nearest known ancestors of each gene segment of H1N1pdm.[1,2]

The mechanisms of pandemic emergence are not clear. It is believed that the pandemics of 1957 and 1968 arose through reassortment of the pre-existing human seasonal influenza virus with avian influenza viruses, and swine have been proposed to be a possible intermediate host where such reassortment between human and avian viruses may take place.[4] The 2009 pandemic was the first to arise for over 40 years and the first to occur after the understanding that pandemics arise from animal influenza viruses.[3] Systematic studies of influenza virus ecology and evolution in swine are, therefore, important in order to understand the dynamics of pandemic emergence. Furthermore, since swine are the likely host within which H1N1pdm virus originated, it was predicted that this virus would readily infect swine and may reassort with endemic swine influenza viruses. These predictions have now been confirmed with reports of H1N1pdm being detected in pigs in many countries and reassortment with endemic swine influenza virus being confirmed.[5] While H1N1pdm has been genetically and antigenically stable in humans, reassortment between H1N1pdm, which is well adapted to transmission in humans, and other avian or swine viruses may lead to the origin of novel viruses posing a threat to public health. In addition to endemic swine virus lineages, avian influenza viruses such as H9N2 and highly pathogenic avian influenza (HPAI) H5N1 have also been occasionally identified in pigs in parts of Asia.[6,7] It has been shown that H1N1pdm readily reassorts with H5N1 to generate viable progeny *in vitro*.[8] It is therefore essential to monitor the ecology, evolution, and biological characteristics of swine influenza viruses so that their continued evolution and zoonotic and pandemic potential can be monitored. There is however, a paucity of surveillance data on swine influenza viruses worldwide. This is in part related to the negative commercial consequences that may arise from detection of influenza in a swine herd leading to a major economic loss to the producer.

Here we outline a surveillance system that has been in place in Hong Kong for the last decade, based on sampling animals arriving at an abattoir in Hong Kong. We demonstrate the feasibility of such surveillance in an abattoir setting and compare methods used for detection influenza viruses in swine.

Materials and methods

Specimens

A central abattoir in Sheung Shui in Hong Kong slaughters approximately 4000 pigs per day, 5–20% of which are raised in Hong Kong, the remainder being imported from mainland China. As part of a systematic virological surveillance of pigs slaughtered at this abattoir since 2002 to date, we collected approximately 252 swabs (152 tracheal swabs and 100 nasal swabs) every month for virus isolation, surveillance being further enhanced since May 2009.

Virus isolation

Virus isolation was carried out by inoculation into MDCK cells and by allantoic inoculation in embryonated eggs as previously described.[6] Virus isolates were subtyped by

haemagglutination inhibition tests using specific antisera and genetically characterized by sequencing and phylogenetic analysis of the haemagglutin gene.[2,6]

Virus detection by RT-PCR

A subset of 1154 recent specimens was tested in parallel by Real Time PCR using the BioRobot Universal System (Qiagen) that enables fully-automated viral nucleic acid extraction and downstream reaction setup in a 96-well plate format. Total viral nucleic acids were extracted in a 96-well plate format with the QIAamp Virus BioRobot MDx Kit (Qiagen) on the BioRobot Universal System (Qiagen) according to the manufacturer's instructions. Briefly, 140 μl of sample was lysed in 280 μl buffer AL, supplemented with 5·6 μg carrier RNA in a S block (Qiagen), which placed the samples into a 96 well plate format. After protease digestion, samples were transferred to silica based membrane in 96 well plate format for binding. Following two washing steps, RNA was eluted in 80 μl of elution buffer (buffer AVE) into a 96 well elution microplate CL (Qiagen).

For the synthesis of cDNA, 10 μl of purified RNA was used in a 20 μl reaction containing 4 μl of 5× buffer, 0·75 nm of each deoxynucleotide triphosphate (dNTP), 10 mm dithiothreitol, 3 μg random primer, 40 U of RNaseOut Recombinant Ribonuclease Inhibitor, and 200 U of Superscript III Reverse Transcriptase (all from Invitrogen). Reactions were performed in the GeneAmp 9700 Thermocycler (Applied Biosystems) with the following parameters: 60 minutes at 50°C, 15 minutes at 72°C, and soak at 4°C. Subsequent to the reactions, 20 μl of cDNA was diluted 1/10 by adding 180 μl of AE buffer (Qiagen).

Real-time PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Briefly, 5 μl of 1/10 diluted cDNA was amplified in a 25 μl reaction containing 12·5 μl of 2× Power SYBR Green PCR Master Mix, 800 nm of forward primer M52C (5′-CTT CTA ACC GAG GTC GAA ACG-3′) and 800 nm of reverse primer M253R (5′-AGG GCA TTT TGG ACA AAG/T CGT CTA-3′). The primers have been designed to amplify the sequences in the conserved region of influenza A virus matrix gene, thereby detecting viruses from different species including swine influenza viruses.[8] Real-time PCR was performed in the ABI 7500 Fast System (Applied Biosystems) with the following cycling conditions: 10 minutes at 95°C once, 30 seconds at 95°C, and 1 minutes at 50°C for 40 cycles, followed by melting curve analysis with 15 seconds at 95°C, 1 minutes at 50°C, and 15 seconds at 95°C. In each assay, serially diluted plasmids containing the full length M gene cloned from A/Vietnam/1204/2004 (H5N1) were included as standards to perform absolute quantification. A manual baseline was set from cycles 3–15 and a manual cycle threshold (Ct) was set at 0·2.

Samples that were positive or unequivocal results from the real-time PCR were confirmed by performing gel electrophoresis on the PCR products. Positive visual identification was made in the presence of the target PCR product at 244 bp in length.

Results

A total of 16 566 tracheal and 11 100 nasal swabs were processed during the years January 2002–April 2010 and yielded 393 influenza virus isolates, an overall virus isolation rate of 1·4%. Of these, 352 were subtype H1 (Classical swine, Eurasian avian-like swine, and triple-reassortant), 25 were human-like H3 viruses, and 16 were Eurasian avian-like swine H3N2 viruses.

Culture in MDCK cells yielded 95% of H1 subtype viruses, 100% of the human seasonal-like H3N2 viruses, and 68·8% of the avian-like Eurasian swine H3N2 viruses. Culture in embryonated eggs yielded 17·9% of the H1 subtype viruses, 0% of the human seasonal-like H3N2 viruses, and 62·5% of Eurasian avian-like swine H3N2 viruses (Figure 1).

Tracheal and nasal swabs each gave comparable overall virus isolation rates (1·4%). However, isolation rates for human-like H3N2 viruses were 4·8 fold higher in nasal swabs (0·17% versus 0·036% respectively; $P = 0·002$) (Figure 1).

A parallel evaluation of RT-PCR and culture was carried out in 1154 specimens. RT-PCR detected 10/12 (83%) of the culture positive specimens. RT-PCR was also positive in 8/1154 (0·7%) culture negative specimens, but all these specimens had very low virus load in the RT PCR tests. Virus could not be cultured from these culture negative specimens even by attempts at virus re-isolation from the frozen specimen.

Discussion

Surveillance in an abattoir setting provides an acceptable yield of influenza viruses and is a feasible method of swine influenza surveillance. Sampling in a large abattoir setting allows surveillance to be carried out anonymously with no negative consequences to the supplier. The supply-chain of pigs to the Hong Kong abattoir involves pigs being trucked in over long distances and may provide opportunity for virus amplification during transport. Thus, virus isolation rates may be lower in more vertically integrated and homogenous production and slaughter systems where less mixing of pigs occurs.

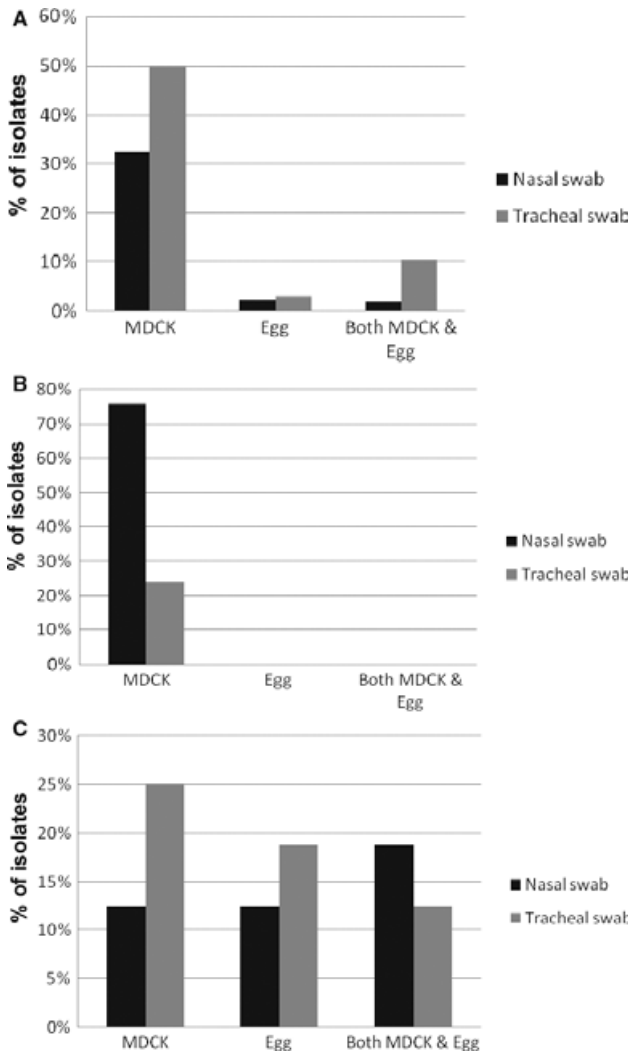

Figure 1. The percentage of (A) H1 (n = 352); (B) H3 (A/Sydney/5/1997-like) (n = 25); and (C) H3 (A/Victoria/75-like) (n = 16) viruses isolated using MDCK cell cultre, allantoic inoculation of embryonated chicken eggs from nasal and tracheal swab samples.

Our results indicate that MDCK cell culture is essential for optimizing virus isolation during swine influenza surveillance. Allantoic inoculation of embryonated eggs by itself is sub-optimal for isolation of swine influenza viruses. It is however possible that inoculation of embryonated eggs by the amniotic route may lead to better isolation rates than allantoic inoculation. RT-PCR detection is an alternative method for virus detection. But the additional specimens detected by RT-PCR did not yield culturable virus, even following attempts at re-isolation and sequential passage. The RT-PCR positive/virus isolation negative specimens had very low virus load, and this may be the explanation for the inability to isolate such viruses. In addition, RT-PCR did not detect all viruses isolated by culture.

Tracheal and nasal swabs gave comparable isolation rates with the exception of human-like H3N2 viruses which were more frequently isolated from nasal swabs. This may suggest that, in contrast to endemic swine influenza virus lineages, these human-like H3N2 viruses are less adapted to replication in the lower respiratory tract.

In summary, collection of nasal or tracheal swabs in an abattoir setting together with virus isolation in MDCK cells provides a feasible approach to surveillance of swine influenza viruses.

Acknowledgements

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Influenza viruses in wild birds in Hong Kong, 2003–2010

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Keywords HPAI, influenza, LPAI, satellite tracking, wild birds.

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Introduction

Wild waterfowl are the natural reservoir of influenza A viruses (AIV), and they play an important role in the genesis of pandemic influenza. It is suggested that the 1918 pandemic virus was purely derived from avian virus, which adapted to humans and caused efficient human-to-human transmission,[1] while the pandemics of 1957 and 1968 had acquired the viral haemagglutinin, PB1 polymerase, and in 1957, the neuraminidase gene segments from the avian gene pool.[2] The major regional outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in Asia, Europe, and Africa highlight the potential role played by migratory waterfowl in disseminating highly pathogenic influenza viruses.[3] Therefore defining the influenza virus gene-pool in wild birds is of vital importance.

Material and methods

Surveillance was carried out 2–3 times weekly from 2003 to 2010 during the winter months of October to April in the Hong Kong Mai Po Nature Reserve and Lok Ma Chau, Hong Kong. The Hong Kong Mai Po Nature Reserve and Lok Ma Chau are along the East Asia-Australian flyway where a peak of more than 30 000 ducks and grebes congregate every winter.[4] Fecal droppings were collected and transported in vials containing 1·0 ml of VTM, which was prepared from M199 (9·5 g/l), penicillin G (2×10^6 U/l), Polymyxin B (10×10^6 U/l), gentamicin (250 mg/l), nystatin ($0{\cdot}5 \times 10^6$ U/l), ofloxacin HCL (100 mg/l), and sulfamethoxazole (1 g/l). An aliquot of 100 μl from each swab sample was inoculated into the allantoic cavity of a 9- to 11-day-old chicken embryonated egg, and incubated for 3 days at 37°C. Positive HA isolates were subtyped using standard antisera [5,6] and RT-PCR was performed with the used of one-step RT-PCR assay (Invitrogen) described earlier,[7] followed by sequencing on ABI PRISM 3730xl DNA analyzer. The determination of species of origin was performed by DNA barcoding of the mitochondrial cytochrome oxidase I gene from DNA extracted from the fecal droppings.[8]

Results and discussion

During the 7-year surveillance period, a total of 193 influenza viruses were isolated from 39 354 samples collected, an overall isolation rate of 0·49%. A total of 192 isolates were obtained from 39 152 specimens collected during the winter period coinciding with the southern migration of waterfowl along the East Asian Flyway and one isolate obtained from 202 samples collected in spring during the period when northern migration of waterfowl took place along the East Asian-Australasian Flyway. The isolation in Hong Kong was slightly lower than a similar study conducted in South Korea in which the isolation rate of migratory birds was 0·8% in 2003–2008.[9] This suggested a slightly lower prevalence of influenza virus present in Hong Kong as the birds migrated southwards. The viruses isolated in Hong Kong, representing hemagglutinin (HA) subtypes of H1-H12 and neuramidinase (NA) subtypes of N1-N9, were all from wild waterfowl (Table 1). Out of the twelve HA subtypes isolated, H10 and H6 were the two subtypes that were isolated frequently every year for H10 and in six out of seven years for H6, respectively. H10 and H6 viruses accounted for 16·7% and 22·4% of all virus isolated, respectively. On the other hand, H3, H9, and H12 were the least prevalence (0·5%) and were only isolated once in 7 years. Of the NA subtypes, N1 and N2 were isolated most often (24·4% and 19·2% of all isolates, respectively) and N5 was the least (0·5%). November was the month that had the highest prevalence of influenza virus (0·83% of samples being positive) compared to only 0·3% in March. The subtype's variation was the most diverse in December during our 7 years of surveillance. This suggested that more of these wild migratory birds may be carrying influenza virus when they arrive in Hong Kong. However the continued isolation of viruses suggests continued circulation of these viruses in the vicinity of Mai Po.

Table 1. The number of different hemagglutinin and neuraminidase subtypes isolation from wild migratory birds in Hong Kong September 2003–April 2010

Hemagglutinin subtype	No. of isolates	Neuraminidase subtype	No. of isolates
H1	11	N1	47
H2	2	N2	37
H3	1	N3	24
H4	17	N4	10
H5	26	N5	1
H6	43	N6	16
H7	33	N7	32
H8	16	N8	8
H9	1	N9	18
H10	32		
H11	10		
H12	1		

The study of DNA barcoding for the mitochondrial cytochrome oxidase I gene retrieved from fecal droppings revealed that the isolates originated mainly (89·2%) from birds of the Order Anseriform, Family Anatidae including Eurasian Wigeon, Northern Shoveler, Northern Pintail, Common Teal, and Garganey. Non-Anseriformes which were found to have shed AIV viruses were Cormorant, Grey heron, and Stint. None of the water samples collected from the ponds where these birds congregate were found to be positive for the virus.

Phylogenetic analyses of the HA gene of the LPAI H5 viruses isolated in this study clustered with that of the other LPAI H5 viruses isolated from Hokkaido, Mongolia, and Siberia and were not closely related to the HPAI H5N1. Satellite tracking of 47 Eurasian Wigeons and Northern Pintails in Dec 2008 and 2009 revealed their flyway from Hong Kong to as far north as Eastern Russia, Eastern Mongolia, and Northern China. No HPAI H5N1 viruses were isolated in this study from apparently healthy birds. However, as part of the surveillance of dead wild birds carried out by the Department of Agricultural, Fisheries and Conservation of the Government of Hong Kong during this same period, over 50 dead wild birds were tested positive for HPAI H5N1 and has been reported elsewhere.[10]

Conclusions

Our influenza surveillance in Hong Kong has revealed a diversity of influenza virus subtypes the migratory waterfowl infected within the region. The result of the phylogenetic analysis correlated with the findings from satellite tracking that viruses isolated in Hong Kong were closely related to those isolated in areas along the migratory route. No healthy bird was isolated with HPAI H5N,1 although dead wild birds have been regularly found to have HPAI H5N1 virus, suggesting that infected birds might not live for a long period.

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Competitive transmissibility and fitness of oseltamivir-sensitive and resistant pandemic influenza H1N1 viruses in ferrets

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Introduction

A novel swine-origin H1N1 influenza virus emerged in Mexico in April 2009 and rapidly spread worldwide, causing the first influenza pandemic of the 21st century.[1] Most confirmed human cases of H1N1/2009 influenza have been uncomplicated and mild, but the increasing number of cases and affected persons worldwide warrant optimal prevention and treatment measures. Today, almost all of the pandemic H1N1/2009 viruses tested are resistant to M2-blockers.[2] Therefore, only the neuraminidase (NA) inhibitors are currently recommended for treatment of this pandemic influenza. For the control of influenza infection, the clinical use of oseltamivir has increased substantially during the 2009 pandemic. To date, the majority of tested clinical isolates have remained susceptible to NA inhibitors, oseltamivir and zanamivir,[2] but oseltamivir-resistant variants with H275Y NA mutation (N1 numbering) have been isolated from individuals taking prophylaxis, from immunocompromised patients, and from a few community clusters.[3,4] In view of the high prevalence of oseltamivir-resistant seasonal H1N1 influenza viruses in 2007–2008, the isolation of resistant H1N1/2009 viruses without known oseltamivir exposure raised great concern about the transmissibility and fitness of these resistant viruses. Here we studied the transmissibility of a closely matched pair of pandemic H1N1/2009 clinical isolates, one oseltamivir-sensitive and one resistant, in both direct contact and respiratory droplets routes among ferrets. Viral fitness was evaluated by co-infecting a ferret with both the oseltamivir-sensitive and -resistant viruses. The viruses were also characterized by full genome sequencing, susceptibility to NA inhibitors, and growth in MDCK and MDCK-SIAT1 cells.

Materials and methods

Oseltamivir-resistant influenza A/Denmark/528/09 (H1N1) virus (A/DM/528/09) was isolated from the throat swab of a patient who had influenza-like symptoms and received post-exposure oseltamivir prophylaxis (75 mg once daily).[5] Wild-type influenza A/Denmark/524/09 (H1N1) virus (A/DM/524/09) was isolated from a patient in the same cluster of infection as the A/DM/528/09 virus. To assess growth kinetics of viruses, confluent MDCK or MDCK SIAT1 cell monolayers were infected with viruses at a multiplicity of infection (MOI) of approximately 2·0 PFU/cell (single-step) or 0·001 PFU/cell (multi-step). Supernatants were collected every 2 h or 12 h p.i. for 6 time points. A modified fluorometric assay using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-d-N-acetylneuraminic acid (MUNANA) was used to determine viral NA activity.[6] The drug concentration required to inhibit 50% of the NA enzymatic activity (IC_{50}) was determined by plotting the percent inhibition of NA activity as a function of compound concentration calculated in the GraphPad Prism 4 (La Jolla, CA) software from the inhibitor-response curve. NA enzyme kinetics were determined by measuring NA activity every 60 seconds for 60 minutes under the same conditions as above, when all viruses were standardized to an equivalent dose of $10^{6\cdot0}$ PFU/ml. The K_m and V_{max} were calculated by fitting the data to the appropriate Michaelis-Menten equations using nonlinear regression in the GraphPad Prism 4 software. Young adult ferrets (4–5 months of age) were obtained from the ferret breeding program at St. Jude Children's Research Hospital. All ferrets were seronegative for influenza A H1N1 and H3N2 viruses and for influenza B viruses. For transmission studies, the donor

ferrets were lightly anesthetized with isoflurane and inoculated intranasally with 10^6 $TCID_{50}$ virus in 1·0 ml sterile PBS. After the donor ferrets were confirmed to shed virus on day 2 p.i., each donor was then housed in the same cage with two naïve direct-contact ferrets. Two additional recipient ferrets were placed in an adjacent cage isolated from the donor's cage by a two layers of wire mesh (approximately 5 cm apart) that prevented physical contact but allowed the passage of respiratory droplets. Ferret weight and temperature were recorded daily for 21 days. Nasal washes were collected from donors and recipients on day 1, 2, 4, 6, 8, 10, 12, and 14 p.i. by flushing both nostrils with 1·0 ml PBS, and $TCID_{50}$ titers were determined in MDCK cells. Serum samples were collected 3 weeks after virus inoculation, and were tested for seroconvention by HI assay.

Results

Full genome sequencing revealed that the pair of H1N1/2009 viruses differed only at NA amino acid position 275, where the pandemic A/DM/528/09 virus had an H275Y amino acid mutation caused by a single T-to-C nucleotide substitution at codon 275. The wild-type A/DM/524/09 was susceptible to oseltamivir carboxylate (mean IC_{50}: 5·0 nm), but the A/DM/528/09 carrying the H275Y NA mutation had IC_{50} values approximately 200–300 times of the wild-type viruses (mean IC_{50}: 972 nm). The IC_{50} of zanamivir was comparable for both viruses and were uniformly low (mean $IC_{50} \leq 1{\cdot}3$ nm). The H275Y NA mutation confers resistance to oseltamivir carboxylate but did not alter susceptibility to zanamivir. To understand the impact of the H275Y mutation on the NA enzymatic properties, NA enzyme kinetics was determined. The NA of the oseltamivir-resistant virus had a slightly higher K_m (mean = 55 μm) and lower V_{max} (mean = 101 U/sec) than NA of the sensitive virus (K_m, mean = 80 μm; Vmax, mean = 86 U/sec). The results suggested that the H275Y NA mutation reduced NA affinity for substrate and NA catalytic activity, although the function of NA was not severely impaired. To further evaluate the impact of the H275Y NA mutation on virus growth *in vitro*, single- and multi-cycle growth studies of both viruses were performed in MDCK and MDCK-SIAT1 cells. In the both single- and multiple-cycle growth curves, the two viruses reached comparable levels eventually, but the initial growth of the resistant virus was significantly delayed by at least 1–2 logs in comparison to that of wild-type virus ($P < 0{\cdot}05$).

The donor ferrets inoculated with wild-type A/DM/524/09 or oseltamivir-resistant virus shed virus productively until day 6 or day 8 p.i., with a peak virus titer comparable to that of A/DM/524/09 virus (Table 1). In A/DM/524/09 virus group, two of 2 direct-contact ferrets

Table 1. Clinical signs, virus replication, and seroconversion in inoculated donor and contact ferrets

	Donor				Direct contact				Respiratory droplets			
	Clinical signs	Virus detection			Clinical signs	Virus detection			Clinical signs	Virus detection		
H1N1 virus	Weight loss	Virus shedding	Last day of shedding	Serum HI titer	Weight loss	Virus shedding	Last day of shedding	Serum HI titer	Weight loss	Virus shedding	Last day of shedding	Serum HI titer
A/DM/524/09	5	7·3	6	1280	2/2 (3·5)	2/2 (8·3)	8, 8	1280, 640	1/2 (6·0)	1/2 (7·2)	10	1280, 320
A/DM/528/09	6·2	6·9	8	640	2/2 (3·3)	2/2 (7·0)	8, 10	1280, 1280	0/2	0/2	NA	<10, <10
Co-inoculation	5·9	7·7	6	640	2/2 (6·0)	2/2 (7·1)	10, 10	1280, 1280	NA	NA	NA	NA

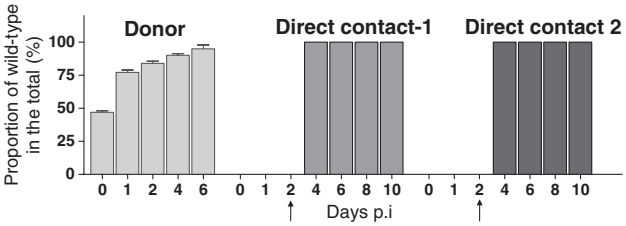
The weight loss in ferrets is the maximum percentage loss compared with the initial weight. Virus shedding is indicated as number of virus-shedding animals/total number; mean peak virus titer ($log_{10}TCID_{50}$/ml) in nasal wash samples is indicated in parentheses. Serum hemagglutination inhibition (HI) titer to homologous virus in ferret serum was determined on day 21 p.i.

and 1 of 2 respiratory droplet-contact ferrets were infected through virus transmission, as indicated by the virus titers and inflammatory cell counts in their nasal washes and also by sero-conversion. Under identical conditions, in A/DM/528/09 group, only 2 of 2 direct-contact ferrets were infected through virus transmission, but neither respiratory droplet-contact ferrets was infected, as confirmed by the absence of sero-conversion (Table 1). Virus shedding in the direct-contact ferrets was lower and peaked after a longer interval in this group than in the oseltamivir-sensitive A/DM/524/09 group (Table 1), but the resistant viruses appeared to cause a similar disease course in ferrets without apparent attenuation of clinical signs. These results showed that an oseltamivir-resistant H275Y mutant of pandemic H1N1 virus, A/DM/528/09 virus could be only transmitted efficiently by direct contact.

To compare the relative fitness, growth capability, and transmissibility of the sensitive and resistant H1N1/2009 viruses within host, a donor ferret was co-inoculated with a 1:1 ratio of the sensitive and resistant viruses, and another two naive ferrets were housed with the donor to test direct contact. During co-infection, the pattern of virus shedding and the clinical signs were similar to those in ferrets inoculated with either A/DM/524/09 or A/DM/528/09 virus (Table 1). In the inoculated donor ferret, the virus population in the nasal washes remained mixed but wild-type viruses outgrew the resistant virus progressively (Figure 1). Two of 2 direct-contact ferrets were infected through virus transmission, but only wild-type virus was detected in both direct-contact ferrets (Figure 1). In summary, oseltamivir-sensitive A/DM/524/09 virus possessed better growth capability in the upper respiratory tract than did resistant A/DM/528/09 virus, and thus had an advantage in direct-contact transmission.

Discussion

Our study determined the comparative transmissibility of two naturally circulating oseltamivir-sensitive and -resistant pandemic H1N1/2009 viruses; we demonstrated inefficient respiratory-droplet transmission of an oseltamivir-resistant H275Y mutant of pandemic H1N1 virus among ferrets, although it retained efficient direct-contact transmission. We suggest that the lower fitness of resistant virus within the host along with its reduced NA function and delayed growth *in vitro* may in part explain its less efficient transmission. Notably, the H275Y mutant of H1N1/2009 used in this study was the first oseltamivir-resistant H1N1/2009 isolate from a patient on oseltamivir prophylaxis to be characterized for transmissibility. Our observation in the animal model is consistent with the epidemiological data collected from humans, which showed no evidence of predominant or continued circulation of oseltamivir-resistant viruses.[4] As this study was undertaken, additional H275Y mutants of H1N1/2009 viruses have emerged in the absence of oseltamivir use.[7,8] The emergence of these viruses should raise concerns as to whether resistant H1N1/2009 viruses will acquire greater fitness and spread worldwide as the naturally resistant H1N1 viruses did during the 2007–2008 season. Two independent studies have evaluated the pathogenecity and transmission of other oseltamivir-resistant pandemic H1N1/2009 clinical isolates in the animal models.[9,10] One of the studies,[9] which also used an oseltamivir-resistant virus isolated from a patient under oseltamivir prophylaxis, observed similar results as ours: although the respiratory-droplet route of transmission was not investigated, it was shown that the resistant isolate was transmitted though direct-contact route and was as virulent as wild-type virus in ferrets.[9] In another study,[10] two oseltamivir-resistant isolates were transmitted through the respiratory-droplet route in ferrets, and the dynamics of transmission were different between the two isolates. Apparently, these two oseltamivir-resistant isolates were still unequal in their transmissibility and were disparate from the resistant isolate in our study. The isolation history of the two resistant isolates was unclear in this study, and this would be an important factor to understand the fitness of drug-resistant viruses. Further studies with more clinical isolates of diverse isolation background are warranted to identify how these novel H275Y mutants of pandemic H1N1/2009 virus have changed to retain their full transmissibility. Taken together, all these related studies underline the necessity of continuous monitoring of drug resistance and characterization of potential evolving viral proteins.



Note. The arrow indicates the day when donor ferret was housed with contact ferrets.

Figure 1. The proportion of wild-type virus in the total virus population recovered from nasal wash samples in the donor ferret and two direct-contact ferrets after co-infection. *Note.* The arrow indicates the day when donor ferret was housed with contact ferrets.

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Disclosure

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Pathogenicity and transmissibility of the pandemic H1N1 2009-related influenza viruses in mice, ferrets, and pigs

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Introduction

Pigs have been considered as hypothetical ''mixing vessels'' facilitating the genesis of pandemic influenza viruses.[1,2] The pandemic H1N1/2009 virus (pH1N1/09) contained a very unique genetic combination and was thought to be of swine origin, as each of its eight gene segments had been found to be circulating in pig populations for more than a decade.[3] However, such a gene constellation had not been found previously in pig herds all around the world. Only after its initial emergence in humans has this virus been repeatedly detected in pigs, and found to further reassort with other swine influenza virus.[3–5] A primary question remaining to be answered is whether the pH1N1/09-like and their genetically related viruses could become established in pig populations, thereby posing novel threats to public health.

Despite the fact that pH1N1/09 first appeared in Mexico and the United States, and six of its eight gene segments were derived from the established North American triple reassortant swine influenza virus (TRIG), its neuraminidase (NA) and matrix protein (M) genes belonged to the Eurasian avian-like swine lineage (EA), which had never been detected in North America previously.[3,6] Likewise, the

TRIG-like viruses were never reported in Europe.[6] In contrast, both lineages of virus were frequently detected in Asia, and reassortants between them have also been documented in recent years.[3,5] This has given rise to a complicated ecological situation, i.e. the simultaneous prevalence of multiple genotypes of H1N1 and H1N2 viruses in pigs.[3,5] Among them, two representative reassortants showed the most similar genotypic characterization to the pH1N1/09 virus, the Sw/HK/915/2004 (H1N2) and Sw/HK/201/2010 (H1N1), which respectively harbor seven and six gene segments closely related to the pandemic strains.[3,5]

To understand their *in vivo* characteristics and zoonotic potential, these two viruses, together with a human prototype strain and a swine pH1N1/09-like isolate, were chosen for a study of their pathogenicity and transmissibility in domestic pigs, ferrets, and mice.

Materials and methods

Viruses

The prototype pH1N1/09 virus, A/California/04/2009 (CA04), was provided by the World Health Organization Collaborating Centers for Reference and Research on Influenza (Atlanta, GA, USA). Three pH1N1/09-related swine influenza viruses were isolated through our surveillance program in South China as previously described.[3,5] The A/Swine/Guangdong/106/2009 (H1N1, GD106) virus was a pH1N1/09-like swine isolate. A/Swine/Hong Kong/915/2004 (H1N2, HK915), the closest pandemic ancestor known to date, possesses an M gene derived from the EA lineage, with the other gene segments from TRIG viruses.[3] A/Swine/Hong Kong/201/2010 (H1N1, HK201), a recent pandemic reassortant progeny, had a pH1N1/09-like NA gene (also belonging to the EA lineage), an EA-like hemagglutinin (HA) gene, and six TRIG-like internal genes.[5] All viruses were propagated in Madin-Darby canine kidney (MDCK) cells for three passages, and their titers were determined by plaque assays. All experiments with live viruses were conducted in biosafety level 3 (BSL-3) containment laboratories.

Animal experiments

Pigs (4–6 week old, $n = 5$–6) and ferrets (5 month old, male, $n = 3$) were intranasally infected with 10^6 PFU of each virus, and mice (8–9 week old, female BALB/c, $n = 10$) with a dose of 10^4 PFU. Naïve uninfected pigs ($n = 2$) were co-housed in the same cage with the inoculated ones from each group. Body weights and clinical signs were recorded daily. Virus replication was determined by titration of the virus in nasal and rectal swabs (pigs), nasal washes (ferrets), as well as from lungs and other organs (pigs and mice). Seroconversion was tested by hemagglutination inhibition (HI) assays. Histopathological and immunohistochemical analysis were performed as previously described.[7]

Statistical analysis

Statistical analysis was performed by Mean Analysis with Pasw Statistics 18 (SPSS Inc., Chicago, IL, USA). The probability of a significant difference was computed using anova (analysis of variance). Results were considered significant at $P < 0{\cdot}05$.

Results

Pathogenicity and virus replication in mice

The pathogenicity of the four viruses tested differed significantly in inoculated mice. Animals infected with 10^4 PFU of HK915 experienced the most severe body weight loss (25·1 ± 4·7%) but started to recover after 8 days post-infection (dpi). HK201 caused similar peak body weight loss (16·9 ± 4·6% on 8 dpi) in mice as did CA04 (17·3 ± 2·4%, on 7 dpi), but the onset of clinical signs and weight loss (on 4 dpi) was 1 day later than those caused by the other three viruses. The GD106-infected group suffered the least body weight loss (6·9 ± 1·9%, 5 dpi) and was the earliest to recover.

Although all four viruses were detected in the lungs with comparable virus titers on 3 dpi ($P > 0{\cdot}05$), mice inoculated with GD106 consistently showed the lowest lung index (lung weight/body weight, %) on 3, 6, and 14 dpi ($P < 0{\cdot}01$), suggesting the slightest injury and consolidation of the lungs. In concordance with the body weight change, the lung index from the HK915 group was higher than that from any other groups on 6 and 14 dpi, indicating the marked virulence of HK915 in mice. Notably, virus titer of HK201 in the nasal turbinate was lower than the other groups both on 3 and 6 dpi ($P < 0{\cdot}01$), but virus replication in the lower respiratory tract was either higher (in the trachea) or similar (in the lungs).

Pathogenicity and virus shedding in ferrets

Observations of the body weight changes caused by infection of pH1N1/09 or its genetically related swine viruses in ferrets have come to a similar conclusion as that for the mouse experiment. After nasal inoculation with 10^6 PFU of each virus, all groups of ferrets experienced transient body weight loss for 2–3 days, except for those infected with GD106, which showed no significant weight loss ($P > 0{\cdot}05$). Although ferrets from the CA04-infected group reached their peak weight loss (6·2 ± 0·8%, 2 dpi) one day earlier than those from the HK201 and HK915 groups, they began to regain body weight quickly thereafter.

HK201-infected ferrets also recovered rapidly and their body weights reached the same level as those of the GD106-infected group at 6 dpi. Comparatively, ferrets inoculated with HK915 had the most retarded body weight recovery, which did not get back to the baseline level until 11 dpi. HK201 was only detectable in the nasal wash on 2 dpi, whereas the duration of virus shedding for GD106, HK915, and CA04 was 4–6 days. By combining the data obtained from the virus titration in the mouse turbinate and ferret nasal washes, a possible conclusion can be made that HK201 may have lower transmissibility than the other three viruses.

Pathogenicity and transmissibility in domestic pigs

After inoculation or exposure by direct contact (physical contact) with the pH1N1/09 virus and its close relatives, most pigs experience no or mild symptoms, such as slight loss of appetite and inactivity. Body weight loss was only recorded in pigs inoculated with HK915 during the second week post-inoculation, but not in their contact pigs or in the other groups.

Diarrhea was observed intermittently in each of the inoculated or contact groups throughout the experiment, and viruses could be recovered in the rectal swabs, saliva, drinking water, and environmental swabs (inner cage walls accessible to the pigs) at various time points. However, virus titers in the positive rectal swabs were just slightly above the detection limit, while those from the environment sometimes could be higher. Whether these viruses can replicate in the digestive tract or were just carried-over by contaminated foods and water requires further investigation.

Although virus could be detected in the nasal swabs of all infected or contact animals, the lowest peak titer was from pigs inoculated or in contact with HK201 (0·5–1·5 log TCID50/ml lower than the other groups), suggesting unfavorable replication in the nasal cavity for this virus. Postmortem examination on 4 and 7 dpi revealed that pigs infected with HK915 had the most extensive gross lesions in the lungs, and histochemical staining of viral nucleoprotein (NP) in lung tissues on 4 dpi also suggested the best replication for HK915 in the lower respiratory tract. On 11 days post-contact (dpc), all pigs exposed to the inoculated animals developed sero-conversions (HI = 80–160) except for one from the GD106 contact group. However, on 17 dpc, its HI titer reached 40, indicating slower seroconversion.

Conclusions

This study revealed that both the 2009 pandemic H1N1 and its genetically related swine viruses could readily infect mice, ferrets, and pigs causing mild to moderate clinical symptoms. They could also transmit efficiently between pigs. When compared with the pandemic stains and its reassortant progeny (HK201), the HK915 (H1N2) virus containing the EA-like M gene in the genetic context of the TRIG virus showed consistently higher virulence in all three mammalian models tested, but it is still unknown what might happen if such a virus further reassorts to obtain the pandemic-like or EA-like NA gene. However, our findings suggest that pigs could likely maintain the prevalence of different genotypes of pandemic-related influenza viruses, and highlight the zoonotic potential of multiple strains of swine influenza virus.

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Transmissibility of pandemic H1N1 and genetically related swine influenza viruses in ferrets

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Introduction

Pandemic influenza viruses emerge from the animal reservoirs.[1] Among the three pandemics that occurred in the last century, we learned that the 1957 H2N2 and the 1968 H3N2 pandemic viruses emerged by reassortment between circulating human virus and avian-origin influenza virus(es).[1] Studies on the emergence of the catastrophic 1918 Spanish H1N1 virus suggest that the virus may have obtained all of its eight gene segments from the avian reservoir,[2,3] or alternatively is a reassortant between mammalian and a previously circulating human influenza virus.[4] Over 40 years since the last pandemic, the first pandemic in the 21st Century arose in 2009 and was caused by a swine-origin influenza virus containing a unique gene combination, with gene segments derived from the circulating North America ''triple reassortant'' (PB2, PB1, PA, HA, NP, and NS) and the ''Eurasian'' (NA and M) swine influenza viruses.[5,6] Analysis of the pandemic H1N1 viruses failed to identify known molecular markers predictive of adaptation to humans.[6] The ''triple reassortant'' swine influenza viruses emerged in late 1990s in North America is a reassortant between classical swine (descendent of the 1918 virus after adaptation in swine population), avian, and human influenza viruses.[7] The Eurasian influenza virus was originally an avian influenza virus that was introduced into the European swine population in the late 1970s.[8,9] While incidents of zoonotic infection with triple reassortant or Eurasian influenza in humans have been reported,[10,11] sustained human-to-human transmission has never been established. These results suggest that the unique gene combination seen with the pandemic H1N1 viruses may confer its transmissibility among humans.

We have carried out systematic prospective surveillance of swine influenza in southern China over that last 12 years through samples routinely collected at an abattoir in Hong Kong. During this time, the surveillance results suggest co-circulation of Classical swine H1N1, triple reassortant H1N2, Eurasian swine H1N1, and a range of reassortants between these three virus lineages.[4,12] Ferrets have been reported as a suitable model for the study of influenza transmission as they are naturally susceptible to influenza infection, exhibit similar clinical signs (including sneezing), and possess receptor distribution in the airway similar to that of humans.[13–15] To identify molecular determinants that enable sustained human-to-human transmission, we compared the pandemic virus with genetically related swine influenza viruses obtained from this surveillance program for their ability to transmit from ferret to ferret by direct contact or aerosol transmission.

Materials and methods

Viruses

Human H3N2 influenza virus [A/Wuhan/359/95 (Wuhan95)] and pandemic H1N1 influenza viruses [A/California/4/09 (CA04)] were included for the study. Swine influenza viruses that are genetically related with the pandemic H1N1 virus were selected from our surveillance system, including classical swine-like influenza virus A/sw/HK/4167/99 (H1N1) (swHK4167), triple reassortant-like A/sw/Arkansas/2976/02 (H1N2) (swAR2976), and one reassortant between triple reassortant and Eurasia swine influenza viruses [A/sw/HK/915/04 (H1N2) (swHK915)]. SwHK915 contains seven gene segments (PB2,PB1,PA,HA,NP,M,NS) closely related to the pandemic H1N1 viruses.

Transmission experiment design

Transmissibility was tested in 4- to 6-month-old male ferrets obtained from Triple F Farm (Sayre, PA); all ferrets were tested to have HI titer ≤40 against human seasonal

influenza H1N1 (A/Tennessee/560/2009), H3N2 (A/Brisbane/10/2007), and influenza B (B/Florida/4/2006) prior the experiments. In each virus group, three ferrets were inoculated with 10^5 $TCID_{50}$ of the virus. At 1 day post-inoculation (dpi), we introduced one naïve direct contact ferret to share the cage with inoculated ferret, and one naïve aerosol contact ferret into the adjacent compartment of the cage separated by a double-layered perforated divider. Nasal washes were collected every other day and tested for influenza virus antigen and to determine viral titers ($TCID_{50}$). Weight changes, temperature, and clinical signs were monitored daily. Transmission is defined by detection of virus from nasal washes and/or by seroconversion (>4-fold rise in the post-sera collected after 16–18 days post contact). Experiments were performed in the P2+ laboratory at St. Jude Children's Research Hospital. 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.

Results

Viral replication in inoculated ferrets

At 10^5 $TCID_{50}$ inoculation dose, all viruses replicated efficiently in the ferret upper respiratory tract with peak titers detected from inoculated ferrets at 2 dpi. Lower peak titers were detected from swHK4167 and swHK915 inoculated ferrets, however, the differences were not statistically significant (Table 1). Tissues collected from inoculated ferrets at 3 dpi showed that pandemic H1N1 and swine influenza viruses replicated both in the upper and lower respiratory tract of the ferrets, while the replication of human seasonal influenza Wuhan95 was restricted in the upper respiratory tract.

Direct contact transmission

Direct contact transmission from inoculated donor ferrets to their cage-mates was observed for all viruses studied, albeit at different efficiency. Human seasonal influenza (Wuhan95) and pandemic H1N1 viruses (CA04) transmitted most efficiently via direct contact route as the virus can be detected on 2 dpi from direct contact ferrets, and the peak titers were detected on 4 dpi from direct contacts. Moderate direct contact transmission efficiency was detected from swAR2976 and swHK915 viruses as the virus can be detected from direct contact ferrets at 4 dpi, with peak titers detected at 4 dpi or 6 dpi. Classical swine-like swHK4167 showed least efficient contact transmission as virus could be detected from all direct contacts only at 6 dpi, and the peak titer detected on 8 dpi.

Aerosol transmission

Aerosol transmission was detected in groups of human seasonal influenza virus Wuhan95 (2/3), pandemic H1N1 influenza virus CA04 (3/3), as well as swine precursor virus swHK915 (1/3). Transmission of Wuhan95 and CA04 to aerosol contacts was detected at 4 dpi or 6 dpi, while transmission of swHK915 was detected later at 8 dpi, suggesting that the swHK915 virus possessed aerosol transmission potential, but may require further adaptation to acquire efficient aerosol transmissibility. In addition to viral detection from nasal washes, we also detected viruses from the rectal swabs of ferrets inoculated or infected with pandemic H1N1 viruses (CA04) or classical swine-like virus (swHK4167), which share the common origin for the HA, NP, and NS gene segments.

Discussion

While many of the swine influenza viruses studied were able to transmit via the direct contact route, swHK915, which shares a common genetic derivation for seven genes with H1N1pdm, possessed capacity for aerosol transmission, albeit of moderate efficiency. SwHK915 differed from swine triple reassortant viruses in the origins of its M gene. It is possible that the M gene derived from Eurasian avian-

Table 1. Transmission of seasonal H3N2, pandemic H1N1, and genetically related swine influenza viruses in ferrets

	Donors				Direct contacts				Aerosol contacts			
Viruses	Nasal wash	Rectal swab	Sero-conversion	Peak titer	Nasal wash	Rectal swab	Sero-conversion	Peak titer	Nasal wash	Rectal swab	Sero-conversion	Peak titer
swHK4167	3/3	1/3	3/3	5·6 ± 0·1	3/3	1/3	3/3	5·9 ± 0·7	0/3	0/3	0/3	NA
swAR2976	3/3	0/3	3/3	6·3 ± 0·5	3/3	0/3	3/3	6·1 ± 0·4	0/3	0/3	0/3	NA
swHK915	3/3	0/3	3/3	5·1 ± 1·0	3/3	0/3	3/3	5·0 ± 0·9	1/3	0/3	1/3	4·5
CA04	3/3	1/3	3/3	6·3 ± 0·3	3/3	1/3	3/3	6·6 ± 0·5	3/3	0/3	3/3	6·2 ± 0·5
Wuhan	3/3	ND	3/3	6·5 ± 0·2	3/3	ND	3/3	6·6 ± 0·4	2/3	ND	2/3	7·3 ± 1·4

like swine viruses also contributes to the transmissibility of H1N1pdm influenza viruses.

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Route of H5N1 exposure in ferrets affects pathogenesis and severity of disease

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Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) of the H5N1 subtype are of extreme concern to global health organisations as human infection can result in severe acute respiratory distress syndrome, multi-organ failure, and coma. HPAI viruses of either H5 or H7 subtypes contain a characteristic multi-basic cleavage site in the hemagglutinin glycoprotein[1] as well as other virulence factors[2] that expand the viral tropism beyond the respiratory tract of poultry. There is also emerging evidence of viral RNA or antigen in multiple organs and the CNS of humans infected with H5N1 that is consistent with systemic infection[3,4] and raises the question of the role of the cleavage site in dissemination of the virus in this species. The majority of human cases with H5N1 have involved contact with sick or contaminated poultry and exposure to respiratory secretions of birds that can be inhaled and ingested. Particular risk factors for H5N1 infection include bathing with sick birds, improper hand washing after handling sick birds, or slaughtering poultry.[5] Viral inoculum may also be consumed directly during a variety of religious and cultural practices, such as drinking contaminated duck blood and kissing of merit release birds.

H5N1 infection is lethal in 60% of human cases, and the pathogenetic mechanisms leading to this level of mortality

are unclear. To date 505 cases have been reported to the WHO, although many more people have potentially been exposed to H5N1 through contact with infected bird populations.[6] Some studies have suggested that genetic factors may predispose an individual to severe H5N1 disease,[7] but little is known about the influence of route of virus exposure on morbidity and mortality. In ferrets, an animal model frequently used to study influenza because of its similar disease profile to humans,[8] Swayne *et al.*[9] observed that exposure to a virulent H5N1 strain A/Vietnam/1203/2004 by intra-gastric gavage did not lead to disease and did not generate an antibody response, whereas ferrets that experienced a more natural exposure by being fed contaminated meat developed severe signs of infection.

In this study we further assessed the disease profile of H5N1 following a natural oral exposure in the ferret model. To achieve this inoculation condition, conscious ferrets voluntarily consumed a liquid inoculum of H5N1 HPAI strain A/Vietnam/1203/2004. As a comparison anesthetised ferrets were exposed by intranasal administration of inoculum and the ensuing disease profiles of the different routes of infection were compared.

Materials and methods

Eight ferrets per group were inoculated with 10^6 egg infectious $dose_{50}$ of A/Vietnam/1203/2004 in a volume of 500 μl that was given to the nares of anaesthetized ferrets to establish a total respiratory tract (TRT) infection or voluntarily consumed by conscious ferrets to establish an oral infection. Ferrets were culled at a predetermined humane endpoint that was defined as either a > 10% weight loss and/or evidence of neurological signs, discussed in[10]; animals that did not reach the humane endpoint were euthanased on day 14 after challenge. Nasal washes and oral swabs collected during the course of infection and organ homogenates were assessed for the presence of replicating virus by growth in embryonated-chicken eggs; viral loads were determined by titration on Vero cells and expressed as $TCID_{50}$. Tissue samples were fixed with formalin and embedded in paraffin for sectioning. Viral lesions were identified by hematoxylin and eosin staining of the sections and the presence of viral antigen in the sections was determined by staining with antibody to influenza A nucleoprotein. Pre- and post-exposure antibody responses were assessed by hemagglutination-inhibition assays using irradiated A/Vietnam/1203/2004 virus.

Results

The majority (75%) of ferrets infected by the TRT route rapidly became inactive, developed severe disease, and were euthanased at the humane endpoint following infection (Figure 1). Ferrets infected orally had an improved chance of survival, as only 25% of animals developed severe disease (Figure 1), and the surviving ferrets were more active than ferrets infected by the TRT throughout the stage of acute infection (data not shown). The improved survival rate and wellbeing of ferrets infected orally was not a result of poor infection rates by this route, as 5 of 6 surviving ferrets developed H5 specific antibodies by day 14 post-infection, and they did not have pre-existing antibodies to H5N1 (data not shown).

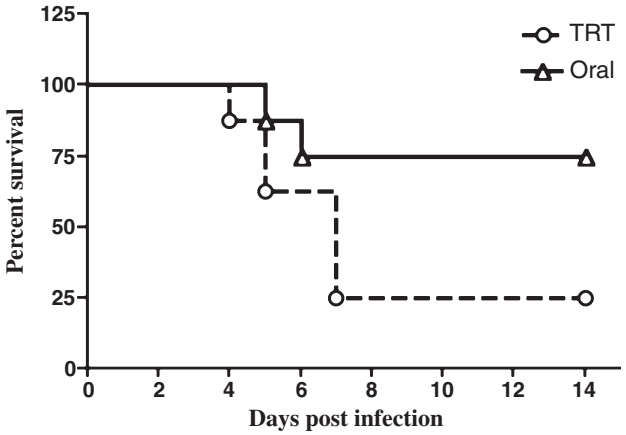

Figure 1. Percentage of ferrets that survived infection after oral or TRT infection. Ferrets were exposed to A/Vietnam/1203/2004 by the total respiratory tract (TRT) route (circles) or the oral route (triangles). The percentages of ferrets that survived infection are indicated at each day following challenge.

The two ferrets that developed severe disease after oral infection had similar disease profiles to ferrets infected by the TRT route; they both progressed to a > 10% weight loss and exhibited neurological signs (data not shown). Viral loads in organs of these two ferrets confirmed dissemination to extra-pulmonary sites (Table 1): replicating virus was detected at high titres in the spleen, pancreas, liver, and brain. Similar findings were recorded in ferrets with TRT infections in this study (not shown) and elsewhere.[10]

Viral load in nasal washes and oral swabs taken at days 3, 5, and 7 post-infection by the oral route did not correlate with the development of severe disease, and virus was isolated only sporadically and at low titre from the nasopharynx of these animals (data not shown). Interestingly, the two ferrets with severe disease after being infected orally had no detectable viral antigen or lesions in the olfactory epithelium and bulb (Table 1), whereas 5 of 6 ferrets culled after infection by the TRT route had lesions and viral antigen in both the olfactory epithelium and bulb (data not shown).

Table 1. Profile of viral replication and antigen in tissues of ferrets experiencing severe disease following oral infection

Tissues	Viral titre ($log_{10}TCID_{50}$/100 μl)		Lesion		Antigen	
Ferret No.	**1**	**2**	**1**	**2**	**1**	**2**
Nasal Turbinates	–	3·00	–	–	–	–
Pharyngeal lymph node	<2·5[b]	–	+	+	+	+
Olfactory epithelium	ND[a]	ND	–	–	–	–
Olfactory bulb	ND	ND	–	–	–	–
Trachea	<2·5	–	–	ND	–	ND
Lung	–	<2·5	+	+	–	+
Spleen	3·75	–	+	+	–	+
Small intestine	–	–	–	–	–	+
Pancreas	–	3·50	+	–	+	+
Liver	4·25	4·75	+	+	+	+
Brain (cerebrum)	5·25	5·50	+	+	+	+

[a]Not done, [b]Low viral titres < $log_{10}2{\cdot}5TCID_{50}$, +Lesion/antigen present, –No lesion/antigen/virus detected.

Discussion

Ferrets exposed orally were more likely to survive H5N1 infection than ferrets exposed to the same dose of virus by the TRT. The improved survival rates that were observed after an oral infection could be a consequence of low-level viral replication in the upper respiratory tract in combination with delivery of a substantial portion of the inoculum directly to the stomach where it may have been inactivated by the harsh environment of the gastro-intestinal tract.[11] Most ferrets infected orally developed an H5-specific antibody response which differs from the studies of Swayne *et al.*[6] in which ferrets gavaged with a liquid inoculum neither developed signs of disease nor an antibody response. However Swayne *et al.* administered virus to anaesthetized ferrets by gastric gavage that would have bypassed the oropharynx. In our study virus was administered to the oral cavity directly and would have had access to the oropharynx. Low level of replication at this site may have been sufficient to trigger an antibody response.

The two ferrets that developed severe disease following oral infection had a similar profile of viral dissemination as ferrets infected by the TRT route. Differences were seen in the olfactory epithelium and bulb as lesions, and viral antigen did not occur in these sites following oral infection, although cerebral involvement was identified. One route of dissemination of H5N1 into the CNS may be by transport within nerves through the olfactory bulb into the cerebrum.[12] Due to the absence of lesions and antigen in these sites following oral infection the spread of virus into the brain in these two animals may be occurring through involvement of other cranial nerves or the hematagenous routes.

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Interactions of oseltamivir-sensitive and -resistant highly pathogenic H5N1 influenza viruses in a ferret model

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Keywords Ferret model, fitness, H5N1 influenza virus, neuraminidase inhibitor, oseltamivir.

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Introduction

The pandemic potential of highly pathogenic H5N1 influenza viruses remains a serious public health concern. While the neuraminidase (NA) inhibitors are currently our first treatment option, the possibility of the emergence of virulent and transmissible drug-resistant H5N1 variants has important implications. Clinically derived drug-resistant viruses have carried mutations that are NA subtype-specific and differ with the NA inhibitor used.[1] The most commonly observed mutations are H274Y and N294S in the influenza A N1 NA subtype (N2 numbering here and throughout the text); E119A/G/D/V and R292K in the N2 NA subtype; and R152K and D198N in influenza B viruses.[2] H5N1 influenza viruses isolated from untreated patients are susceptible to the NA inhibitors oseltamivir and zanamivir,[3] although oseltamivir-resistant variants with the H274Y NA mutation have been reported in five patients after[4,5] or before[6] drug treatment; and the isolation of two oseltamivir-resistant H5N1 viruses with N294S NA mutation from an Egyptian girl and her uncle after oseltamivir treatment were described.[6] The impact of drug resistance would depend on the fitness (i.e., infectivity *in vitro*, virulence, and transmissibility *in vivo*) of the drug-resistant virus. If the resistance mutation only modestly reduces the virus' biological fitness and does not impair its replication efficiency and transmissibility, the effectiveness of antiviral treatment can be significantly impaired.

Materials and methods

The recombinant wild-type H5N1 influenza A/Vietnam/1203/04 (VN-WT), A/Turkey/15/06 (TK-WT) viruses, and oseltamivir-resistant viruses with H274Y NA mutation (VN-H274Y and TK-H274Y) were generated by using the 8-plasmid reverse genetics system. Susceptibility to NA inhibitors was tested by using a fluorescence-based NA enzyme inhibition assay with MUNANA substrate at a final concentration of 100 μm. Viral fitness was studied *in vivo* in a ferret model: groups of three ferrets were lightly anesthetized with isoflurane and inoculated intranasally with VN-WT, VN-H274Y, or mixtures of the two at a different ratios at a dose of 10^2 PFU in 0·5 ml PBS; they were inoculated with TK-WT, TK-H274Y, or mixtures of the two at a different ratios at a dose of 10^6 PFU in 0·5 ml PBS. Respiratory signs (labored breezing, sneezing, wheezing, and nasal discharge), neurologic signs (hind-limb paresis, ataxia, torticollis, and tremor), relative inactivity index, weight, and body temperature were recorded daily. Virus replication in the upper respiratory tract (URT) was determined on days 2, 4, and 6 p.i. The competitive fitness (i.e., co-inoculation of ferrets with different ratios of oseltamivir-resistant and -sensitive H5N1 viruses) was evaluated by the proportion of clones in day-6 nasal washes that contained the H274Y NA mutation. NA mutations were analyzed by sequence analysis of individual clones (~20 clones/sample) created by ligation of purified PCR products extracted from nasal wash samples into a TOPO vector.

Results

Introduction of the H274Y NA mutation conferred high resistance to oseltamivir carboxylate *in vitro*; the mean IC_{50} of the VN-H274Y and TK-H274Y viruses was 3375 and 1208 times, respectively, that of the corresponding wild-type viruses. The oseltamivir IC_{50} of the TK-WT virus was ~16 times that of the VN-WT virus. All four recombinant H5N1 viruses were susceptible to zanamivir. Introduction of the H274Y NA mutation reduced ~90% and 60% of the NA activity of VN-H274Y and TK-H274Y viruses,

respectively, as compared to the wild-type virus activity ($P < 0{\cdot}01$; two-tailed *t*-test).

All ferrets inoculated with either VN-WT or VN-H274Y virus exhibited acute disease signs (high fever, marked weight loss, anorexia, extreme lethargy), rapid progression, and death by day 6–7 p.i., and no differences in clinical signs and replication in the URT of ferrets were observed between wild-type and oseltamivir-resistant viruses (Table 1). Both of the TK viruses caused milder illness than did the VN viruses, despite a much higher dose (10^6 PFU/ferret), and the TK-H274Y virus caused less weight loss and fever than the TK-WT virus (Table 1).

However, competitive fitness experiments revealed a disparity in the growth capacity of VN-H274Y and TK-H274Y viruses as compared to their wild-type counterparts: clonal analysis established the uncompromised fitness of VN-H274Y virus and the impaired fitness of TK-H274Y virus (Table 2). Although, the trend towards an increase/-decrease in the frequency of the H274Y NA mutation relative to the wild-type was statistically significant ($P > 0{\cdot}05$) for two studied groups only. Mutations within the NA catalytic (R292K) and framework (E119A/K, I222L, H274L, N294S) sites or near the NA active enzyme site (V116I, I117T/V, Q136H, K150N, A250T) emerged spontaneously (without drug pressure) in both pairs of viruses (results not shown). The NA substitutions I254V and E276A could exert compensatory effect on the fitness of VN-H274Y and TK-H274Y viruses.

Discussion

The lethality and continuing circulation of H5N1 influenza viruses warrants an urgent search for an optimal therapy. Our results showed that the H274Y NA mutation affects the fitness of two H5N1 influenza viruses differently: the oseltamivir-resistant A/Vietnam/1203/04-like virus outgrew its wild-type counterpart, while the oseltamivir-resistant A/Turkey/15/06-like virus showed less fitness than its wild-type counterpart.

We used a novel approach to compare the fitness of oseltamivir-sensitive and -resistant influenza viruses that included analysis of virus–virus interactions within the host (competitive fitness) during co-infection with these viruses. Although mixed populations were present in the URT of ferrets on day 6 p.i., the fitness of VN-H274Y virus was uncompromised as compared to that of its drug-sensitive counterpart, while that of TK-H274Y virus was impaired. A minor population of NA inhibitor-resistant variants may gain a replication advantage under suboptimal therapy in two ways: (i) preexisting variants less sensitive to the drug are selected from the quasispecies population, leading to an increase of the number of resistant clones, and (ii) outgrowing variants may acquire additional compensatory mutations that enhance their fitness. It is possible that use of antiviral drugs (particularly at suboptimal concentration) against mixtures of oseltamivir-resistant and sensitive viruses will promote the spread of drug-resistant variants

Table 1. Pathogenicity of oseltamivir-sensitive and -resistant recombinant H5N1 influenza viruses in ferrets

	No. showing indicated signs(s)/total no				Nasal wash titer ($log_{10}EID_{50}$/ml, mean ± SD)		
H5N1 virus or mixture	Weight loss (%, mean ± SD)	Temperature increase, (°C, mean ± SD)	Respira- tory signs	Neurologic signs	2	4	6
A/Vietnam/1203/04*							
VN-WT	22·3 ± 6·7	1·4 ± 1·9	3/3	2/3	†	4·9 ± 1·2	4·2 ± 1·2
VN-H274Y	22·9 ± 1·0	2·1 ± 0·4	3/3	1/3	3·3**	5·2 ± 0·8	5·3 ± 1·7
10%VN-WT: 90% VN-H274Y	21·9 ± 4·2	2·0 ± 0·3	3/3	1/3	2·8**	3·8 ± 0·4	4·5 ± 1·0
50%VN-WT: 50% VN-H274Y	17·5 ± 1·7	1·9 ± 0·5	3/3	1/3	2·8**	4·0 ± 0·4	4·4 ± 1·0
90%VN-WT: 10%VN-H274Y	19·3 ± 5·2	2·2 ± 0·5	3/3	1/3	†	3·8 ± 0·6	3·5 ± 0·3
A/Turkey/15/06***							
TK-WT	16·7 ± 5·8	2·8 ± 0·8	1/3	0/3	2·9 ± 0·3	3·5 ± 1·2	1·8 ± 0·7
TK-H274Y	4·8 ± 5·0	1·7**	0/3	0/3	2·8 ± 0·0	1·8 ± 0·7	2·3 ± 1·3
50%TK-WT: 50% TK-H274Y	6·4 ± 3·3	1·8 ± 0·5	0/3	0/3	4·1 ± 0·5	3·2 ± 0·6	2·1 ± 1·0
90%TK-WT: 10% TK-H274Y	19·2 ± 2·2	2·4 ± 0·5	1/3	0/3	3·6 ± 1·1	2·8 ± 0·4	2·4 ± 0·3

*Ferrets in all groups inoculated with A/Vietnam/1203/04 virus died by day 6–7 p.i. and were observed once daily for 7 days.
**Results obtained from one ferret.
***Ferrets in all groups inoculated with A/Turkey/15/06 virus survived the infection and were observed once daily for 14 days.
†below lower limit of detection (<0·75 $log_{10}EID_{50}$/ml).

Table 2. Sequence analysis of virus populations in the inoculum and in inoculated and co-inoculated ferrets

	In inoculum	H274Y no clones/total no. (%) in ferret nasal washes on day 6 p.i.				
H5N1 virus or mixture	H274Y no. clones/total no. (%)	Other mutations (%)	F#1	F#2	F#3	Mean H274Y frequency (%)
A/Vietnam/1203/04						
VN-WT	0/18 (0)	I117T (6)	0/20 (0)	0/20 (0)	0/20 (0)	0
VN-H274Y	20/20 (100)	–	20/20 (100)	18/20 (90)	20/20 (100)	97
10%VN-WT : 90% VN-H274Y	20/30 (67)	K150R (3)	15/20 (75)	15/20 (75)	18/20 (90)	80
50%VN-WT : 50% VN-H274Y	15/30 (50)	–	14/20 (70)	13/20 (65)	16/20 (80)	72
90%VN-WT : 10%VN-H274Y	2/30 (7)	–	11/20 (55)	10/20 (50)	10/20 (50)	53*
A/Turkey/15/06						
TK-WT	0/20 (0)	–	0/20 (0)	0/18 (0)	0/20 (0)	0
TK-H274Y	20/20 (100)	N294G (5)	8/20 (40)	14/18 (78)	5/20 (25)	48
50%TK-WT : 50% TK-H274Y	6/20 (30)	–	5/38 (13)	1/24 (4)	3/34 (9)	9*
90%TK-WT : 10% TK-H274Y	2/20 (10)	–	4/22 (18)	1/26 (4)	0/26 (0)	7

–, no neuraminidase mutations were detected. F, ferret.
*$P < 0{\cdot}05$, unpaired two-tailed *t*-test with Welch's correction.

by inhibiting drug-sensitive variants that are competing with them for the dominance in the infected host.

The influence of multiple genes on the fitness of viruses carrying H274 NA mutation cannot be excluded. In our study we focused on additional NA mutations, and sequence analysis of individual NA clones [7] was done to identify potential host-dependent and compensatory NA mutations. We found that the NA mutations E119A and N294S, which confer cross-resistance to oseltamivir and zanamivir,[1,2] can emerge spontaneously in clade 2.2 H5N1 influenza virus in ferrets. Further, we observed that mutations at NA catalytic (R292K) and framework (I222L and N294S) sites and in close proximity to the NA enzyme active site (V116I, I117T/V, Q136H, K150N, A250T) emerged without drug pressure in both pairs of H5N1 viruses. Compensatory mutations in NA or other genes may mitigate any fitness cost imposed by resistance mutations. Our study identified six potential compensatory NA changes (D103V, F132S, I254V, E276A, H296L, and F466S) that may affect the fitness of viruses with the H274Y NA mutation. We suggest that NA mutations at residues I254V and E276A are of importance. Interestingly, we observed differences in predominance of I254V and E276A NA mutations in different genetic backgrounds: I254V mutation was identified in A/Vietnam/1203/04 (H5N1)-like and E276A in A/Turkey/15/06 (H5N1)-like genetic background. Moreover, I254V NA mutation was identified only when ferrets were inoculated with the mixtures of VN-WT and VN-H274Y viruses, but not in ferrets inoculated with VN-H274Y virus. None of the potential compensatory NA mutations was identified in the original inoculum used to infect ferrets. The H274Y NA mutation causes a large shift in the position of the side chain of the neighboring E276 residue,[8] which must form a salt bridge with R224 to accommodate the large hydrophobic pentyl ether group of oseltamivir. Residue I254 is located near the NA active site, and although it does not alter polarity, it results in a shorter side-chain and, thus, may indirectly affect the residues in the NA active site.

We suggest that antigenic and genetic diversity, virulence, the degree of NA functional loss, and differences in host immune response and genetic background can contribute to the observed differences in the fitness of H5N1 influenza viruses. Therefore, the risk of emergence of drug-resistant influenza viruses with uncompromised fitness should be monitored closely and considered in pandemic planning.

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Disclosure

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Unique characteristics of long-acting neuraminidase inhibitor laninamivir octanoate (CS-8958) that explains its long-lasting activity

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Abstract

We speculate that there are two unique characteristics that explain the long action of CS-8958: one is a stable binding of its active metabolite laninamivir to neuraminidase of influenza virus; another is the long lasting effect of laninamivir in the respiratory tract after intranasal administration of CS-8958. In this manuscript, we report that the stable binding of laninamivir to pandemic (H1N1)2009 virus was also confirmed. In addition, the hypothetical mechanism of the long lasting characteristics in mouse respiratory tract based on the localization of the enzyme hydrolyzing CS-8958 to laninamivir is presented.

Introduction

Laninamivir (R-125489) is a strong NA inhibitor against various influenza viruses, including oseltamivir-resistant viruses.[1–6] We discovered a single intranasal administration of laninamivir octanoate (CS-8958), a prodrug of laninamivir, showed a superior anti-virus efficacy in mouse and ferret infection models compared to repeated administration of oseltamivir and zanamivir.[2–4,7] This suggested that CS-8958 works as a novel long-acting NA inhibitor of influenza virus *in vivo*. A single inhalation of CS-8958 proved noninferiority in adult patients[8] and significantly superior in child patients,[9] compared to an approved dosage regimen of oseltamivir for treatment. CS-8958 has been commercially available as an inhaled drug, Inavir®, for the treatment of influenza in Japan since October 2010.

The long-acting characteristics of CS-8958 are explained by several reasons. First, CS-8958 was quickly hydrolyzed to an active metabolite, laninamivir, after an intranasal administration to mice, and was retained for a long time as laninamivir in target organs, such as lung and trachea.[10] However, with an intranasal administration of laninamivir, it disappeared quickly and did not demonstrate its long-lasting characteristics.[10] Another reason is a strong binding of laninamivir to NAs of seasonal influenza viruses compared to other three NA inhibitors, oseltamivir carboxylate, zanamivir, and peramivir.[3]

In the following, the tight-binding ability of laninamivir to pandemic (H1N1)2009 NA, as well as to the seasonal

influenza virus NAs, was demonstrated. In addition, we present a hypothesis of the mechanism of the long-lasting property of CS-8958 in mouse based on a localization of an enzyme that hydrolyzes CS-8958 to laninamivir.

Materials and methods

Binding stability of NA inhibitors to virus NAs

The influenza viruses, pandemic(H1N1)2009 (INF139), A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Mie/1/93 were treated with excess NA inhibitors, such as oseltamivir carboxylate, zanamivir, peramivir, and laninamivir, and then unbound NA inhibitors were removed from the mixtures with a Bio-Spin column Bio-Gel P-6 (Bio-Rad Laboratories, Hercules, CA, USA). The NA substrate, 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (nacalai tesque, Japan) was added to the virus-NA inhibitor complex, and the NA activities were followed for 6 hours at room temperature by measuring the fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

CS-8958 hydrolyzing enzyme

The enzyme which hydrolyzes CS-8958 to laninamivir was partially purified from rat lungs using ion exchange column chromatography, and almost all bands separated by an SDS-polyacrylamide gel electrophoresis were identified by mass spectrometry. The gene expression profiles of the enzyme were investigated by the BioExpress database (GeneLogic Inc., Gaithersburg, MD, USA). The enzyme gene cloned from mouse lung mRNA was transiently expressed in COS cells. Antiserum to the esterase was prepared by immunizing rabbits, and immunostaining was done using HistoMouse-TM-MAX kit (Invitorgen Corp., Carlsbad, CA, USA) according to the manufacturer's manual.

Results

Binding stability of NA inhibitors to the four viruses are shown in Figure 1. Considerable differences in the dissociation rates of the NA inhibitors among the four test virus strains were observed. Binding stabilities of four NA inhibitors to pandemic(H1N1)2009 INF139 were similar to those of the seasonal H1N1 virus, A/New Caledonia/20/99 (H1N1). Laninamivir bound to NAs of H1N1 and B viruses more stably than three other NA inhibitors. NA activity of H3N2 virus was severely inhibited by all test NA inhibitors.

The enzyme that hydrolyzes CS-8958 to laninamivir in rat lungs was identified as carboxyesterase. This esterase was shown to be expressed in epithelial cells of rat lung by *in situ* hybridization.[11] The mouse homolog of the rat

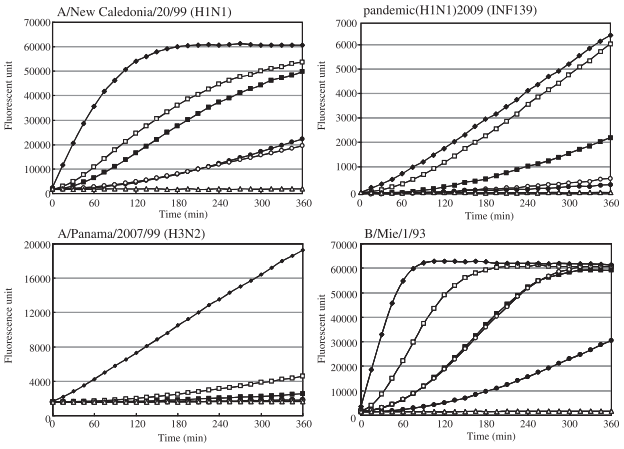

Figure 1. Difference of binding stabilities of various NA inhibitors to influenza virus neuraminidases. The NA substrate was added to the influenza virus-NA inhibitor complex (oseltamivir carboxylate, ■; zanamivir, □; peramivir, ○; laninamivir, •; distilled water, ♦), and the NA reaction was followed for 360 minutes. The background (only the NA substrate [Δ]) is also shown. A part of data from.[3]

esterase was carboxylesterase 3 (CES3). The mRNA of the mouse CES3 was shown to be highly expressed in lung and liver by the gene expression profile, and CES3 was also found to contain signal sequences for retention in endoplasmic reticulum (ER) and Golgi at the C-terminus. The cloned CES3 gene and the CES3 gene lacking the signal sequence were exogenously expressed in the COS cells. The CS-8958-hydrolyzing activity associated with the COS cells expressing CES3 was recovered from the culture sup of the COS cells expressing CES3 lacking the retention signal sequence. Localization of CES3 was immunohistologically confirmed inside the airway epithelium cells of mice, which are the target cells for influenza virus infection.

Conclusions

The long acting property of intranasal administration of CS-8958 in mice can be explained both by the long retention of laninamivir in the respiratory tract and by the stable binding of laninamivir to influenza virus NA. Again, stable binding of laninamivir to NA of pandemic (H1N1)2009 virus was also observed similar to that of seasonal H1N1 virus.

The following are speculated as the mechanisms for the long-lasting characteristics of CS-8958 in mice. We explain the mechanism by clarifying a CS-8958 hydrolyzing enzyme and its localization inside cells. The hypothesis of the mechanism is presented in Figure 2. Briefly, hydrophilic laninamivir may not enter easily inside cells, whereas hydrophobic CS-8958 may enter inside cells. CES3 with ER/Golgi retention signal hydrolyzes octanoate of CS-8958

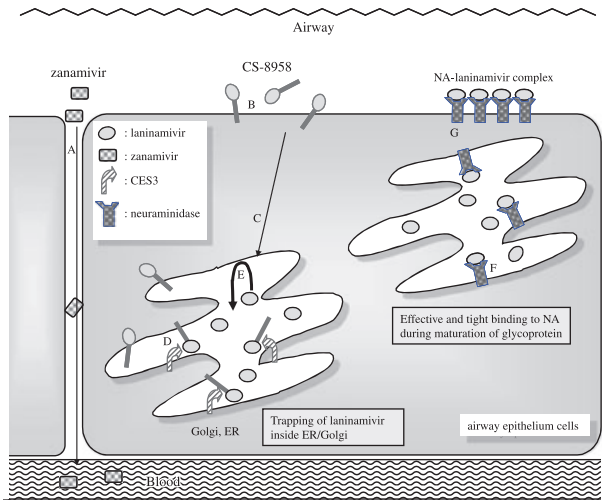

Figure 2. Hypothesis of the mechanism of long-lasting and long-acting characteristics of intranasally administered CS-8958 in mouse respiratory tract. (A) Mainly hydrophilic zanamivir and laninamivir will not enter into cells due to their hydrophilic property, and they disappear quickly from the air way. Therefore, twice daily inhalation for 5 days is required for zanamivir. (B) Major portion of CS-8958 will associate with the cell membrane via the hydrophobic moiety. (C) Cell associated CS-8958 will move to endoplasmic reticulum (ER)/Golgi by an unknown mechanism. (D) Carboxylesterase 3, the active site of which faces inside ER/Golgi, hydrolyzed octanoate of CS-8958 to generate laninamivir. (E) Once generated, laninamivir acquires a hydrophilic property, so that it is difficult to leak outside the ER/Golgi. (F) NA is a glycoprotein that matures in the ER/Golgi, where NA meets laninamivir to efficiently make a complex. Laninamivir binds NA stably. (G) NA-laninamivir complex moves to the cell membrane to assemble progeny viruses.

to generate the hydrophilic drug, laninamivir, and then it is trapped inside ER/Golgi because of its high hydrophilicity. The glycoprotein, NA, which matures in ER/Golgi, meets laninamivir there and efficiently makes a stable complex with it.

There are some questions that remain. How does CS-8958 move from the cell membrane to ER/Golgi? Is laninamivir indeed trapped inside ER/Golgi, and does it make a complex with NA in mice? We are now making an attempt to clarify these concerns.

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Potent inhibition of pandemic influenza H1N1 (2009) virus propagation by novel chemically synthesized compounds

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Keywords Chemical compounds, MDCK cell line, pandemic influenza H1N1.

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Abstract

Introduction Influenza viruses continue to pose a severe threat worldwide, causing thousands of deaths and enormous socio-economic loss. The recent emergence of a novel pandemic influenza H1N1 (2009) strain has demonstrated the rapid and unpredictable nature of influenza virus evolution and demands effective therapeutics and vaccines to control such outbreaks. In view of this, antiviral approaches with novel mechanisms of action are required to combat resistant influenza strains.

Objectives Our study aimed to determine the toxic concentrations of the newly synthesized compounds on madin-darby canine kidney (MDCK) cells and thereby assess their median effective dose able to inhibit the virus propagation in the cells.

Materials and methods We performed *in vitro* studies to evaluate the activity of two newly synthesized chemical compounds against the pandemic influenza H1N1 (2009) virus. MDCK cell line was used for studying the antiviral activity of the compounds. Inhibition of the virus by these novel compounds was also analyzed by cell viability assays, viral plaque assay, real time RT-PCR, and western blotting.

Results The percentage cell viability of the viral infected MDCK cells increased by 52% and 45% in the presence of MeUH and FLH, respectively, and by 60% when used in combination. The real time RT-PCR and immunoblotting showed 50% inhibition in expression of the HA gene of the virus.

Discussion Our study shows these novel compounds to possess potent antiviral activity, which could be used as potential therapeutic agents for prevention and treatment of influenza virus infection.

Introduction

Influenza viruses cause the most prevalent respiratory tract infection in humans. Every year, almost 10–20% of the world population suffers from influenza virus. The recent emergence of a novel pandemic influenza H1N1 (2009) strain has caused massive destruction on the socio-economic front and has raised the pandemic alarm all over the world.[1–3] The pandemic influenza H1N1 (2009) strain is the first example of triple reassortment of virus capable of human to human transmission. The two surface proteins, haemagglutinin (HA) and neuraminidase (NA), are of swine influenza virus origin, but the other internal genes of the virus have originated from influenza viruses of swine, avian, and humans.[4] Such outbreaks caused by the emergence of novel influenza viruses call for effective therapeutics, vaccines, and antiviral strategies for efficient control for treating patients with virus infection. Also, the newly emerging drug resistant strains have posed serious problems in the implementation of effective therapeutic strategies. Thus, there is an urgent need for the development of newer and effective drugs to combat with the changing patterns of the influenza virus.

In our study, we have explored the antiviral potential of two newly synthesized compounds to provide protection against the novel pandemic influenza virus H1N1 (2009) strain. The compounds were reconstituted in dimethylsulphoxide (DMSO), and so the initial studies began with cytotoxicity determination of solvent on uninfected and untreated madin-darby canine kidney (MDCK) cells. On obtaining an upper limit for DMSO, the compounds were tested for estimation of their maximum non-toxic dose to the MDCK cells. Thereafter, the effective dose of the compounds was evaluated and validated by a number of assays and gene expression profiling at both nucleic acid and protein level. We found that these newly synthesized compounds possess potent inhibitory activity towards the novel pandemic influenza H1N1 (2009) virus. These findings are being evaluated *in vivo* for a better understanding of their inhibitory capabilities and also their effect on the host metabolism. This will be

required in the course of development of new drugs for use in the prophylaxis and treatment against the influenza virus.

Materials and methods

Cell culture

The MDCK cell line (from NCCS, Pune) was maintained in 1× DMEM media (Sigma, St. Louis, MO, USA) supplemented with 10% Fetal Calf Serum and antibiotics viz. 100 unit/ml penicillin and 100 μg/ml streptomycin at 37°C/5% CO_2.[5]

Inhibitors

The synthesized compounds used in this study were kindly provided by the Department of Chemistry, University of Delhi, Delhi, India.

Virus stocks

The pandemic influenza H1N1 (2009) virus was isolated and propagated in the allantoic cavities of embryonated chicken eggs during the pandemic period. The virus stocks were prepared and stored at −80°C.

Plaque assay

Plaque assay was performed as previously described by Hui *et al.*, 2003.[6] Briefly, $0{\cdot}5 \times 10^6$ MDCK cells/ml were seeded in six-well plates and maintained in DMEM for 24 hours at 37°C/5%CO_2. The monolayer of the cells was inoculated with serially diluted virus samples for 45 minutes at 37°C/5%CO_2. Subsequently, a mixture of agar overlay was added, and the plates were incubated at 37°C for 5 days or until formation of plaques. The plaques were visualized after removal of the agar plug and staining with 0·1% crystal violet or neutral red solution. The virus titre was expressed as plaque forming unit (PFU) per milliliter.

Cytotoxicity screening of compounds

The *in vitro* cytotoxicity analysis was performed to determine the 50% cytotoxic concentration (CC_{50}) of the compounds on MDCK cells. The compounds were dissolved in dimethylsulfoxide (DMSO), and so a prior cytotoxicity analysis was performed to determine the toxic concentration of DMSO on the cells. Various concentrations of compounds were mixed with DMEM containing 1% FCS before addition to the preformed monolayer of MDCK cells in 96-well plates. A series of suitable controls for *in vitro* CC_{50} determination was included in every plate, and the plates were incubated in the optimum environment for MDCK cell culture. The CC_{50} of test compounds was analyzed by estimation of percentage cell viability of the compound- and mock-treated MDCK cells by performing a colorimetric assay using tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) at end-point of 48 hours post-incubation.

Cell viability assay

The assay was performed as described by Mosman 1983.[7] Briefly, MTT stock at a concentration of 0·5 mg/ml was prepared in 1× PBS. The media was aspirated from the wells and 100 μl of MTT dye from the stock was added to each well. Following incubation at 37°C/5% CO_2 for 2–4 hours, the dye was very carefully removed from the wells, and the cells were incubated with 100 μl of stop solution (DMSO) per well at 37°C/5% CO_2 for 1 hour. The absorbance of the supernatants from each well was measured at 540 nm, and the percentage cell viability was calculated.

$TCID_{50}$ of virus

Madin-darby canine kidney cells were maintained overnight in a 12-well tissue culture plate at 37°C/5% CO_2. The cells were inoculated with various virus dilutions at 37°C/5% CO_2 for 45 minutes and observed for cytopathic effect (CPE). The media from the experimental wells were aspirated after 48–72 hours of infection and were subjected to plaque assay. The percentage cell viability was determined by performing MTT assay. The results of both these tests were used to assess $TCID_{50}$ of the virus.

Estimation of antiviral activity of compounds

The pre-formed monolayer of MDCK cells was inoculated with the 10-fold dilution corresponding to $TCID_{50}$ of the virus for 1 hour at 37°C/CO_2. The experimental setup included control wells for the cells, virus, and compound. Meanwhile, the concentrated stocks of the synthesized compounds were diluted with DMEM (with1% FCS) to various concentrations within their respective CC_{50} ranges. One hour post-infection, the cells were incubated with these diluted solutions. The cells were observed at various time intervals post-inoculation for CPE, and 200 μl media was collected from each experimental well for performing hemagglutination test. After 48 h, the media was collected for plaque assay and the cells were subjected to MTT cell viability assay.

Real time PCR

Preformed monolayers of MDCK cells were infected with virus and treated with the respective inhibitory concentration of the compounds. Forty-eight to 72 hours post-incubation, total cellular RNA was isolated using Ribozol (Amresco, Solon, OH, USA) and treated with 50 μg/ml of DNase (Promega, Madison, USA). The concentration and

quality of the RNA from each well were determined by measuring their absorbance at 260 and 280 nm. One microgram of the cDNA synthesized from each RNA sample was used for SYBR green-based real-time PCR detection of the HA gene of pandemic influenza H1N1 (2009) virus. As a control, human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was also amplified using gene specific primers.[8,9]

Immunoblotting

Immunoblotting was performed to further validate the antiviral potential of the compounds. The experimental protocol was the same as for real time RT-PCR analysis. The cells were harvested 48 hours post-treatment with the compounds to prepare whole cell lysates in mammalian cell lysis buffer [0·1 m NaCl, 0·01 m Tris Cl (pH 7·6), 0·001 m EDTA (pH 8·0), 1 m m protease inhibitor cocktail, 100 μg/ml PMSF]. The protein concentration was determined by BCA protein assay. The cell lysates were fractionated on 12% polyacrylamide for western blotting. The blot was developed using sheep monoclonal antibody (Santa Cruz Biotechnology, CA, USA) against HA protein of influenza virus and horseradish peroxide conjugated rabbit-anti sheep IgG (1:1000 dilutions) as secondary antibody.[10]

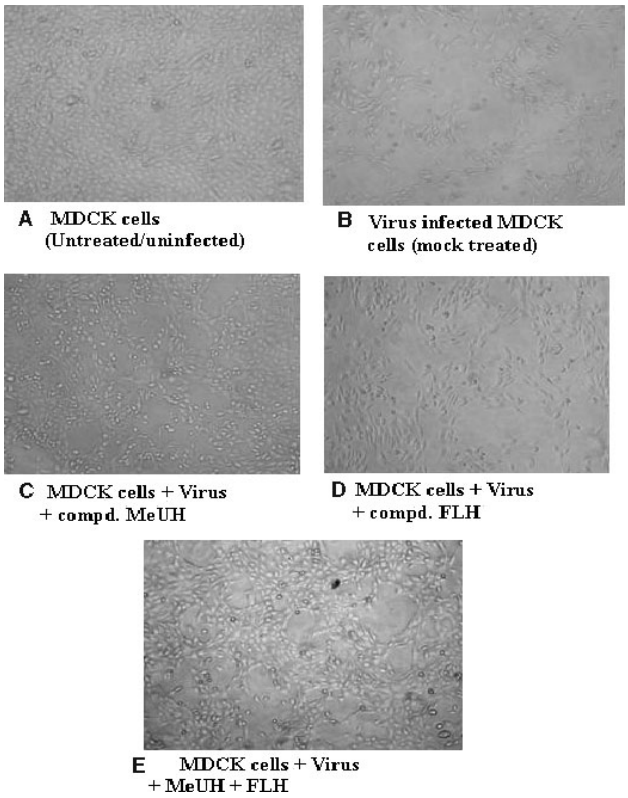

Figure 1. Compounds showing potent antiviral effect on the pandemic influenza H1N1 (2009) virus propagation in madin-darby canine kidney cells.

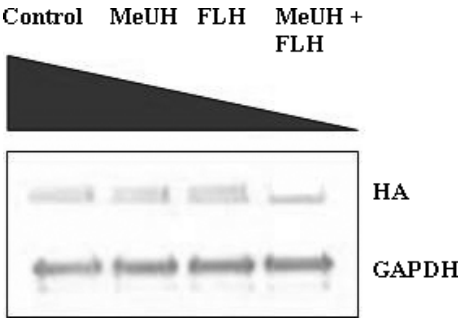

Figure 2. Decreased expression of HA protein of pandemic influenza H1N1 (2009) virus as seen through immunoblotting.

Results

Fifty percent cytotoxic concentration (CC_{50})

The median cytotoxic concentration for compound MeUH came out to be 150 μm, and that for FLH was 160 μm.

Reduced cytopathic effect (CPE)

Compounds showing potent antiviral effect on the pandemic influenza H1N1 (2009) virus propagation in madindarby canine kidney cells (Figure 1).

Plaque assay

The viral titres remained constant in cells treated with the compounds, while they increased in the untreated virus infected cells.

Median effective dose (ED_{50})

ED_{50} for the compounds MeUH and FLH were 150 and 160 μm, respectively. Fifty-two percent (MeUH) and 45% (FLH) inhibition against the pandemic influenza H1N1 (2009) virus was achieved using ED_{50} of the test compounds.

Inhibition of viral gene expression

Both the compounds were able to reduce the RNA levels of the HA gene by approximately 40–45%, whereas approximately 50% inhibition was seen when both the compounds were used in combination. Similar results were obtained by the immunoblotting analysis (Figure 2).

Discussion

Antiviral therapy has shown to be a promising tool in the management of various respiratory diseases, including those caused by influenza viruses. We have already shown inhibition of influenza virus replication in our earlier studies using catalytic nucleic acids,[11] which can be used as an approach in the development of new therapeutic strategy. These therapies are very useful as the influenza virus

vaccines need annual renewals due to frequent genetic drifts in the viral surface proteins. In pandemic situations the existing vaccines do not provide complete protection against the novel virus as the population generally remains naïve for the newly mutated surface antigens. The antiviral drugs play an important role in the control of novel viral strains for which there are no vaccines available. However, the key obstruction in the extensive use of antiviral drugs is their cost and relative therapeutic efficacy provided. Two classes of drugs were being used for treatment and control of the influenza virus infection in humans, the M2 ion-channel blockers[12,13] (amantadine and rimantadine), which prevent viral uncoating, and the neuraminidase inhibitors[14,15] (zanamivir and oseltmivir), which prevent the release of influenza virions from the cytoplasmic membrane. But widespread resistance to these antiviral drugs[16,17] has limited their use. Thus, novel drugs are required for the effective therapy against the emerging strains of influenza virus.

The novel chemical compounds used in our study were tested for their antiviral efficacy against the pandemic influenza H1N1 (2009) virus. A reduction in the CPE in compound treated virus infected MDCK cells indicated presence of antiviral activity in chemical compounds. The persistence of constant viral titers in the compound treated cells provided evidence for the interference posed by the compounds in the replication of influenza virus. Inhibition in the HA gene expression further validated our hypothesis for the antiviral effect of compounds. The efficacy of these compounds in animal models is currently being validated in our laboratory. Further, molecular studies are required to ameliorate the awareness regarding the mode of action of these chemical compounds against the viruses.

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Susceptibility of human influenza viruses to neuraminidase inhibitors (season 2008–2009)

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Keywords Oseltamivir, pandemic H1N1, peramivir, seasonal influenza A and B, zanamivir.

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Abstract

Antiviral drugs play an essential role in managing infections caused by seasonal and pandemic influenza viruses. Due to the high prevalence of damantine resistance among seasonal influenza A viruses circulating in certain geographic regions, neuraminidase inhibitors (NAIs) are presently the only effective antiviral drugs for treatment and chemoprophylaxis of both seasonal and pandemic influenza infections. NAI susceptibilities of virus isolates collected during the 2008–2009 influenza season were assessed in the chemiluminescent neuraminidase inhibition (NI) assay. Among seasonal influenza A (H1N1) viruses tested, ~90% were outliers for oseltamivir and harbored the oseltamivir-resistance conferring H275Y mutation in the neuraminidase (NA), while only ~1% of pandemic 2009 influenza A (H1N1) viruses (H1N1pdm) were resistant to oseltamivir. All influenza A (H3N2) and B viruses were sensitive to oseltamivir, except for one A (H3N2) virus with D151V mutation, and an influenza B virus with D197E (D198E in N2 numbering) mutation in the NA. All viruses were sensitive to zanamivir, except for some seasonal influenza A (H1N1) and A (H3N2) outliers, which had no apparent changes in the NA besides the cell culture induced mutations at residue D151. All viruses tested for peramivir were sensitive to the drug, with the exception H275Y variants among seasonal A (H1N1) and H1N1pdm isolates, which exhibited reduced susceptibility. This study summarizes baseline NAI susceptibility profiles of seasonal and pandemic influenza viruses and contributes further criteria for defining resistance to NAIs.

Introduction

Adamantanes (M2 ion channel blockers) and neuraminidase inhibitors (NAIs) are two classes of drugs currently licensed by the US Food and Drug Administration (FDA) for prevention or treatment of influenza A virus infections.[1] The effectiveness of adamantanes is, however, compromised by resistance among influenza A viruses circulating in certain geographic areas.[2,3] Two FDA-approved NAIs, oseltamivir and zanamivir, are presently the only antiviral drugs effective against seasonal and pandemic influenza infections. An investigational NAI, peramivir, was recently prescribed in the United States (US) for treatment of 2009 pandemic influenza H1N1 infections under an emergency use authorization[4] and is now licensed in Japan,[5] while another, laninamivir, is being developed as an inhaled prodrug.[6]

Resistance to NAIs among circulating influenza viruses was previously low (<1% worldwide).[7–9] However, the 2007–2008 influenza season was marked by a worldwide emergence of oseltamivir-resistant seasonal influenza A (H1N1) viruses with the H275Y (H274Y in N2 numbering) in the NA.[9–14] The prevalence of oseltamivir resistance was even higher in the subsequent 2008–2009 influenza season with many countries reporting up to 100% oseltamivir resistance,[15,16] emphasizing the critical need for NAI susceptibility surveillance of influenza viruses circulating globally.

This study presents NAI surveillance data of seasonal influenza A and B viruses collected globally during the 2008–2009 influenza season (October 1, 2008 to September 30, 2009), as well as H1N1pdm viruses collected between April 2009 and September 30, 2009. The study defines baseline NAI susceptibility patterns of seasonal and pandemic influenza viruses, and seeks to contribute further criteria for evaluating NAI resistance.

Materials and methods

Seasonal and pandemic influenza viruses collected globally between October 1, 2008 and September 30, 2009 were submitted to the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the Centers for Disease Control and Prevention (CDC) in Atlanta,

Table 1. Neuraminidase inhibitor susceptibility of seasonal and pandemic influenza viruses (2008–2009)

NAI	Type/Subtype	Isolates analyzed (*n*)*	IC50 (nm)** Range	Mean (±SD)	Median	Statistical Cutoff***
Oseltamivir	Seasonal Influenza A (H1N1) *H275 wildtype*†	98	0·10–0·49	0·23 ± 0·08	0·21	0·58
	Seasonal Influenza A (H1N1) *H275Y variants*††	1431	34·69–1023·68	123·32 ± 65·90	105·13	–
	2009 Influenza A (H1N1) *H275 wildtype*	2243	0·05–1·78	0·25 ± 0·11	0·24	0·52
	2009 Influenza A (H1N1) *H275Y variants*	14	54·21–155·00	87·57 ± 25·53	80·30	–
	Influenza A (H3N2)	833	0·04–1·38	0·24 ± 0·15	0·20	0·74
	Influenza B	913	0·59–7·75	3·41 ± 0·99	3·38	7·82
Zanamivir	Seasonal Influenza A (H1N1) *H275 wildtype*†	102	0·13–1·03	0·35 ± 0·15	0·31	1·05
	Seasonal Influenza A (H1N1) *H275Y variants*††	1424	0·07–3·49	0·51 ± 0·27	0·45	–
	2009 Influenza A (H1N1) *H275 wildtype*	2243	0·08–1·03	0·31 ± 0·08	0·30	0·69
	2009 Influenza A (H1N1) *H275Y variants*	14	0·27–0·53	0·38 ± 0·08	0·36	
	Influenza A (H3N2)	812	0·22–4·24	1·23 ± 0·80	0·97	4·31
	Influenza B	911	0·27–8·77	3·34 ± 1·31	3·27	10·06
Peramivir	Seasonal Influenza A (H1N1) *H275 wildtype*†	19	0·05–0·17	0·10 ± 0·03	0·10	0·28
	Seasonal Influenza A (H1N1) *H275Y variants*††	215	2·46–510·57	30·56 ± 66·06	12·95	–
	2009 Influenza A (H1N1) *H275 wildtype*	538	0·03–0·35	0·08 ± 0·04	0·07	0·22
	2009 Influenza A (H1N1) *H275Y variants*	11	8·1–12·91	10·40 ± 1·37	10·29	–
	Influenza A (H3N2)	219	0·05–0·94	0·17 ± 0·10	0·14	0·40
	Influenza B	52	0·24–1·10	0·56 ± 0·18	0·56	1·37

*Outliers and mixes (comprising wildtype and variant populations) were excluded from the calculation of mean, SD, and median of IC_{50} values.
**Determined in the chemiluminescent neuraminidase inhibition assay.
***Statistical cutoff of IC_{50} values for NAI susceptibility, determined by $X_{0·75}$ + 3IQR. Outliers with IC_{50} above this cutoff and >10 times the mean IC_{50} for each drug were characterized as extreme outliers; those with known drug-resistance mutations such as H275Y were classified as resistant and analyzed separately.
†H275 wildtype, oseltamivir-susceptible isolates.
††H275Y variants, oseltamivir-resistant virus isolates.
IQR, interquartile ranges; NAI, neuraminidase inhibitors.

GA, USA, and propagated in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA, USA). Reference viruses representative of oseltamivir-sensitive and -resistant seasonal and pandemic viruses were also propagated in MDCK cells.

Susceptibilities of virus isolates to the NAIs oseltamivir carboxylate (Hoffman-La Roche, Basel, Switzerland) and zanamivir (GlaxoSmithKline, Uxbridge, UK) were assessed in the chemiluminescent NI assay using the NA-Star™ kit (Applied Biosystems, Foster City, CA, USA) as previously described.[9] Additionally, subsets of virus isolates were tested for susceptibility to peramivir (BioCryst Pharmaceuticals, Birmingham, AL, USA).

Fifty percent inhibitory concentration (IC_{50}) values were calculated using JASPR curve fitting software, an in-house program developed at CDC. Curve fitting in JASPR was done using the equation: $V = Vmax \times (1 - ([I]/(Ki + [I])))$, where Vmax is the maximum rate of metabolism, [I] is the inhibitor concentration, V is the response being inhibited, and Ki is the IC_{50} for the inhibition curve.

Box-and-whisker plot analyses[7] of log-transformed IC_{50}s were performed for each virus type/subtype and NAI using SAS 9.2 software (SAS Institute, Cary, NC, USA) to identify viruses with extreme IC_{50} values (outliers). Outliers were characterized based on a statistical cutoff of IC_{50} greater than three interquartile ranges from the 75th percentile.

Outliers were subjected to genetic analysis by pyrosequencing[17] and/or conventional sequencing[18] to detect known or novel markers of NAI resistance. Those harboring previously characterized mutations in the NA associated with NAI resistance were considered drug-resistant; their descriptive statistics were determined separately from NAI-susceptible viruses.

Descriptive statistics to compute the mean, median, and standard deviation (SD), and a one-way analysis of variance were performed on original scale IC_{50} data, using SAS 9.2 software (SAS Institute) for each NAI and virus

type/subtype, excluding outliers, with statistical significance set at $\alpha = 0{\cdot}05$. Virus isolates comprising mixes of both wildtype and variant populations were also excluded from the descriptive statistical analyses.

Results

Among seasonal influenza A (H1N1) viruses tested for oseltamivir susceptibility ($n = 1533$), 1431 (93·3%) were outliers for the drug (Table 1) and harbored the oseltamivir-resistance conferring H275Y mutation in the NA. By contrast, only a small proportion (0·7%) of tested H1N1pdm viruses ($n = 2259$) were resistant to oseltamivir. All influenza A (H3N2) viruses ($n = 834$) were sensitive to oseltamivir except for one outlier, A/Ontario/RV0442/2009 with D151V mutation in the NA, whose IC_{50} of 2·50 nm was beyond the statistical cut-value off and >10-fold the mean IC_{50} for the drug (0·24 nm). All influenza B viruses ($n = 914$) were sensitive to oseltamivir with exception of an outlier B/Texas/38/2008, with D197E (D198E in N2 numbering) mutation in the NA, whose IC_{50} was beyond the cut-off, but only fourfold greater than the mean IC_{50} for the drug.

All virus types/subtypes tested for zanamivir were sensitive to the drug (Table 1), except for some outliers among seasonal influenza A (H1N1) and A (H3N2) outliers. The seasonal influenza A (H1N1) outliers included A/Thailand/1035/2008 (H1N1) and A/Hawaii/20/2008 (H1N1), both with combined H275Y and D151D/G mutations in their NA. The presence of concurrent mutations at NA residues H275 and D151 in seasonal influenza A (H1N1) virus isolates substantially enhances resistance to oseltamivir and peramivir and/or zanamivir, however, the changes at D151 are typically cell-derived and not present in clinical specimens.[19]

Influenza A (H3N2) outliers for zanamivir included A/Ontario/RV0442/2009 with D151V mutation in the NA, as well as A/Maryland/02/2009 and A/Vladivostok/53/2009 with D151G and mixed D151D/G mutations, respectively. Some mild outliers for zanamivir among A (H3N2) viruses with IC_{50} beyond the statistical cutoff but <10-fold mean IC_{50} for the drug were also identified; their genetic analysis revealed presence of wildtype and mutant sequences at residue 151 namely, D151D/G, D151D/N, or D151D/A. Mutations at residue D151 of the NA are associated with reduced susceptibility to zanamivir in A (H3N2) viruses,[9] but were reported to be cell-culture derived in recent H3N2 viruses.[20]

All virus isolates tested for peramivir ($n = 1058$) were sensitive to the drug, except for H275Y variants among seasonal influenza A (H1N1) and H1N1pdm viruses, which exhibited reduced susceptibility to the drug. In addition, one influenza A (H3N2) isolate, A/Ontario/RV0442/2009 with D151V mutation in the NA, showed reduced susceptibility to peramivir.

Discussion

The IC_{50} values determined in functional NI assays provide valuable information for detection of resistant viruses, but should not be used to draw direct correlations with drug concentrations needed to inhibit virus replication in the infected human host, as clinical data to support such inferences are inadequate.[21]

Nevertheless, combining elevated IC_{50} values with the presence of established molecular markers of resistance in the NA of virus isolates and their matching clinical specimens provides a reliable and reasonably comprehensive approach of identifying NAI-resistant isolates for surveillance purposes. In this study, outliers with elevated IC_{50} values for oseltamivir among seasonal influenza A (H1N1) and H1N1pdm viruses were confirmed to be oseltamivir-resistant based on the presence of the H275Y mutation in the NA. Outliers for oseltamivir and/or zanamivir among influenza A (H3N2) viruses in this study were shown to harbor mutations at D151, which were earlier associated with reduced susceptibility to zanamivir,[9] and were cell-culture derived.[20] The effects of D151 mutations on NAI susceptibility appear to be strain-specific; however, there are no conclusive supporting data and further investigations are required.

Outliers among the Influenza A viruses in this study exhibited changes in the NA, derived naturally or through cell-culture, which altered their susceptibility to NAIs. However, mild outliers for oseltamivir and/or zanamivir among influenza A viruses with slightly elevated IC_{50}s, but without apparent changes in the NA are sometimes identified. In such instances it is imperative to exclude the potential presence of influenza B among such outliers, using conclusive genetic tests such as real time PCR, since Influenza B viruses exhibit higher IC_{50} values for oseltamivir and zanamivir than Influenza A viruses.[21] Viruses exhibiting such mixes are typically excluded from statistical analyses of IC_{50}s for respective drugs and virus type/subtype.

Establishment of a clinically relevant IC_{50} cutoff value which could be used to differentiate statistical outliers from truly resistant viruses is imperative. Global surveillance for NAI susceptibility of influenza viruses circulating globally should be sustained to reflect the impact of seasonal and pandemic of influenza, given the limited pharmaceutical options available for control of influenza infections.

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Disclaimer

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention (CDC).

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Antiviral drug resistance study of influenza viruses isolated in Mongolia in recent years

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Introduction

Antiviral resistance of influenza viruses is a decisive factor for an effective clinical management of the disease. During the 2007–2008 season, increased levels of resistance to oseltamivir among influenza A(H1N1) isolates were reported in Europe and North America.[1–3] WHO recommended to National Influenza Centers to test antiviral resistance of representative strains isolated in each member country. Our previous study has been shown a dramatic increase of amantadine-resistant influenza A(H3N2) and A(H1N1) viruses from 2006 to 2008 in Mongolia.[4] Neuraminidase (NA) inhibitor resistance of influenza virus strains isolated in Mongolia has not been published so far. In this study we report the antiviral susceptibility of influenza virus strains isolated in 2008–2010 in Mongolia to NA and M2 ion channel inhibitors.

Material and methods

Specimen collection and virus isolation

Nasopharyngeal swab specimens from patients with acute respiratory infection were collected at 164 influenza sentinel surveillance units (outpatient and hospital-based) all over Mongolia. Specimens were transported to the Virology Laboratory, NCCD, Ulaanbaatar, and rt-RT PCR positive samples were grown in a MDCK cell culture according to the protocol developed by CDC.[5] Isolate viruses were kept frozen at −80°C until the antiviral testing. We have selected randomly 317 strains [37 A(H1N1) seasonal influenza virus strains isolated in 2008/2009, 262 strains of A(H1N1)pdm, and 18 B viruses isolated 2009/2010] for oseltamivir susceptibility testing, and 16 pandemic A(H1N1) strains for a screening of antiviral resistance mutations in the influenza virus M gene.

Amantadine resistance testing was performed by influenza virus M2 gene pyrosequencing according to Bright, *et al.* (9 strains),[6] and influenza virus gene segment 7 (M genes) sequencing (7 strains- GenBank accession numbers: CY053364, CY053365, CY054547, CY054549, CY055171, CY065990, and CY065998) and influenza virus gene segment 6 (NA gene) sequencing (2 strains GenBank accession numbers: CY073447 and CY073448) by the standard methods with Applied Biosystems 3130xl Genetic Analyzer using primers supplied by WHO Collaboration Centers.

NA inhibition assay

A chemiluminescent NA inhibition assay was performed with Veritas Microplate Luminometer using the commercially available kit, NA-Star (Applied Biosystems, Foster City, CA, USA), according to the manufacturers protocol. The NA inhibitor susceptibility of influenza virus isolates was expressed at the concentration of NA inhibitor needed to reduce NA enzyme activity by 50% (IC_{50}). Oseltamivir carboxylate, was provided by F. Hoffman-La Roche Ltd (Basel, Switzerland). NA inhibition assay data were analyzed using Robosage software comparing test data with the data produced by the reference NA inhibitor sensitive and resistance strains, which were provided by the WHO Influenza Collaboration Center, Melbourne, Australia.

Results

All viruses tested were sensitive to oseltamivir with two exceptions: a seasonal influenza virus A/Ulaanbaatar/1735/2009(H1N1) with 137·1 nm IC_{50} value and a pandemic influenza virus A/Dundgovi/381/2010(H1N1) with 65·6 nm IC_{50} value (Figure 1). There was oseltamivir resistance detected in 2·7% (37/1) of seasonal A (H1N1) and in 0·4% (262/1) of A (H1N1) pdm viruses. The oseltamivir-resistant viruses were collected from untreated patients. In total, 18 influenza B viruses were analyzed by NA inhibition assay and all were sensitive to oseltamivir.

The NA of both oseltamivir-resistant strains contained H275Y mutation based on the sequencing analysis. The difference in the NA amino-acid sequences between the

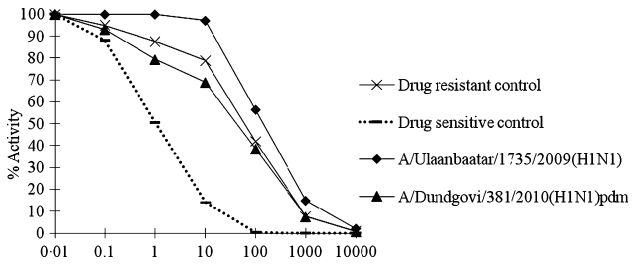

Figure 1. Results of the chemiluminescent NA inhibition assay with NA-resistant influenza virus strains.

Mongolian oseltamivir-resistant viruses and the respective oseltamivir-sensitive reference viruses is shown in Table 1. The sequence of A/Dundgovi/381/2010(H1N1) pdm (ProteinBank accession number ADM33442) differs from that of the A/California/07/2009 (H1N1) virus at amino acid residues 106, 248, and 275. The seasonal A/Ulaanbaatar/1735/2009(H1N1) had substitutions at amino acid resides 275 and 354 (ProteinBank Accession number ADM33443) compared to the A/Brisbane/59/2007 (H1N1) virus.

All 37 A(H1N1) viruses analyzed for M2 channel inhibitor resistance by pyrosequencing contained the S31N mutation and, thus, were resistant to this class of anti-influenza drugs. The Segment 7 sequencing revealed that 6 seasonal A(H1N1) viruses possess the common S31N mutation. Of note, a single strain A/Zavkhan/8299/2009(H1N1) contained an unusual S31D change in the M2 protein.

Discussion

Our study shows that the same prevalence [2·7% (1/37)] of seasonal A(H1N1) viruses with H275Y mutation in 2008/2009 season in Mongolia with the published data for 2007/2008 season from Japan.[7,8] However the prevalence of oseltamivir resistance in Japan has dramatically increased in 2008/2009 season to 100% (77/77). The observed double mutations: H275Y and D354G in A/Ulaanbaatar/1735/2009(H1N1) strain, which have been also found in Japan in 2008/2009 season.[7] The patient from whom the oseltamivir resistant seasonal influenza H1N1 virus has been isolated was a 1-year-old boy, living in Ulaanbaatar, the Capital City, without history of using oseltamivir. The patient from whom the oseltamivir resistant A(H1N1)pdm virus was isolated was a 59 year-old man, residing in the Dundgovi, the southern Province, also without history of antiviral treatment. According to the WHO data, isolation of the pandemic viruses carrying H275Y change from untreated patients has been uncommon.

Table 1. The comparison of NA amino acids of pandemic and seasonal A(H1N1) strains

Location of amino acids	106	248	275	354
Pandemic A(H1N1)				
A/California/07/2009(H1N1)	V	N	H	–
A/Dundgovi/381/2010(H1N1)	I	D	Y	–
Seasonal A(H1N1)				
A/Brisbane/59/2007(H1N1)	–	–	H	D
A/Ulaanbaatar/1735/2009(H1N1)	–	–	Y	G

Circulation of amantadine-resistant seasonal A (H1N1) viruses has been increasing in Mongolia since 2006/2007 influenza season.[4] All pandemic influenza A(H1N1) strains (16) tested were resistant to M2 channel inhibitors due to the presence of the S31N mutation in the M2 protein. Among seasonal A(H1N1) viruses, one contained a S31D change whereas the others had S31N, the well established marker of resistance to both amantadine and rimantadine.

This is the first report of detecting the S31D change in the seasonal A(H1N1) viruses. According to the CDC data (unpublished), the S31D change conferred the drug resistance in the A(H3N2) viruses according to the virus yield reduction assay.

It is essential to continue the antiviral resistance surveillance of influenza virus strains circulating in Mongolia to ensure the efficiency of a proper clinical management of influenza patients.

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Adamantane and oseltamivir dual resistance in seasonal influenza A (H1N1) viruses

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Keywords Adamantanes, neuraminidase inhibitors, resistance, seasonal A(H1N1).

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Introduction

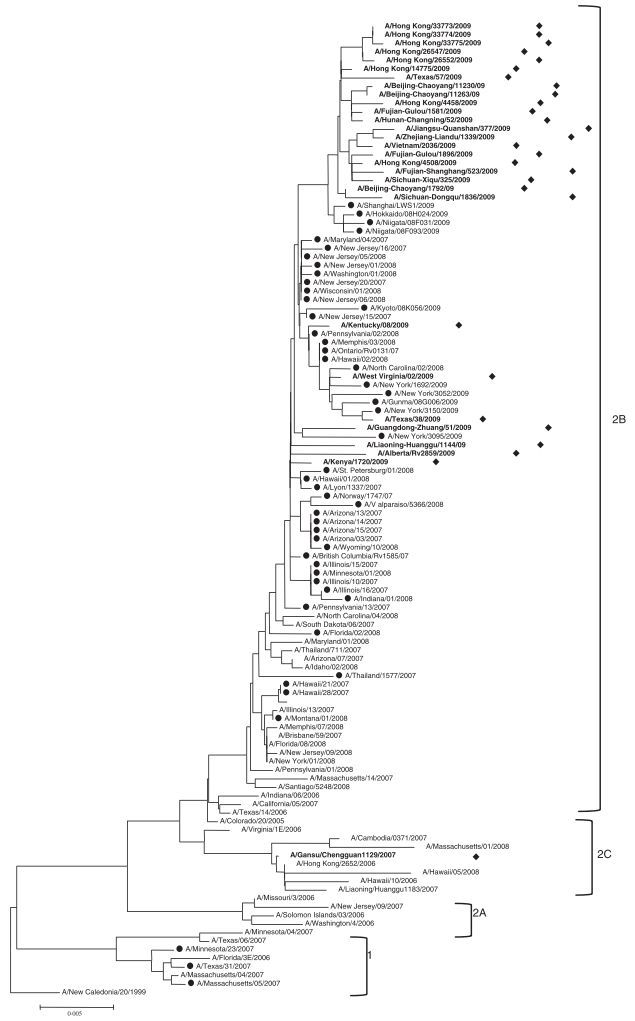
Seasonal influenza A (H1N1) viruses collected from the United States and globally from 2008 to 2010 were tested for resistance to the FDA approved antivirals: NAIs (zanamivir and oseltamivir) and adamantanes (amantadine and rimantadine). In recent seasons, two distinct genetic clades have co-circulated: clade 2B, which included oseltamivir-resistant and adamantane-susceptible viruses, and clade 2C, which contained viruses resistant to adamantanes and susceptible to oseltamivir. Although resistance to both adamantanes and oseltamivir was uncommon, we report 28 viruses with dual antiviral drug resistance from five countries: Canada, China, Kenya, Vietnam, and the United States.

Materials and methods

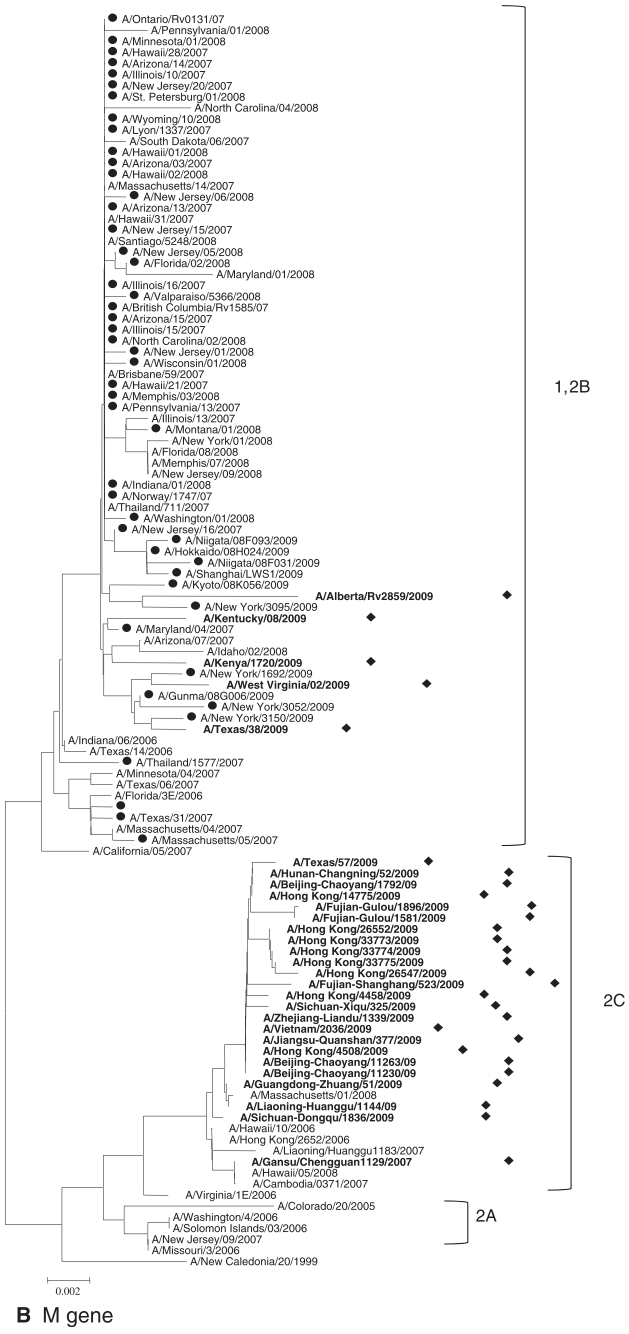
Seasonal (pre-pandemic) influenza A (H1N1) virus isolates collected globally during the 2008–2009 and 2009–2010 influenza seasons were submitted to the CDC for virus strain characterization and antiviral resistance surveillance as previous described.[1,2] Testing for NAI resistance was performed in the NA inhibition assay using the NAStar^{TM} kit (Applied Biosystems, Carlsbad, CA, USA) with a chemiluminescent substrate as previously described.[2] IC_{50} values (the inhibitor concentration required to reduce enzyme activity by 50%) were determined using CDC in-house curve-fitting software (JASPR). For seasonal influenza A (H1N1) viruses, resistance to adamantanes and the presence of the oseltamivir-resistance conferring mutation, H275Y in the NA, was analyzed using pyrosequencing on the PyroMark Q96 ID platform (Qiagen, Valencia, CA, USA) as previously described.[1] Full genome sequencing (PB2, PB1, PA, HA, NP, M, and NS gene segments) of seasonal influenza A (H1N1) was performed using the conventional Sanger sequencing method[3] on the 3730xl DNA analyzer (Applied Biosystems). Sequences were submitted to GISAID (accession numbers: EPI89455–EPI89457, EPI89861–EPI89863, EPI90085–EPI90087, EPI211257–EPI211259, EPI211263–EPI211265, EPI215523–EPI215525, EPI215535–EPI215536, EPI215538–EPI215540, EPI232853–EPI232855, EPI241608–EPI241610, EPI244128–EPI244130, EPI244149–EPI244157, EPI244243–EPI244245, EPI249557–EPI244560, EPI266810–EP1266935). Phylogenetic analysis of each gene was performed using Tamura-Nei neighbor-Joining method in MEGA version 4. Phylogenetic trees were rooted to A/New Caledonia/20/1999. To illustrate reassortment events, NA and M genes are shown (Figure 1A, B). Phylogenetic trees for additional genes (PB2, PB1, PA, HA, NP, and NS) are available upon request. Epidemiological and clinical information for the United States cases of dual resistance was collected with the assistance of the State Health Departments (Table 1).

Results

A total of 28 dual resistant seasonal influenza A (H1N1) viruses were detected. Oseltamivir-resistance in each of the dual resistant viruses was confirmed by elevated oseltamivir IC_{50} values and by the presence of the H275Y mutation in the NA gene. Adamantane-resistance was confirmed by the presence of one of the known markers in the M2 protein. The dual resistant viruses belonged to four genetic backgrounds based on phylogenetic analysis of full genome sequences.[3] In genotype 1, all segments were similar to circulating clade 2B viruses; genotype 2 viruses were reassortants with seven gene segments from clade 2B and a M gene characteristic of circulating clade 2C viruses; genotype 3 contained the 2C M and NS genes in a clade 2B background; and genotype 4 was entirely 2C with the exception of a clade 2B NA gene. Of the 28 dual resistant viruses, five were genotype 1 and were from the US ($n = 3$), Canada ($n = 1$), and Kenya ($n = 1$). Twenty-one viruses, from the



A NA gene



B M gene

Figure 1. Phylogenetic analysis of seasonal influenza A (H1N1) viruses by gene segment. Phylogenetic trees for the dual resistance seasonal influenza A (H1N1) viruses. Adamantane resistance is indicated by an open triangle preceding strain designation, oseltamivir resistance is indicated by a black circle preceding strain designation, and dually-resistant viruses are in boldface type and indicated by a black diamond following strain designation. (A) Phylogenetic analysis of seasonal influenza A(H1N1) neuraminidase (NA) gene. (B) Phylogenetic analysis of seasonal influenza A (H1N1) matrix (M) gene.

Figure 1. (Continued)

US (n = 1), China (n = 11), Hong Kong (n = 8), and Vietnam (n = 1), were genotype 2 reassortants. Only two single instances of genotypes 3 and 4 were detected, both of which were from China.

Of the US cases, A/Texas/38/2009 (H1N1) and A/Kentucky/08/2009 (H1N1) were identified in severely immunocompromised patients after adamantane treatment (Table 1). Both of these viruses contained the less commonly seen V27A adamantane-resistance conferring mutation. While the other genotype 1 virus, A/West Virginia/02/2009 (H1N1), was isolated from a patient who was not severely immunocompromised and received no antiviral agents, suggesting either spontaneous acquisition of the S31N in the M2 or transmission from a person treated with adamantane drugs (Table 1). The genotype 2 virus, A/Texas/57/2009 (H1N1), was from a patient with an influenza infection unassociated with adamantane treatment; however, reassortment demonstrated by the presence of the 2C M gene would explain adamantane resistance

Table 1. Description of patient infections with oseltamivir and adamantane resistant seasonal influenza A (H1N1) viruses in the United States, October 1, 2008–January 15, 2010

Patient	Strain designation	Underlying condition	Antiviral treatment, dates and drugs	Date first specimen collected	Testing results	Date second specimen collected	Testing results
1	A/Texas/38/2009	Leukemia	01/31/2009–02/04/2009: oseltamivir + amantadine	01/31/2009	R-oseltamivir S-adamantanes	2/8/09	R- oseltamivir R -adamantanes
2	A/West Virginia/02/2009	6 weeks pregnant	None	03/18/2009	R-oseltamivir R-adamantanes		
3	A/Kentucky/08/2009	Post bone marrow transplant	04/27/2009: oseltamivir 04/28/2009: zanamivir 05/05/2009–05/11/2009: zanamivir + amantadine	04/27/2009	NA	5/11/09	R- oseltamivir R-adamantanes
4	A/Texas/57/2009	Seizure disorder, mental retardation, quadriplegia (LTCF resident)	08/12/2009–08/17/2009: oseltamivir	08/12/2009	R-oseltamivir R-adamantanes		

None of the patients reported travel during the 7 days before onset of illness and none reported illness in a family member or close contact during the week before or after their illness; R, resistant; S, susceptible; NA, not available; LTCF, long term care facility.

(conferred by the S31N mutation). Of note, the genotype 2 and genotype 3 dual resistant viruses from Asia appear to be genetically similar to those previously reported dual resistant viruses from Hong Kong, SAR.[4,5] The genotype 4 virus was the only dual resistant virus with a nearly complete 2C genome. Oseltamivir-resistance for this virus appears to be the result of a reassortment as demonstrated by the presence of the oseltamivir-resistant clade 2B NA gene.

Discussion

Although the detection of dual resistant seasonal influenza A (H1N1) viruses is still rare, there has been an increased prevalence of dual resistance viruses during the last three seasons: 0.06% (1 of 1753 tested in 2007–2008), 1.5% (21 of 1426 in 2008–2009), and 28% (7 of 25 in 2009–2010) (χ^2 $P < 0.001$). While the continued circulation or co-circulation of seasonal A (H1N1) viruses is uncertain, the emergence of dual resistant influenza viruses in five countries does present a public health concern, especially since dual resistant viruses would limit the options for antiviral treatment to a single licensed antiviral drug: zanamivir. Moreover, the markers of resistance seen in seasonal A (H1N1) viruses also confer resistance in the more widely circulating 2009 pandemic A (H1N1) virus. And, since the acquisition of mutations in influenza A viruses typically occur through drug selection, spontaneous mutation, or genetic reassortment with another drug resistant influenza A viruses, the detection of influenza A (H1N1) viruses that are resistant to both adamantanes and oseltamivir warrants close monitoring, even if only detected at low frequency. New antiviral agents and strategies for antiviral therapy are likely to be necessary in the future.

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Standardization of the classic MUNANA-based neuraminidase activity assay for monitoring anti-viral resistance in influenza

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Keywords Antiviral drugs, neuraminidase assay, neuraminidase inhibitors, NI surveillance, NISN.

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Abstract

We describe a MUNANA-based fluorescent assay kit, the NA-Fluor™ Influenza Neuraminidase Assay kit, which provides standardization and reproducibility of IC_{50} methodology for this traditional and highly popular neuraminidase enzyme assay. The assay utilizes conditions such as MUNANA substrate concentration, assay buffer, and run time comparable to protocols that have been used historically to generate influenza neuraminidase inhibitor and antiviral resistance data. The assay was benchmarked against MUNANA-based protocols provided on the Neuraminidase Inhibitor Susceptibility Network (NISN) website, http://www.nisn.org/au_about_us.html. The NA-Fluor™ assay gave similar IC_{50} values for oseltamivir carboxylate and zanamivir when compared to NISN protocols for several tested oseltamivir sensitive and resistant viral strains. The large differential in IC_{50} values between resistant and sensitive viral strains using this assay allowed for detection of resistant mutant virus in mixed viral populations. The assay is easy-to-use, highly reproducible, and the fluorescence signal is stable for hours making this assay compatible with processing many viral isolates in a surveillance screen or for high throughput screening for drug lead identification. The NA-Fluor™ assay also provides user flexibility. The assay can be performed in either the traditional end-point format or using a real-time, kinetic read-out and is compatible with typical methods to reduce viral contamination, such as the addition of detergents or ethanol.

Introduction

Although neuraminidase inhibitors (NIs) remain an effective treatment for influenza virus strains currently considered world health threats, such as Influenza virus A which includes pandemic H1N1 and Influenza virus B, viral resistance to these drugs threatens their usefulness for future pandemics. Key antivirals oseltamivir carboxylate (Tamiflu®) and zanamivir (Relenza®) target the viral enzyme neuraminidase (NA), which is responsible for release of newly generated viral particles from an infected cell's surface, a process necessary for transmission of the virus to other cells within the organism and for transmission between organisms. Oseltamivir resistance in seasonal H1N1 increased from 12·3% during the 2007–2008 flu season to 98·5% in the 2008–2009 season,[1] heightening concern that drug resistance will likewise become prominent in pandemic viral strains and highlighting the need for antiviral drug resistance surveillance. The H275Y mutation in H1N1 neuraminidase is the most common mutation conferring resistance. However, due to the high mutation rates of viruses, new mutations can be expected that will also render viral neuraminidase less sensitive to antiviral drugs. PCR methods can be used to detect previously identified mutations; however, functional neuraminidase enzyme activity inhibition testing is necessary for detecting drug resistance that results from novel mutations. The two neuraminidase enzyme inhibition assays using either the fluorescent MUNANA or chemiluminescent NA-*Star*® substrate are robust tools for NI susceptibility testing. The MUNANA-based assay is broadly used by many groups, including many regional health organizations for NI susceptibility testing, yet no standardized protocol or dedicated kit has been in place for this assay, making comparison of data generated between different laboratories difficult. Borrowing from multiple Neuraminidase Inhibitor Susceptibility Network (NISN)-published MUNANA-based neuraminidase assay protocols,[2] we have developed a kit-based fluorescent neuraminidase assay that offers both standardization and off-the-shelf quality-controlled reagents for NI susceptibility testing and other neuraminidase assay applications.

Materials and methods

All experiments, unless otherwise noted, were conducted using the standard NA-Fluor™ Influenza Neuraminidase Assay Kit protocol. Viruses were titered according to the NA-Fluor™ assay protocol. For IC50 determination, NIs were prepared in 10-fold serial dilutions at 4× final concentration in assay buffer (33·3 mm MES, 4 mm CaCl2, pH 6·5), and 25 μl were added to wells of a black flat-bottom 96-well microplate (NUNC). Viral samples were diluted

(1:3–1:100 depending on virus strain and titer) in assay buffer, and 25 μl were added to the NI serial dilutions for pre-incubation for 30 minutes at 37°C. NA-Fluor™ Substrate was diluted to 2× final concentration in assay buffer, and 50 μl was added to each well for a final assay concentration of 100 μm MUNANA. The neuraminidase assay was conducted at 37°C for 60 minutes. Non-virus controls were included on each assay plate. The assay was terminated by addition of 100 μl of NA-Fluor™ Stop Solution, and plates were read on a SpectraMax® M5 or M2 (Molecular Devices, Sunnyvale, CA, US) plate reader using Ex 355 nm/Em 460 nm settings. For data analysis, no-virus control assay well RFUs were subtracted from viral data RFUs, and data was processed using either Microsoft Excel® or GraphPad® Prism® software. For real-time detection, the assay was performed as above, but without the stop solution addition, and assay reads were taken at 5 minute intervals. The slope of the kinetic read at each NI concentration was plotted for IC50 determination using real-time fluorescent read data.

Influenza-A/WS/33 (H1N1) (VR-1520™) and influenza B/Lee/40 (VR-1535™) were purchased from ATCC. Influenza A/Texas/36/91 (H1N1) wild-type and H274Y strains were kindly provided by Dr. Larisa Gubareva (Influenza Branch, CDC, USA). Oseltamivir carboxylate was kindly provided by Hoffman-La Roche Inc. (Basel, Switzerland), and zanamivir was kindly provided by GlaxoSmithKline (Research Triangle Park, NC, USA).

Results

Overview of neuraminidase inhibition assay workflow

The NA-Fluor™ Influenza Neuraminidase Assay reagents and protocols were optimized in comparison to published NISN protocols according to the criteria of assay performance, ease-of-use, consideration of historically used assay conditions, reagent storage stability, and environmental impact. Our optimized assay conditions consists of 100 μm MUNANA, 33·3 mm MES, 4 mm $CaCl_2$, and pH 6·5 in a 100 μl assay volume, and performing the assay for 60 minutes at 37°C following a 30 minutes preincubation of drug with the virus. These conditions are consistent with the majority of published influenza NI screening data in publication.[2]

The standard NA-Fluor™ assay workflow for screening viral isolates for sensitivity to NIs includes first titering the viral sample by neuraminidase activity to determine optimal virus concentration to be used in subsequent IC_{50} determination assays. The NA-Fluor™ assay is an ideal tool for titering virus based on neuraminidase activity in the viral coat. Titering of viral samples prior to running the IC_{50} determination assays insured that assays would be performed within the fluorescence detection dynamic range of both the assay and the fluorometric instrument being used. Viral titers giving RFUs in the range of 2000–5000 were used for subsequent assays.

Comparison to traditional MUNANA assays

A primary goal of developing a standardized MUNANA assay was to provide a standardized protocol and set of reagents that would allow for comparison of NI surveillance data between laboratories and over time. In addition, the assay should provide data comparable to historical data sets based on traditional MUNANA-based protocols. To insure that our newly developed NA-Fluor™ assay met these criteria we performed side-by-side comparisons of the NA-Fluor™ assay to MUNANA-based NISN protocols, as well as our NA-XTD™ and NA-*Star*® chemiluminescent neuraminidase assays to compare assay sensitivity and dynamic range and for NI IC50 determination with multiple viral isolates. For all assay comparisons, assays were performed according to respective published protocols. For direct comparison of results, an equivalent amount of virus (and concomitant neuraminidase activity) was used for each assay.

The NA-Fluor™ Assay provides low-end sensitivity (by signal to noise ratio) and dynamic range similar to NISN-published, MUNANA-based protocols (data not shown). These assays all show a low-end detection of approximately 0·002 U/well and dynamic range of 2–3 orders of magnitude when performed simultaneously side-by-side using serial dilutions of bacterial (*Clostridium perfringes*) neuraminidase. These assays show approximately onefold less dynamic range and approximately fivefold less low-end sensitivity than chemiluminescent assays under these conditions.

Given the large amount of archived NI inhibition data for viral isolates over the past decade, it is very important for a standardized assay to generate data similar to established protocols so that data can be compared in relative terms. When run side-by-side, NA-Fluor™ Assay provided oseltamivir carboxylate and zanamivir IC_{50} values similar to NISN-published, MUNANA-based protocols. IC_{50} values vary somewhat for MUNANA assays versus chemiluminescent assays depending on the viral isolate, as previously described.[3] The NA-Fluor™ assay also exhibited similar sensitivity for detecting NI sensitive virus compared to NISN-published fluorescent assays as shown in Figure 1.

The large shift in IC_{50} values between oseltamivir-sensitive and resistant virus using the NA-Fluor™ assay enables detection of mutant virus in mixed viral samples (Figure 2). This capability is critical for identifying resistant virus in clinical isolates presenting mixed populations of resistant and sensitive virus during NI susceptibility surveillance.

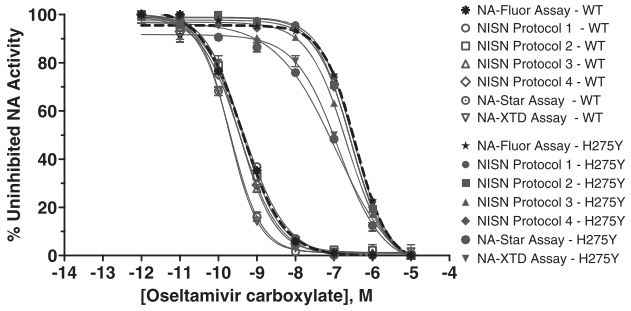

Figure 1. The NA-Fluor™ assay provides IC_{50} values and sensitivity for detecting sensitive virus similar to Neuraminidase Inhibitor Susceptibility Network-published, MUNANA-based protocols. All protocols were run in parallel to determine the IC_{50} values of influenza-A/Texas/36/91 (H1N1) sensitive and oseltamivir-resistant mutant (H275Y) strains for oseltamivir carboxylate. The NA-Fluor™ assay results are represented by the dotted line.

Compatibility for high throughput processing

Several characteristics of the NA-Fluor™ assay make it an ideal assay for processing large numbers of viral isolates for NI sensitivity surveillance or for using the assay for high throughput screening for lead discovery of new antiviral reagents. The NA-Fluor™ Assay signal was found to remain stable for up to 4 hours after stop solution addition when stored at room temperature and for several days when stored at 4°C (data not shown). IC_{50} values did not change over these times, indicating that the assay is compatible with processing many samples in a short time frame.

The NA-Fluor™ assay was also found to be highly reproducible giving a Z' of 0·78 or above indicating that the assay can be used confidently to identify NIs in high throughput screening mode.[4] The assay can tolerate up to 5% DMSO, a common compound delivery reagent used in high throughput screens (data not shown).

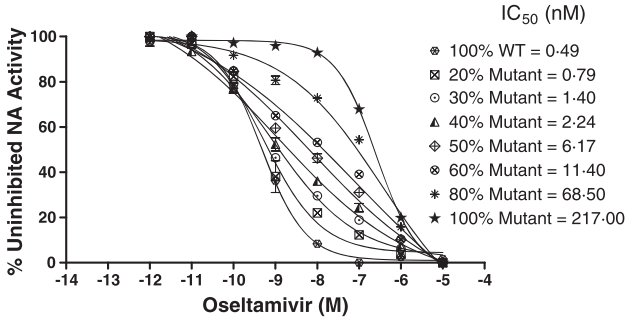

Figure 2. Detection of neuraminidase inhibitors-resistant H1N1 virus in mixed populations. Sensitive and oseltamivir-resistant (H275Y) influenza A/Texas/36/91 (H1N1) strain dilutions were normalized by NA activity. IC_{50} determination was performed using GraphPad® Prism® software. Subpopulations displaying drug-resistant mutations can be detected in mixtures of 50:50 sensitive and resistant virus by IC_{50} values alone.

Assay flexibility

We have developed a standardized NA-Fluor™ assay suggested protocol that gives data similar to established MUNANA protocols. However, we have also found that several protocol adaptations can be made that generate comparable data while allowing the user more flexibility in assay mode, use of additional reagents, and to meet user-specified assay time requirements.

The NA-Fluor™ assay can be run in either the standard 60 minutes/37°C endpoint mode described above or as real-time kinetic assay[5] with repeated reads taken over time without the addition of stop solution, which both serves to terminate neuraminidase activity and to enhance the fluorescence of the product. For typical NI-sensitive viral strains, the rate of MUNANA substrate turnover at 37°C is linear for at least 4 hours (data not shown). As would be expected, rates of substrate turnover decrease in the presence of NIs reflected in a decreased slope exhibited by real-time kinetic reads. Real-time acquired RFUs are typically 5–6 fold lower than RFUs acquired after addition of stop solution at the same time point. IC_{50} values obtained using slope analysis for real-time assays are similar to values obtained by end-point analysis. Whether run in real-time or end-point mode, the linear rate of substrate turnover allows the user to run the assay for shorter or longer assay times than the standard protocol without compromise to assay performance.

The NA-Fluor™ assay is also compatible with standard methods used in many laboratories to inactivate virus.[6] We have shown that NI IC_{50} values for multiple viral strains remain unchanged when the assay is performed in the presence of 0·1% NP-40 or 1% Triton X-100 (data not shown). Similar results are also obtained by adjusting the NA-Fluor™ Stop Solution to 40% ethanol prior to addition for assay termination.

The assay is unaffected by phenol red concentrations present in cell culture media.

Discussion

We have developed a standardized MUNANA-based fluorescent neuraminidase assay, the NA-Fluor™ Influenza Neuraminidase Assay kit, which has been optimized for NI susceptibility screening. The assay provides data that can be compared to data generated using traditional MUNANA-based protocols. The assay is economical, highly reproducible, easy to use, and environmentally friendly. The assay is flexible and amendable to user-specific adaptations including assay mode, assay timing, and reagent compatibility.

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Protection against H5N1 influenza by immunoglobulin single variable domains

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Keywords Antiviral drugs, H5N1, Nanobodies®, VHH

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Introduction

Zoonotic infections with highly pathogenic H5N1 influenza viruses have a high case fatality rate and remain very difficult to treat. Neuraminidase inhibitors such as oseltamivir and zanamivir are licensed for the treatment of uncomplicated influenza caused by seasonal influenza virus strains have also been used to treat H5N1-infected patients. Such treatment appears to improve patient survival, but the selection of oseltamivir-resistant H5N1 viruses is a major concern.[1,2] Novel antiviral drugs are desired to prevent or treat patients that have been infected with highly pathogenic H5N1 viruses and influenza in general. Here, we assessed the therapeutic potential of camelid-derived immunoglobulin single variable domains (VHHs or Nanobodies®,[3]) that are specific for the hemagglutinin (HA) of A/Vietnam//1194/2004 (H5N1) virus in a mouse model.

Materials and methods

Seven to 9 weeks old, SPF-housed BALB/c mice were treated intranasally with recombinant H5N1 HA-specific (H5-VHHb) or with control (RSV-VHHb) VHH derived Nanobodies® and challenged with mouse-adapted NIBRG-14ma virus. NIBRG-14 is a 2:6 reverse genetics derived reassortant between A/Vietnam/1194/2004 (H5N1) and A/PR/8/34 (H1N1) viruses. Nanobodies® were obtained as described (Hultberg *et al.*, in press) and used here as homobivalent recombinant proteins for *in vivo* experiments. H5-VHHb or RSV-VHHb were administered as a single dose ranging from 60 to 100 mg per mouse, depending on the experiment. Nanobodies® were administered up to 48 hours before or 72 hours after NIBRG-14ma challenge. In all experiments, mice were weighed daily after challenge and loss of 25% of body weight was used as the endpoint for euthanizing moribund mice. Lung virus titers were

determined by end-point dilution in MDCK cells. Nanobody®-escape viruses were selected by growth of NIBRG-14ma virus in the presence of monovalent H5-VHHm or bivalent H5-VHHb in MDCK cells and subsequently plaque-purified. The nucleotide sequence of independently isolated H5-VHH-escape viruses was determined and used to deduce the position in the H5-HA crystal structure that confers resistance.

Results

To test the prophylactic potency of H5-VHHb, mice were treated intranasally with PBS, 100 μg of H5-VHHb, or negative control RSV-VHHb at 4, 24, or 48 hours before infection with one LD_{50} of NIBRG-14ma virus. Body weight loss was monitored daily, and on day 4 mice were sacrificed to determine the viral load in the lungs. All mice that received H5-VHHb retained their original body weight, whereas those receiving PBS or RSV-VHHb gradually lost weight (data not shown). Intranasal administration of H5-VHHb at 4 or 24 hours before challenge resulted in undetectable lung virus titers. When animals were treated with H5-VHHb 48 hours before challenge, virus titers were 50-fold lower compared to PBS and RSV-VHHb treated mice, and three out of seven animals still had undetectable virus titers (Figure 1).

We next determined if H5-VHHb Nanobody® could be also be used therapeutically. We administered 60 μg of this Nanobody® intranasally to mice up to 72 hours after challenge with 1 LD_{50} of NIBRG-14ma virus. Four days after challenge, animals that received H5-VHHb 4, 24, or 48 hours after challenge had significantly higher body weight (data not shown) and lower lung virus loads than control mice. Although mice treated with H5-VHHb Nanobody® 72 hours after challenge were not clinically protected compared to control mice, they had significantly lower lung virus titers (Figure 1).

To identify the HA amino acid residues that are potentially involved in H5-VHH binding, escape viruses were selected by growth and plaque purification of NIBRG-14ma virus in the presence of H5-VHHm or H5-VHHb Nanobodies®. The HA sequences of six independently isolated H5-VHHm escape viruses revealed substitution of a lysine by a glutamic acid residue at position 189 in HA1 (H5 numbering). In addition, two H5-VHHm escape mutants carried an N154D and four carried an N154S substitution. The three-dimensional structure of NIBRG-14 HA shows that N154D/S and K189E are close to each other as part of the corresponding antigenic site B in H3 HA.[4,5] Interestingly, the N154D/S mutations remove an *N*-glycosylation site, which is surmised to have evolved in H5N1 HA as a strategy to mask an antigenic site.[6] Escape viruses selected in the presence of H5-VHHb carried K189N ($n = 4$) or K189E ($n = 2$) substitutions. These results indicate that residues in antigenic site B, at the top of HA and very close to the receptor binding domain (RBD), are essential for neutralization of the virus by H5-VHHm/b Nanobodies® (Figure 2).

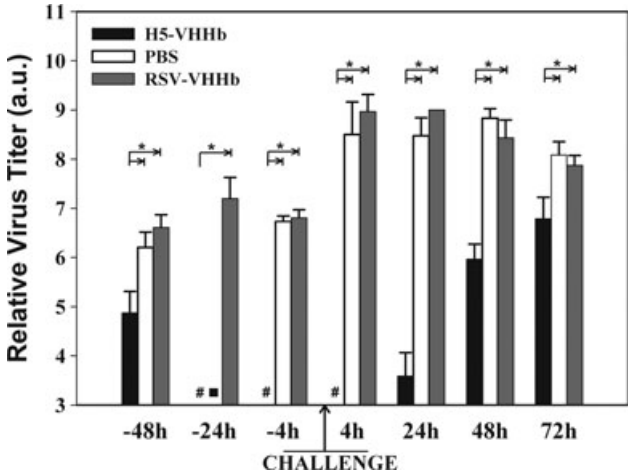

Figure 1. H5-VHHb inhibits H5N1 virus replication when administered 48 hours before and up to 72 hours after challenge with NIBRG-14 ma. The virus titer was measured in lung homogenates prepared on day 4 after challenge. The *X* axis refers to the time points in hours relative to the challenge (time = 0 hours) when HA-specific nanobodies (H5-VHHb), control nanobodies (RSV-VHHb) or PBS was administered to the mice. # below detection limit, ■ not determined [$n = 4$–7 mice per condition: P values < 0·05 (*)].

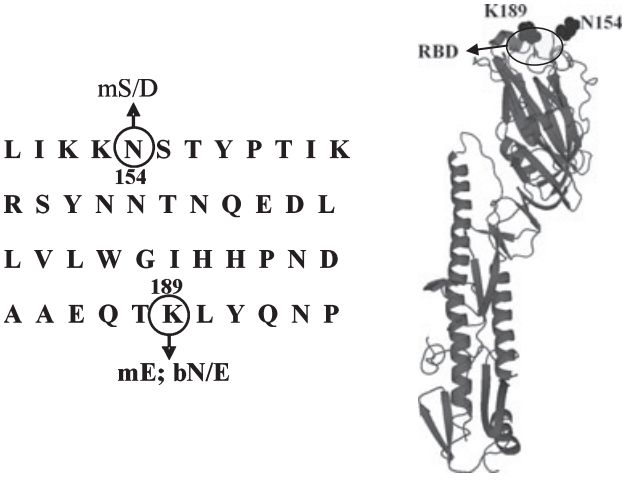

Figure 2. Amino acid sequence of *in vitro* selected H5-VHH escape mutant NIBRG-14ma viruses. Mutated sites are encircled and showed in ribbon diagram. Escape mutants that were selected in the presence of mono or bivalent H5-VHH Nanobodies® are denoted "m" and "b", respectively. RBD: receptor binding domain.

Discussion

Here we demonstrated that prophylactic and therapeutic treatment with llama-derived immunoglobulin single variable domain fragments is effective to control infection with H5N1 influenza virus in a mouse model. We demonstrate that pulmonary delivery is a highly effective route of administration to treat or prevent influenza virus infection. In addition, we demonstrate that a homobivalent H5-VHHb has powerful H5N1-neutralizing activity *in vivo*. It is important to note that we used a mouse-adapted derivative of the non-highly pathogenic NIBRG-14 virus in our challenge model. Nevertheless, this virus induces severe morbidity and lethality in mice. Compared to conventional neutralizing monoclonal antibodies, VHHs offer the advantage that they are easy to produce in *Escherichia coli*, typically with high yield. In addition, their small size (15 kDa for a monovalent VHH) and high folding capacity allow the generation of oligovalent VHH derivatives.

In vitro escape selection revealed that a K189E substitution in HA1 abolished the neutralizing effect of H5-VHHm/b. A Lys or Arg residue at this position is conserved in all human H5N1 virus isolates. Of note, all selected escape mutants contained a glutamic acid or serine residue at position 189, which suggests that the conserved positively charged amino acid is important for neutralization by H5-VHH Nanobodies®. Interestingly, escape mutants selected with H5-VHHm also carried an N154D/S co-mutation that removes an *N*-glycosylation site in this antigenic site of HA. The predicted *N*-glycosylation site at N154 in A/Hong Kong/156/97 HA was shown to be glycosylated and may have evolved to mask an antigenic site near the RBD.[7,8] The selected amino acid changes are located near the receptor binding site of HA. Therefore, it is possible that enhanced receptor binding properties of these escape viruses contribute to or are responsible for the loss of neutralizing activity of H5-VHH nanobodies®.[9,10] We conclude that influenza virus neutralizing Nanobodies® have considerable potential for the treatment of H5N1 virus infections. Although we focused on VHHs that presumably recognizes an epitope near the RBD, it is possible to select VHH molecules that bind to other epitopes in HA, including more conserved domains.

Disclosure

Data presented in this manuscript has been accepted for publication in Journal of Infectious Diseases, ''Nanobodies with/*in vitro*/Neutralizing Activity Protect Mice Against H5N1 Influenza Virus Infection,'' Lorena Itatí Ibañez, Marina De Filette, Anna Hultberg, Theo Verrips, Nigel Temperton, Robin A Weiss, Vandevelde Wesly, Bert Schepens, Peter Vanlandschoot, Xavier Saelens, 2011.

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Oseltamivir-resistant infection of pandemic (H1N1) 2009 influenza virus in South Korea

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Introduction

Antiviral drugs are essential for the preparedness and treatment against influenza. Most pandemic influenza A (H1N1) 2009 viruses were found to be amantadine-resistant, but oseltamivir-susceptible. However, oseltamivir-resistant pandemic viral infections have been reported worldwide (285 cases, 14 April 2010). Therefore, we conducted the antiviral monitoring, particularly focusing on the patients who remained uncured after reasonable antiviral treatment. A total of 1085 samples of patients were tested, and 11 (1·0%) cases were confirmed to be resistant to oseltamivir. These viruses were found in samples from the patients who were suffering from chronic diseases or immune system suppression. The viruses contained the variation H275Y in the neuraminidase (NA) gene. Further-

Table 1. Epidemiological information of patients infected with oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus in South Korea

Patient	Age/Sex	Antiviral treatment	Date of specimen collection	Type of clinical specimen	Underlying conditions
A	5/M	Oseltamivir 30 mg (7 days) Oseltamivir 60 mg (5 days)	6 November 2009	Nasopharyngeal swab	Mitochondria cytopathy (bedridden)
B	46/M	Oseltamivir 150 mg (10 days) Zanamivir (11 days)	18 November 2009	Nasopharyngeal swab	Leukemia
C	1/F	Oseltamivir 30 mg (6 days) Oseltamivir 60 mg (17 days)	21 November 2009 22 November 2009	Nasopharyngeal swab	Brain damage (Fatal)
D	2/M	Oseltamivir 90 mg (5 days) Oseltamivir 180 mg & Amantadine 65 mg (6 days)	26 November 2009 2 December 2009	Nasal suction	None
E	3/F	Oseltamivir 30 mg (5 days) Oseltamivir 30 mg (5 days)	01 December 2009 07 December 2009 12 December 2009	Oropharyngeal swab Nasopharyngeal swab	Asthma
F	3/F	Oseltarnivir 45 mg (6 days) Oseltarnivir 75 mg (5 days)	18 December 2009	Oropharyngeal Swab	Delayed development
C	1/F	Oseltamivir 30 mg (5 days) Peramivir 75 mg (7 days)	1 December 2009 5 December 2009 15 December 2009	Nasal oropharyngeal swab	Myelodisplasia (Fatal)
H	63/M	Oseltamivir 150 mg (13 days) Zanamivir (5 days)	16 December 2009	Oropharyngeal Swab	Diabetes
I	58/M	Oseltamivir 150 mg (3 days) Peramivir 600 mg & Amantadine 100 mg& ribavirion 300 mg (7 days) Zanamivir 10 mg (7 days)	26 December 2010	Nasopharyngeal swab	Cancer
J	60/M	Oseltamivir 75 mg (3 days)	December 2010	Viral RNA	Diabetes, cardiac disorders (Fatal)
K	54/F	Oseltamivir 75 mg (13 days)	14 January 2010	Nasopharyngeal swab	Tuberculosis

Table 2. Amino acid differences in the HA an NA of genes from 10 patients infected with oseltamivir-resistant

Cases		Amino acid sequences																
		NA		M2	HA (Hemagglutinin)													
No. (spencimen)		275	117	31	22	56	83	116	119	120	128	134	155	197	203	249	283	321
Kor/01*,**		H	I	N	K	N	S	S	I	T	S	A	G	A	S	V	K	V
A*	1	Y	I	N	K	N	S	S	I	T	S	A	G	T	T	V	K	V
B	1	Y	I	N	K	N	S	N	I	I	S	A	G	A	T	V	K	V
C*	1	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	V	K	V
	2	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	V	K	V
D	1	H	I	N	K	N	S	S	I	T	P	A	G	A	T	M	K	V
	2	Y	I	N	K	N	S	S	I	T	P	A	G	A	T	M	K	V
E	1	H	I	N	K	N	S	S	I	T	S	A	G	A	T	L	K	V
	2	H	I	N	K	N	S	S	I	T	S	A	G	A	T	L	K	V
	3	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	L	K	V
F	1	Y	I	N	K	N	S	S	I	T	S	T	G	A	T	V	K	V
G	1	H	M	N	R	N	S	S	I	T	P	A	G	A	T	V	K	V
	2	H	M	N	R	N	S	S	I	T	P	A	G	A	T	V	K	V
	3	Y	M	N	R	S	S	S	I	T	P	A	E	A	T	V	K	V
H	1	Y	I	N	K	N	S	S	M	T	S	A	G	A	T	V	K	V
I	1	Y	I	N	K	N	S	S	I	T	S	A	G	A	T	V	E	V
J	1	Y	I	N.A	N.A													
Cal/07***		H	I	N	K	N	P	s	I	T	s	A	G	T	S	V	K	I
Cal/04†		H	I	N	K	N	P	s	I	T	s	A	G	A	S	V	K	I

*Indicate that viral isolation and phenotypic assay were implemented.
**Denotes the first Korean isolate of (H1N1) pdm.
***and †indicate A/California/07/2009 and A/California/04/2009 respectively.
N.A, not available. NA sequence numbering uses N1 nomenclature.

more, a novel NA (I117M) substitution was discovered in a series of specimens from a patient. For the amantadine resistance, 1113 samples were tested, and all of them were confirmed to be resistant.

Methods and methods

We collected respiratory specimens from patients who had been clinically refractory to antiviral treatment since October 2009 upon ethical approval from the relevant institutions. To investigate the resistant pattern, sequence analysis to the NA and Matrix (M2) genes were conducted by reverse transcription (RT)-PCR and sequencing reaction. The obtained sequences were analyzed by the Influenza Sequences and Epitopes Database, which was developed in Korea.

Results

Eleven patients were found to be having oseltamivir-resistant pandemic (H1N1) 2009 viruses with the H275Y substitution in the viral NA genes (Tables 1 and 2). Some cases were associated with oseltamivir treatment on the basis of H275Y change from the oseltamivir-sensitive genotypes to oseltamivir-resistant genotypes in consecutive samples from the same patient. Furthermore, a novel NA (I117M) substitution that may be associated with oseltamivir resistance was detected in specimens from one patient (patient G) who had myelodysplasia and received oseltamivir and peramivir (Tables 1 and 2). In addition, we obtained viruses from clinical specimens (patients A and C) and evaluated antiviral susceptibility by measuring the dose of oseltamivir and zanamivir required for 50% inhibition (IC_{50}) of NA activity. These viruses (from patients A and C) were resistant only to oseltamivir (IC_{50} 713·2 and 359·4 nmol/l, respectively). Susceptibility to zanamivir was not altered whether NA contained Y275 or H275 (IC_{50} 0·13 and 0·78 nmol/l, respectively). One isolate of pandemic (H1N1) 2009 virus with an oseltamivir-sensitive genotype (H275 in its NA) was susceptible to oseltamivir (IC_{50} 1·18 nmol/l) and zanamivir (IC_{50} 0·42 nmol/l). Patients with oseltamivir-resistant pandemic (H1N1) 2009 were treated during hospitalization with oseltamivir alone or with a combination of other antiviral drugs (Table 2). Active surveillance that evaluated spread of oseltamivir-resistant viral infections among hospital staff, family members, and other patients who had contact with or cared for the patients showed no evidence of virus transmission in the hospitals.

Conclusion

We found 11 patients of oseltamivir resistance with H275Y mutation in the NA gene of pandemic (H1N1) 2009 virus through the surveillance of patient refractory to antiviral treatment. In addition, novel amino acid change (I to M) at position 117 in the NA gene, which might influence oseltamivir susceptibility, was detected in sequential specimens of a patient. These data showed that generation of oseltamivir resistance could be associated with oseltamivir treatment. Therefore, it needs to strengthen the antiviral monitoring by supplementation of the clinical data including antiviral treatment.

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A strategy to provide influenza antiviral resistance testing for patients with a clinical suspicion of resistance, United States 2009–2010

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Introduction

Oral oseltamivir or inhaled zanamivir, US FDA approved neuraminidase (NA) inhibitors (NAI), were recommended for treatment and chemoprophylaxis for 2009 pandemic influenza A (H1N1) (pH1N1) virus infections during 2009–2010.[1,2] With rare exceptions, pH1N1 viruses were susceptible to oseltamivir. Although rarely reported, oseltamivir-resistant pH1N1 viruses all had the H275Y mutation in the neuraminidase. All pH1N1 viruses were sensitive to zanamivir and resistant to adamantanes.[3,4]

During the pandemic, oral oseltamivir was the primary antiviral medication used for treatment of hospitalized patients with pH1N1 infection. Many physicians worried that clinical deterioration or failure to respond to treatment with oseltamivir was due to either oseltamivir resistance or oseltamivir failure. In the United States, two investigational intravenous (IV) NAIs were available during 2009–2010: peramivir through Emergency Use Authorization and zanamivir by Investigational New Drug application. Peramivir would be an option for patients with oseltamivir failure, but would not be appropriate for patients infected with H275Y oseltamivir resistant mutants. IV zanamivir was available in limited supply, but would be appropriate for severely ill patients infected with an oseltamivir-resistant pH1N1 virus. During the pandemic, clinicians had few options for antiviral resistance testing in the United States. To respond to this need, the US Centers for Disease Control and Prevention (CDC) offered antiviral resistance testing for patients suspected to have clinical failure due to oseltamivir resistance. We describe the methods that CDC used to prioritize patients for testing during the pandemic and to detect markers for oseltamivir resistance, as well as the results from this testing.

Materials and methods

To facilitate decisions on which patients to test, we developed testing algorithms that were shared with state laboratories, epidemiologists, and the Emergency Operation Center at CDC. We prioritized patients who might benefit the most from antiviral testing given the inherent delay in providing antiviral results, e.g. patients who might have prolonged pH1N1 shedding. Patients that were critically ill [intensive care unit (ICU) admission] or patients with severe immunocompromising conditions with clinical evidence for oseltamivir treatment failure (persistent detection of virus and clinical unresponsiveness to the drug) were prioritized. In addition, we tested specimens from patients that failed oseltamivir chemoprophylaxis. Standard forms with information regarding specimen and minimal clinical information were collected on all patients. All protocols were validated and approved by Clinical Laboratory Improvement Amendments, e.g. quality standards to ensure accuracy, reliability, and timeliness of patient test results. Information collected on patients was deemed public health response, not research, at CDC.

Clinical specimens, confirmed as 2009 pandemic influenza A (H1N1), were tested for the H275Y mutation in the NA using pyrosequencing.[5] Results were returned to sender within 24–48 hours of specimen receipt.

Results

From October 2009 until July 2010, a total of 116 specimens from 73 patients were submitted for testing. Viruses from 26 (36%) of 73 patients had H275Y mutation in the NA in at least one submitted specimen. Clinical information was available for 66 patients (Table 1). Most patients had received oseltamivir for treatment prior to obtaining the specimen sent for antiviral testing. Four patients received oseltamivir for chemoprophylaxis, all were immunosuppressed, and all had the H275Y mutant; duration of chemoprophylaxis until pH1N1 infection was detected varied (4–39 days). Among the patients with an H275Y mutant who were treated with oseltamivir, the median time on oseltamivir prior to collection of specimen with H275Y mutation was 18 days (range 3–36 days). Three patients

Table 1. Characteristics of patients with specimens submitted for testing for clinical use

	Total (n = 66) No. (%)	Patients with H275Y virus infection (n = 25) No. (%)
Age, median years (range)	43 (0–82)	45 (0–67)
Female	31 (47)	13 (52)
Severe Immunosuppression	32 (48)	23 (92)
ICU without immunosuppression	34 (52)	2 (8)
Oseltamivir use prior to testing	63 (95)	22 (88)
Chemoprophylaxis	4 (6·1)	4 (16)
Treatment	57 (86)	18 (72)
Unknown dose or use	2 (3)	0
No oseltamivir use*	3 (4·5)	3 (12)

*Three patients had exposure to another patient with resistant virus infection and were part of a hospital cluster.

were part of a hospital cluster of oseltamivir-resistant virus infections and were infected with H275Y mutants prior to oseltamivir treatment.[6]

Patients with immunocompromising conditions accounted for almost half of all patient specimens tested, but they accounted for the majority of oseltamivir-resistant pH1N1 virus infections (Table 1); among 32 individuals with severe immunocompromising conditions and clinical failure while on oseltamivir therapy, 23 (72%) had the H275Y mutant detected. Among the immunosuppressed patients with an oseltamivir-resistant virus, 16 (70%) had hematologic malignancies reported. In contrast, among the subset of ICU patients without immunocompromising conditions and clinical failure while on oseltamivir therapy, we found little resistance: 2 (5·9%) of 34 ICU patients had oseltamivir resistance detected.

Discussion

During the 2009 pandemic, we were able to provide timely and useful information to clinicians regarding suspected cases of oseltamivir resistance. Our testing algorithm limited the number of specimens to specimens from the highest risk patients that would benefit the most from antiviral treatment. Such an approach allowed us to offer this service without compromising our public health duties. In addition, the information we collected on patients from this service complimented our data on the national surveillance for antiviral resistance.

We also performed national antiviral resistance surveillance from April 2009 to July 2010. Overall, 67 resistant pH1N1 viruses were identified from April 2009 to July 2010 in the United States among 6812 tested samples, including specimens described above, surveillance specimens, and resistant viruses reported in the literature.[7]

Further studies to understand risk factors for oseltamivir-resistant pH1N1 infection in patients with severe immunocompromising conditions are needed. While efforts to provide antiviral testing technology and materials to state laboratories are ongoing, clinicians still have limited options for such testing. Rapid and inexpensive assays that could be performed by clinical laboratories, especially those caring for immunosuppressed patients, would be useful to inform patient care.

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The NA-XTD™ Influenza Neuraminidase Assay Kit: extended-glow chemiluminescence assay system for highly sensitive influenza neuraminidase assays

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Introduction

The Applied Biosystems® NA-XTD™ Influenza Neuraminidase Assay Kit provides the next-generation NA-XTD™ 1,2-dioxetane chemiluminescent neuraminidase (NA) substrate, together with all necessary assay reagents and microplates, to quantitate sensitivity of influenza virus isolates to neuraminidase inhibitors. Like the NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit, the NA-XTD™ Influenza Neuraminidase Assay provides highly sensitive detection of influenza neuraminidase activity. In addition, the NA-XTD™ assay provides extended-glow light emission that eliminates the need for reagent injection and enables signal measurement either immediately or up to several hours after assay completion. The NA-XTD™ assay is also used to quantitate influenza NA activity directly in cell-based virus cultures to monitor viral growth or inhibition.

Global monitoring of influenza strains for resistance to neuraminidase inhibitors (NIs) is essential for understanding their efficacy for seasonal, pandemic, or avian influenza, and studying the epidemiology of viral strains and resistance mutations. Functional neuraminidase inhibition assays enable detection of any resistance mutation, making them extremely important for global monitoring of virus sensitivity to NIs. The first-generation chemiluminescent NA-*Star*® Influenza Neuraminidase Inhibitor Resistance Detection Kit has been widely used for virus NI sensitivity assays,[1–8] including identification of A/H1N1 pandemic virus resistant to oseltamivir.[9,10] In addition, this assay has been used for identification of new NI compounds,[11] NI characterization,[12] studies of virus transmission,[13] drug delivery,[14] NA quantitation of virus-like particles,[15] and cell-based virus quantitation.[16]

Neuraminidase assays performed with chemiluminescent 1,2-dioxetane substrates, including NA-*Star*® and NA-XTD™ substrates, typically provide 5-to-50-fold higher sensitivity by signal-to-noise ratio than assays performed with the fluorescent MUNANA substrate. In addition, chemiluminescent assays provide linear results over 3–4 order of magnitude of neuraminidase concentration compared to 2–3 orders of magnitude with the fluorescent assay.[2] The high assay sensitivity achieved with chemiluminescent assays enables use of lower concentrations of viral stocks, and the wide assay range minimizes the need to pre-titer virus stocks prior to IC_{50} determination.

Materials and methods

The NA-XTD™, NA-*Star*® and NA-Fluor™ assay kits are supplied by Life Technologies. The NA-XTD kit includes (i) NA Sample Prep Buffer (Triton X-100-containing), (ii) NA-XTD Assay Buffer for dilution of virus, neuraminidase inhibitors (NIs), and substrate, (iii) NA-XTD Substrate, (iv) NA-XTD Accelerator, and (v) NA-*Star* Detection Microplates (white 96-well). Influenza strains used include type A/H1N1 (VR-1469™ (human), VR-1520™ (human), VR-1682™ (swine) from ATCC (Manassas, VA) and A/H1N1/Texas/36/91, wild-type and H275Y oseltamivir-resistant mutant strains, (provided by the CDC, Atlanta, GA, USA), and B/Lee/40 (VR-1535™, ATCC) viruses. NIs used include oseltamivir carboxylate (F. Hoffmann-La Roche Ltd, Basel, Switzerland) and zanamivir (Glaxo-SmithKline, Research Triangle Park, NC, USA). Assays were performed according to the NA-XTD assay protocol.

Sample prep

1. Add 10% volume of NA Sample Prep Buffer to virus stock.
2. Dilute virus as appropriate.

Assay protocol

25 μl diluted virus.

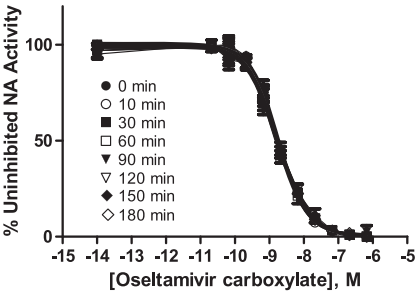
25 μl NI dilution (in NA-XTD Assay Buffer), pre-incubate × 30 minutes at 37°C.
25 μl 1:1000 NA-XTD Substrate, incubate × 30 minutes.
60 μl NA-XTD Accelerator, read 1 second/well.
Assays were performed with the SpectraMax M5 multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA). IC_{50} analysis was performed using GraphPad Prism Software dose-response curve-fitting.

Results

Chemiluminescent reactions result in conversion of chemical energy to light energy, as light emission. The NA-XTD™ substrate is a 1,2-dioxetane structure bearing a sialic acid cleavable group. To perform the NA-XTD assay, virus dilutions (from cell culture supernatant) are pre-incubated in the presence of neuraminidase inhibitor. Then NA-XTD Substrate is added and incubated for 30 minutes for substrate cleavage to proceed. Finally, light emission is triggered upon addition of NA-XTD Accelerator, which provides a pH shift and a proprietary polymeric enhancer, both required for efficient light emission. Chemiluminescent assays are performed in solid white microplates, and light emission is measured in a luminometer. The NA-XTD™ substrate has a single structural difference from the NA-*Star*® substrate that provides a much longer-lasting chemiluminescent signal, with a signal half-life of approximately 2 hours (not shown), compared to ~10 minutes with the NA-*Star* assay, eliminating the need for luminometer instruments equipped with reagent injectors and enabling more convenient batch-mode processing of assay plates. The NA-XTD™ Assay Kit also provides a new accelerator solution, containing a next-generation polymer enhancer, and a Triton® X-100-containing sample prep buffer providing enhanced NA activity.

Read-time flexibility is demonstrated by determination of oseltamivir IC_{50} values using data collected over 3 hours after addition of NA-XTD™ Accelerator. Although signal intensity slowly decreases over time, the IC_{50} curves and values are identical at each time point, shown using influenza B/Lee/40 (Figure 1).

Triton X-100 detergent at 1% has been shown to inactivate flu virus while increasing neuraminidase activity.[17] The addition of NA Sample Prep Buffer (containing 10% Triton X-100) to virus stocks (at 1/10 volume, achieving a final concentration of 1%) provides increase in NA activity up to fourfold, but is not consistently observed, and seems to be most effective with more concentrated virus stocks. IC_{50} values are unaffected by the addition of Triton X-100 to the virus stock prior to virus dilution (not shown), so the assay is compatible with known virus inactivation reagents. Assay sensitivity and IC_{50} values determined with the NA-XTD assay have been compared to those obtained



Read time	IC50 (nM)
0 min	1·84
10 min	1·86
30 min	1·77
60 min	1·59
90 min	1·88
120 min	1·84
150 min	1·83
180 min	1·71

Figure 1. Extended-glow light emission kinetics enables assay read-time flexibility, with identical IC_{50} values obtained with read times up to 3 hours after NA-XTD Accelerator addition.

with both the chemiluminescent NA-*Star* assay and the fluorescent NA-Fluor assay (not shown). The chemiluminescent assays provide 2- to 50-fold higher sensitivity by signal-to-noise ratio, depending on the virus strain, wider assay dynamic range, and better low-end detection limit than the fluorescent assay. The wide assay range with the chemiluminescent assays enables determination of IC_{50} values over a range of virus concentrations, eliminating the need to titer virus prior to performing IC_{50} determination assays. IC_{50} values obtained with the NA-XTD assay are nearly identical to those obtained with the NA-*Star* assay, with both oseltamivir and zanamivir neuraminidase inhibitors, and tend to be slightly lower than IC_{50} values obtained with the fluorescent assay.

Viral NA quantitation provides a convenient read-out to measure viral growth or inhibition, including inhibition in the presence of inhibitory compounds or antibodies, described as Accelerated Viral Inhibition with NA as read-out assay (AVINA).[16] MDCK cell cultures in a 96-well microplate were infected with different virus strains (B/Lee/40 (VR-1535™), A/WS/33 (H1N1) (VR-1520™), A/Swine/1976/31 (H1N1) (VR-1682™), A/Texas/36/91 (H1N1) wild-type and H275Y mutant), followed by incu-

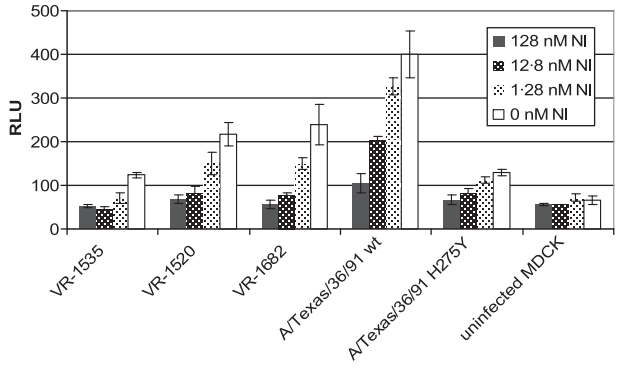


Figure 2. NA-XTD assay for cell-based virus growth inhibition application.

bation in the presence of varying concentrations of oseltamivir carboxylate. Samples of culture media were assayed 24 hours later. Quantitation of NA activity with the NA-XTD™ assay demonstrates inhibition of viral growth by oseltamivir carboxylate in cell culture (Figure 2).

Different volumes of culture media were assayed with the NA-XTD assay, either in the culture plate or in a separate assay plate (not shown). Performing the assay using the entire well contents (100 μl) reduces assay sensitivity due to the high concentration of phenol red. Assaying a smaller volume of culture medium (either in culture plate or a separate assay plate) provides higher sensitivity, and enables temporal monitoring or use of remaining culture medium for other assays.

Discussion

The Applied Biosystems® NA-XTD™ Influenza Neuraminidase Assay Kit is a next-generation chemiluminescent neuraminidase assay providing high assay sensitivity and ''glow'' light emission kinetics for improved ease-of-use. The Applied Biosystems® NA-Fluor™ Influenza Neuraminidase Assay Kit, based on the fluorescent MUNANA substrate, has also been developed to complement the NA-XTD™ and NA-*Star*® chemiluminescence assays, for users lacking luminometer instrumentation or choosing to use fluorescence assay detection. Together these kits offer:

- Standardized reagents and protocols
- Choice of detection technology
- Simple instrumentation requirements
- High sensitivity for use with low virus concentrations
- Compatibility with batch-mode processing and large-scale assay throughput
- Broad specificity of influenza detection
- Flexibility in assay format
- Additional NA assay applications – cell-based viral assays, screening for new NIs, detection of NA from other organisms

Functional neuraminidase inhibition assays enable detection of any resistance mutation and are extremely important in conjunction with sequence-based screening assays for global monitoring of virus isolates for NI resistance mutations, including known and new mutations. Together, these assays provide highly sensitive, convenient and versatile assay systems with standardized assay reagents, and simple assay protocols for influenza researchers.

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Diagnosis of bacterial complications of seasonal and pandemic influenza in hospitalized adults; can serum procalcitonin (ProCT) help?

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Abstract

Background: Influenza pandemics have been associated with high morbidity and mortality in part due to bacterial complications. Accurate bacterial infection rates complicating 2009 H1N1 infection are limited, primarily because of difficulty collecting reliable respiratory samples. Procalcitonin (ProCT), a serum biomarker, may aid in distinguishing viral and bacterial infections. Materials and methods: Adults with respiratory illnesses admitted to the hospital between November and June (2008–2010) were recruited. Patients underwent testing for influenza and bacterial infection. Serum ProCT was measured on admission and day 2 of hospitalization. Results: Fifty-one subjects were diagnosed with influenza infection, 20 seasonal and 31 H1N1 2009 (pandemic). Demographics and illness characteristics of seasonal and pandemic infected patients were similar. Bacterial infections were diagnosed in 8 (16%) subjects; none were bacteremic. Mean serum ProCT (ng/ml) levels in seasonal versus pandemic patients on admission and day 2 were not significantly different. Reliable sputum samples were collected in 22 (43%) subjects. Of these, ProCT was ≥0·5 ng/ml in two with influenza alone and three associated with bacterial infection, and <0·5 ng/ml in 12 with influenza alone and five associated with bacterial infection. The sensitivity of a ProCT ≥0·5 ng/ml for bacterial infection was 38% and specificity 86%. Mean ProCT values were significantly higher in patients with infiltrates versus those with atelectasis or no acute disease on chest radiographs (CXR) (2·4 ± 4·8 ng/ml versus 0·36 ± 1·2 ng/ml, $P = 0{\cdot}02$). Combining patients with ProCT values ≥0·5 ug/ml and those with a positive bacterial test, seasonal and pandemic influenza bacterial infection rates were 20% and 26%, respectively. Conclusions: Bacterial infections occur in approximately ¼ of adults hospitalized with influenza with no significant difference in seasonal and pandemic influenza infection rates. Given the difficulties with bacterial diagnosis, elevated ProCT values may be helpful to identify patients at high risk for invasive disease.

Introduction

Over 200 000 hospitalizations and 36 000 deaths in the US annually are attributable to seasonal influenza, primarily in chronically ill persons and the elderly.[1–3] Following the emergence of pandemic 2009 H1N1 influenza, severe illnesses have also been observed in children and young healthy adults.[4] The occurrences of staphylococcal and pneumococcal pneumonia complicating influenza pandemics are well described.[5–7] Although temporal associations of bacterial pneumonia and influenza circulation have been reported, there is little precise data on rates of bacterial complications of seasonal or pandemic influenza. The study of bacterial lung infection has been hampered by insensitive tests for invasive disease and the difficulty of interpreting routinely obtained sputum culture results.[8,9]

Procalcitonin (ProCT), the prohormone of calcitonin, can discriminate viral and bacterial infections.[10] This 116-aminoacid precursor protein normally produced by neuroendocrine cells of the lungs and thyroid gland was first shown to be elevated in bacterial infections in patients with pulmonary injury and pneumonitis.[11] Stimuli of ProCT include TNF-α, endotoxin, and other bacterial products.[12] Several studies indicate that bacterial infections commonly induce hyperprocalcitonemia, but that viral infections, including 2009 H1N1, are associated with only minimal increases.[10,12,13] Of note, ProCT induction is attenuated by viral-induced interferon-γ.[14] A meta-analysis of studies comparing ProCT and CRP as markers for bacterial infection found that ProCT was more sensitive and specific than CRP for differentiating bacterial from other causes of inflammation.[15,16] Therefore, we measured ProCT levels in patients with seasonal and pandemic influenza and compared results with conventional methods for bacterial diagnosis.

Materials and methods

Patient population

Adults ≥21 years of age admitted to Rochester General Hospital (RGH) from November 1st to June 30th for two winter seasons (2008–2010) with an admitting diagnosis compatible with acute respiratory tract infection were recruited for the study. Patients were screened within 24 hours of admission, and those with prior antibiotic use, immunosupression, or pregnancy were excluded. Subjects or their legal guardian provided written informed consent. The study was approved by the University of Rochester and RGH Research Subjects Review Board.

Illness evaluation

At enrollment demographic, clinical and laboratory information was collected. Influenza testing included nose-throat swabs (NTS) for rapid antigen, viral culture, and reverse transcription-polymerase chain reaction (RT-PCR) and serology. Testing for bacterial pathogens included blood cultures, sputum for culture and gram stain, NTS for *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* PCR, *S. pneumoniae* antigen testing, and pneumococcal serology. If patients were unable to expectorate, sputum was induced with normal saline and bronchodilators. Specimens were considered adequate by the standard criteria of >25 neutrophils (PMNs) and <10 epithelial cells per high power field. Serum was collected at admission and hospital day 2 for ProCT measurements.

Laboratory methods

Influenza diagnostics

Influenza infection was defined a positive result for any of the following tests:

1. Rapid Antigen- Influenza rapid antigen tests were performed using QuickVue® Flu A & B (Quidel, San Diego, CA, USA).
2. Viral Culture- R Mix™ (Diagnostic HYBRIDS, Athens, OH, USA) was used for influenza culture.
3. RT-PCR: NTS and sputum was processed for influenza viruses using established methods.[17] The primers and probes used for influenza strains are as follows: Influenza A(Matrix gene) Forward primer 5′GACCRATCCTGTCACCTCTGAC3′, Reverse Primer 5′AGGGCATTY TGGACAAAKCGTCTA3′, Probe[FAM]TGCAGTCCTC GCTCACTGGGCACG[BHQ1], Influenza B (NS1 gene) Forward primer 5′TCCTCAAYTCACTCTTCGAGCG3′, Reverse Primer 5′CGGTGCTCTTGACCAAATTGG3′, Probe [FAM]CCAATTCGAGCAGCTGAAACTGCGGTG [BHQ1]. 2009 H1N1 proprietary primers and probe sequences were supplied by Dr Stephen Lindstrom of the Center for Disease Control and Prevention, Atlanta, GA.

Serology: Whole viral culture lysates were used as antigen. The circulating strains of A and B influenza virus were obtained from the clinical laboratory. Influenza A was typed by the New York State Department of Health laboratories. The titer of IgG to each virus in acute and convalescent serum was determined using enzyme immunoassay (EIA), and a positive result was defined as a greater than or equal to fourfold rise in titer.[17]

Bacterial assays

Standard microbiological testing

Blood cultures, sputum gram stain and culture were performed by the clinical microbiology laboratory at RGH. Only adequate sputum samples were analyzed.

S. pneumoniae Psp A serology

Psp A antigens were obtained form the Bacterial Respiratory Pathogen Reference Laboratory at the University of Alabama [Available at http://www.vaccine.uab.edu/ (Accessed 14 October 2010)]. Cloned proteins were coated on EIA plates at 2 ug/ml in bicarbonate buffer. After overnight incubation, plates were washed and two-fold dilutions of serum were incubated overnight at room temperature. Plates were washed and incubated with alkaline phosphatase conjugate for 3 hours, followed by substrate. A greater than or equal to fourfold rise in titer was considered evidence of infection with *S. pneumoniae*.

Urinary antigen for S. pneumoniae

Samples were assayed for antigen using the Binax NOW kit. (Binax Inc, Scarborough, ME, USA).

Procalcitonin (ProCT)

The ProCT was measured using time resolved amplified cryptate emission technology (Kryptor PCT; Brahms, Henningsdorf, Germany). Functional sensitivity is 0·06 ng/ml (normal levels are 0·033 ± 0·003 ng/ml).[18]

Mycoplasma and chlamydia PCR

Real-time PCR targeting the P1 adhesion gene for *M. pneumoniae* and the *ompA* gene for *C. pneumoniae* was used to detect atypical bacteria.[19]

Results

Fifty-one of 529 (9·6%) illnesses evaluated tested positive for influenza virus. Of these, 20 were due to ''seasonal influenza'' (16 influenza A/H3N2 and 4 influenza B), and

Table 1. Procalcitonin values on the first and second Hospital Day

	Day 1		Day 2	
	Seasonal ***N* = 20**	**Pandemic** ***N* = 31**	**Seasonal** ***N* = 20**	**Pandemic** ***N* = 31**
Mean ± SD	0·14 ± 0·17	1·05 ± 2·86	0·17 ± 0·17	1·34 ± 4·37
Range	0·02–0·84	0·05–13·7	0·02–0·67	0·05–20·1
Median	0·08	0·09	0·11	0·12

31 were identified as ''pandemic influenza'' (2009 H1N1). Demographics of both groups were similar: mean ages 55 ± 18 and 50 ± 11 years, respectively, and equivalent sex and racial characteristics. Other than a higher incidence of underlying lung disease in the seasonal group (75% versus 45%, $P = 0{\cdot}05$), pre-existing medical conditions including obesity were similar. Symptoms, physical findings, and discharge diagnoses did not differ, and chest radiographs (CXR) showed infiltrates in 20% and 13% of seasonal and pandemic subjects, respectively. Two pandemic and one seasonal influenza patient developed respiratory failure, and none died.

Overall, bacterial infections were diagnosed in 8 (16%) subjects (4- seasonal and 4-pandemic), and none were bacteremic. Bacterial infections included: 5- *S. pneumoniae*, 1- *M. pneumoniae*, 1-*S. aureus,* and 1- *H. influenzae.* All seasonal patients were diagnosed with asthma or bronchitis, whereas three pandemic patients had pneumonia.

Mean serum ProCT (ng/ml) levels in seasonal versus pandemic patients on admission and day 2 were: 0·14 ± 0·17 versus 1·05 ± 2·86 and 0·17 ± 0·17 versus 1·39 ± 4·37, respectively, and were not significantly different (Table 1).

Several patients in the pandemic group had high ProCT levels, and there was a trend toward more pandemic patients having admission ProCT values ≥0·5 ng/ml than seasonal subjects [1 (5%) versus 6 (19%), $P = 0{\cdot}22$] (Figure 1A, B). Of the four patients with ProCT > 1·5 ng/ml, two had dense infiltrates on CXR, one had a peripheral WBC of 17 200/ml with a threefold increase in *S. pneumoniae* antibody, and one developed respiratory failure associated with COPD exacerbation.

Reliable sputum samples (within 6 hours of antibiotics) were collected in only 22 (43%) subjects. Of these, ProCT was ≥0·5 ng/ml in two with influenza alone and three associated with bacterial infection, and <0·5 ng/ml in 12 with influenza alone and five associated with bacterial infection. In the 22 with reliable sputa and accepting the conventional bacterial diagnosis, sensitivity of a ProCT ≥ 0·5 ng/ml for bacterial infection was 38%, specificity

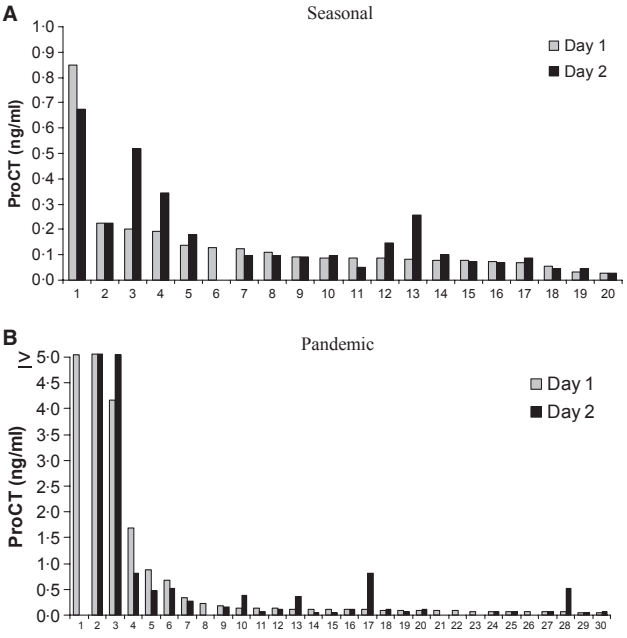

Figure 1. Procalcitonin values for individual study participants on Admission (gray bar) and Day 2 (black bar). Patients with seasonal influenza are shown in (A) and pandemic patients in (B).

86%, positive predictive value 60%, and negative predictive value 71%. Notably, one patient considered to have influenza alone (ProCT – 0·87 ng/ml) had group A streptococcus and *S. aureus* in a contaminated sputum and bilateral infiltrates on CXR. Three of five patients with bacterial infections and ProCT < 0·25 ng/ml had a clinical diagnosis of bronchitis. Mean ProCT values were significantly higher in patients with infiltrates versus those with atelectasis or no acute disease on CXR (2·4 ± 4·8 ng/ml versus 0·36 ± 1·2 ng/ml, $P = 0{\cdot}02$).

Combining patients with ProCT values ≥0·5 ng/ml with those having positive bacterial tests, rates of bacterial infection associated with seasonal and pandemic influenza were 20% and 26%, respectively. Notably, antibiotics were administered to 88% of subjects despite 73% having no acute disease on CXR.

Discussion

In our study, bacterial infections were diagnosed in approximately 25% of adults hospitalized with influenza with no significant difference in rates noted between seasonal and pandemic influenza infected subjects. Previous reports of bacterial infection rates of 4–15% with seasonal influenza are difficult to compare with recent studies of pandemic influenza, because the latter tended to focus on more severely ill patients.[20–22] Bacterial pneumonia has been suspected or diagnosed in 13–24% of patients in intensive care associated with 2009 H1N1 infection and up to 38% of patients who died.[23,24] Despite aggressive pursuit of specimens for bacterial testing, diagnoses could be confirmed in only 8 (15·6%) of patients using conventional methodology.

Given the difficulty in establishing a diagnosis of bacterial infection, elevated ProCT values may be helpful to identify patients at high risk for invasive disease. In a study of 25 patients with severe 2009 H1N1 or bacterial infection necessitating intensive care, a threshold ProCT level of 0·8 ng/ml, demonstrated 100% sensitivity and 62% specificity for bacterial infection.[25] Among 38 patients with 2009 H1N1 associated pneumonia, many of whom had respiratory failure, a threshold ProCT value of 0·5 ng/ml provided a sensitivity of 100% and specificity of 53% for bacterial infection.[26] Access to samples from lower airways in ventilated patients in these studies may have improved recovery of bacteria and account for the different results we observed. It should be noted that none of our patients were bacteremic, which is a very strong stimulus for ProCT release. ProCT levels have been used successfully to guide therapy in community acquired pneumonia, and our data showing high ProCT levels in patients with infiltrates on CXR suggests ProCT may be most useful for excluding invasive disease.[27,28]

Elevated ProCT levels were not observed in patients with purulent sputum and clear CXR. It is notable that a ProCT level of <0·25 ng/ml did not exclude patients with bacterial bronchitis since ProCT has been used to guide antibiotic therapy in COPD exacerbations.[29] While it could be argued that healthy patients with bacterial bronchitis do not require antibiotic treatment, physician behavior in our study indicates antibiotics are frequently prescribed.

Combining patients with ProCT values ≥0·5 ng/ml and those with a positive bacterial test, approximately 25% in patients in our study had bacterial complications associated with influenza infection. Efforts should be made to curtail antibiotic use in hemodynamically stable patients with clear CXRs. Given physician discomfort regarding discontinuing antibiotics, ProCT measurements in combination with routine bacterial cultures should be useful tools to guide therapy.

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Influenza, MRSA, cytokines: diagnosis, treatment, prevention – a possible strategy for outpatient care

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Keywords Cytokines, double infection, humoral inflammation status, influenza, MRSA, outpatient, prevention, rapid test.

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Background

We started the antiviral treatment of influenza in humans using neuraminidase inhibitors on January 29, 1999 in a successful attempt to cure a 96-year-old patient. Since then, we have used the inhalant antiviral drug zanamivir, and later (October 1, 2002) changed to the use of oseltamivir with systemic bioavailability for treating patients with influenza. After 10 years of experience with antiviral treatment of outpatients, we highlight the importance of early diagnosis and early treatment.[1] The necessity of an earliest possible diagnosis was confirmed in the pandemic of 2009. Large hospitals reported that patients with an H1N1/09 infection had to be treated with extracorporeal membrane oxygenation. We are convinced this is due to delayed recognition of infection in most cases. Valuable time is lost when the patient with a sudden onset has to be brought to a hospital for emergency treatment. The point at which the patient goes to the doctor is decisive, and this problem of timing and the delivery of early treatment is not specific to Germany.

Material and methods

In our medical office, we assessed patients with suspected influenza (to date 262 seasonal infections, and in 2009, 25 H1N1/09) through clinical diagnosis,[2] and then proven by point of care rapid test (QuickVue; Quidel, San Diego, CA, USA) followed by PCR. All of the patients undergo concomitant lab tests: leukopenia, serum iron level, and the humoral inflammation status [sum of the C-reactive protein (CRP) and fibrinogen levels]. Because of the constant threat of a bacterial superinfection, a bacterial swab and antibiogram is carried out on every patient. In all cases positive for influenza, oseltamivir was given immediately. Nowadays it is important that a double infection with influenza and MRSA must be recognized immediately and treatment started at once with antivirals and, when

appropriate, with a suitable antibiotic. We pay particular attention to an extremely low iron level (Signum mali ominis). In addition we monitor oxygen saturation and the course of the humoral inflammation status every 1–2 hours for every of our outpatients.

Results

Among our patients with seasonal influenza, we saw 132 within 12 hours, 103 within 36 hours, and 27 within 48 hours after disease onset. For pandemic influenza, it was 12 patients within 12 hours, 11 within 24 hours, and two within 36 hours. For all patients, we measured CRP < 3·5 mg and fibrinogen < 350 mg/dl (12 hours), CRP < 10 mg and fibrinogen < 450 mg/dl (36 hours), and CRP > 10 mg and fibrinogen < 500 mg/dl (48 hours, only seasonal cases). Antibiotics were necessary in 130 cases, heparin and oxygen administration in 27 cases. One hundred forty-eight patients had a superinfection following influenza. The most common strains were *Haemophilus parainfluenzae* and *Staphylococcus aureus*. The subsequent use of a suitable antibiotic was only necessary in 50% of the patients. In all cases diagnosed, treatment (including heparin and oxygen administration) and monitoring were conducted in our medical office. None of our patients (seasonal and pandemic) had to be admitted to hospital.

Discussion

The early decision of whether or not antiviral and antibacterial treatment is taking effect is the only way the threat of a cytokine storm can be averted. Not only does the primary care physician have to be aware of the pathophysiology involved, but also the necessary diagnostic and therapeutic options have to be made available to him. The result will lead to a saving of both lives and healthcare costs. This applies both in epidemic as well as in pandemic times. Today we know that influenza leaves behind a defenceless immune system, and that the proteases of *S. aureus* contribute to influenza associated pneumonia. Mark von Itzstein, who discovered neuraminidase inhibitors, emphasized the synergistic cooperation of viruses and bacteria (personal communication, 2009). MRSA and influenza viruses are posing problems worldwide.

The case of a 17-year-old boy with H1N1/09 infection demonstrates how fatal developments can be prevented. Due to his constantly recurring colds, we had already detected the MRSA colonization years earlier and had always worked on boosting his general health and resistance. Both the patient and his family were included in dealing with the problem. The patient was, and is, always vaccinated early with a virosomal vaccine (Baxter). During the Oktoberfest in Munich in September 2009, when H1N1 infections were increasingly occurring, we learned that our patient had come down with an extremely acute feverish illness. With the help of the rapid test, we diagnosed an H1N1/09 virus infection and started treatment with oseltamivir immediately. The humoral inflammation status, which had increased very rapidly to more than 4·5 mg/dl within hours, was treated with the effective cotrimoxazol from the antibiogram. At the same time, the patient was heparinized. The following day the patient had no fever and was symptom-free. It was only through our early knowledge of what could develop pathophysiologically that we were in a position to make the right decision at the right time.

Every doctor treating outpatients can follow this procedure if he is familiar with the pathophysiology of the disease and has the available tests on hand: virus rapid test, additional laboratory parameters (leukopenia, iron), and the humoral inflammation status. The decisive factor, however, is the constant clinical alertness towards the course of every acute feverish cold with acute onset. The patient has to remain in the care of the attending physician, and the chosen treatment has to be administered and monitored. This means constant SpO2 measurements and checking the humoral inflammation status every 2 hours. If a clinical worsening occurs during monitoring, the treatment regime has to be changed immediately, which means the administration of an appropriate antibiotic. This outpatient care on the part of the doctor has to be available 7 days a week so that no time will be lost. Reports from The Netherlands and Denmark show that, with the help of this preventive strategy under the motto 'search and destroy,' the dangerous, fatal course of infections reported in Germany with at least four deaths a day, can be avoided. However, the doctor has to be adequately remunerated for the elaborate amount of time this intensive outpatient care requires. With our strategy, we have moved from divergence to convergence in the care of our patients. We reported on our 10 years of clinical experience with this approach at the Antivirals Congress in Peking. Our main message was early diagnosis and early treatment. We were able to demonstrate this in 287 outpatients with seasonal influenza and 25 H1N1/09 outpatients. Our creed is: as much outpatient care as possible and as little hospitalization as possible.

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Virological and autopsy findings in suspected and confirmed fatal cases of 2009 H1N1 pandemic influenza in the Czech Republic – preliminary results

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Keywords Autopsy, influenza, mortality, pandemic.

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Introduction

Influenza viruses cause substantial morbidity and mortality. Pandemic influenza may have a serious impact on certain (mainly younger) age groups in comparison with seasonal flu. Influenza is one of few viral infections capable of causing a pneumonia that is difficult to cure and/or leads to sudden death. The aim of this study was to analyze and compare virological and autopsy findings in patients who died with suspected or confirmed 2009 H1N1 pandemic influenza virus infection.

Material and methods

There were 2477 virologically confirmed cases of pandemic influenza and 102 deaths in the Czech Republic during pandemic wave. More than 400 influenza strains belonging to the new pandemic variant were isolated in the National Influenza Reference Laboratory. Postmortem biological samples were collected from any patient who died with suspected influenza infection to test for respiratory viruses. The samples were screened for 2009 H1N1 pandemic influenza virus by real-time PCR (RT PCR), and when RT PCR positive, by virus isolation assay. No immunohistochemical staining for influenza antigen was done on the RNA PCR positive cases. Other important respiratory viruses such as respiratory syncytial virus, parainfluenza viruses, and adenoviruses were detected by virus isolation assay in a suitable cell culture. Epidemiological analysis of postmortem histopathologic findings in the airway tissue was carried out in 57 of 61 fatal cases. Virological findings were subsequently correlated with histological changes and available demographic and clinical data. Statistical analysis was performed by *t*-test using SPSS software.

Results

Sixty-one deaths (34 males, 27 females) were analyzed. The RNA of the 2009 H1N1 pandemic influenza virus was detected by PCR in 38 cases, while 23 cases remained negative. Five respiratory syncytial viruses and two adenoviruses were detected in the influenza negative group. The mean age of 38 confirmed 2009 H1N1 pandemic influenza victims was 46·0 years, age range 7–74 years and median 47·5 years. The mean age of 23 influenza negative victims was 55·1 years, age range 1–89 years and median 57·0 years. The 95% CI for the difference in the age between the two groups is −1·7; 19·9. The test is statistically significant at the 10% level. The obtained significance ($P = 0{\cdot}07$) can be explained by the relatively small size of the study group.

The most common postmortem histopathologic finding in the lung tissue of the 2009 H1N1 pandemic influenza virus-positive victims was diffuse alveolar damage (often bilateral) and/or hyaline membrane formation, possibly with signs of respiratory distress syndrome (in 18, i.e., 51·4%, of 35 autopsied patients). In the 2009 H1N1 pandemic influenza virus negatives, the most common finding was pneumonia or bronchopneumonia with the detection of various bacterial species (in 12, i.e., 54·5% of 22 autopsied patients). The cause might be either primary bacterial infection or superinfection following primary infection with influenza virus that remained undetected.

Discussion

The 2009 H1N1 pandemic influenza victims were younger than the patients who died with suspected but undetected

2009 H1N1 pandemic influenza. The majority of deaths were primarily linked to rapidly developing respiratory failure. This result supports the previous reports of severe respiratory outcomes in younger age groups that are typically linked to the spread of a pandemic strain of influenza.[1] Due to limited amount of pandemic vaccine, especially at the beginning of pandemic, it is advisable to assess experiences with antiviral treatment, mainly dosing, and way of antiviral administration.

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Rapid detection of reassortment of pandemic H1N1/2009 influenza virus

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Keywords Influenza, molecular diagnosis, pandemic H1N1, quantitative RT-PCR, reassortment.

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Abstract

Background: Influenza viruses with novel genotypes can arise from gene reassortment events during co-infections. Pandemic H1N1/2009 virus has circulated in humans globally for a period of time and was repeatedly detected in pigs. Concerns are raised about the possibility of reassortment between H1N1/2009 virus and other influenza viruses. New reassortants might have altered virulence and/or transmissibility and result in unpredictable consequences. Thus, there is urgent need for rapid diagnostic tools that can identify reassortants in full genome influenza surveillance. Materials and methods: We employed real-time RT-PCR-based strategy for the detection of reassortment of pandemic H1N1/2009 virus. Eight singleplex SYBR green-based RT-PCR assays specific for each gene segment of pandemic H1N1/2009 were designed and evaluated with influenza viruses of different genetic backgrounds. Results: Of human influenza virus isolates tested with theses assays, all pandemic H1N1 ($n = 67$) and all seasonal ($n = 104$) isolates were differentiated effectively for all eight segments. For the 59 swine isolates from our on-going surveillance program of influenza viruses in pigs, 10 were positive in all eight viral segments reactions. They were confirmed to be of pandemic H1N1 origin by sequencing, showing that these were caused by zoonotic transmissions of H1N1/2009 virus from humans to pigs. The other 49 non-pandemic H1N1/2009 swine viruses had one to seven gene segments positive in the tests. Further characterization of these viruses indicated that these PCR positive genes are the precursor genes of pandemic H1N1/2009 virus in the contemporary triple reassortant (TR) or Eurasian avian-like swine influenza virus lineages. The melting curve analysis of hemagglutinin (HA) assay can differentiate the pandemic (H1N1) 2009 virus from other contemporary swine viruses with the same HA lineage. Most interestingly, using these established assays, a reassortant between pandemic H1N1/2009 and swine H1 viruses were identified in our on-going surveillance study. Conclusions: The result highlighted that our assays are able to detect pandemic H1N1/2009 virus in humans and its re-introduction in pigs. These assays might be useful screening tools for identifying viral reassortants derived from pandemic H1N1/2009 or its precursor viruses, thereby enhancing our pandemic influenza surveillance plans.

Introduction

Influenza A virus is a member of the *Orthomyxoviridae* family. Its genome consists of eight negative sense RNA segments, namely polymerase basic protein 2 (PB2), PB1, polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M), and nonstructural protein (NS). Influenza viruses can exchange gene segments with each other in co-infected cells and give rise to progeny with novel gene combinations in the event of reassortment, which is responsible for the antigenic shifts of influenza virus. In 2009, it was a surprise that a pandemic was caused by a novel influenza virus of swine origin of the H1 subtype and not from the expected avian source. This pandemic H1N1/2009 virus is a reassortant of avian, swine, and human viruses.[1,2] We previously developed several molecular tests for rapid diagnosis of this virus.[3] As there are concerns about the possibility of reassortment events between pandemic H1N1 and other influenza viruses, it is important to detect evidence of reassortment by rapidly assessing the genetic origin of each of the eight gene segments of the virus. Pandemic H1N1 has co-circulated with seasonal H1 and H3 viruses in global human population for a period of time. Far more worrying was repeated reports of zoonotic transmissions of the virus from humans to pigs.[4–6] This provides the virus many other options for reassortment, thereby increasing chances to generate reassortant with altered pathogenicity and/or transmissibility.[7,8] Therefore, there is an urgent need for a

rapid diagnostic test that can identify novel reassortants of pandemic H1N1/2009.

Materials and methods

Primers specific for each of the eight genes of pandemic H1N1/2009 were adopted from assays as described previously to discriminate against seasonal human H1N1 and H3N2 viral segments (Table 1).[9] The primers were allowed to cross-react specifically with the sister clade viral segments of pandemic H1N1/2009.[2] The method we employed in this study was a 2-step singleplex SYBR Green-based real-time RT-PCR. This approach helped lower the running cost of the assays and facilitated downstream molecular analyses (e.g., sequencing) by using screened cDNA samples. Viral RNA was extracted from viral cultures or clinical samples as described [3,10] and was converted to cDNA in a universal RT-PCR. Each 10 μl RT reaction containing 5·5 μl of purified RNA, 2 μl of 5× first-strand buffer (Invitrogen), 100 U of Superscript II reverse transcriptase (Invitrogen), 0·1 μg of Uni12 (5′-AGCAAAAGCAGG-3′),[11] 0·5 mm of deoxynucleoside triphosphates and 10 mm of dithiothreitol was incubated at 42°C for 50 minutes, followed by 72°C for 15 minutes for heat inactivation. For each segment-specific real-time PCR, the 20 μl reaction contained 1 μl of a 10-fold diluted cDNA samples, 10 μl of Fast SYBR Green Master Mix (Applied Biosystems), and 0·5 μm of the corresponding primer pair. The thermocycling conditions of all eight segment-specific PCRs were optimized as 95°C for 20 seconds, followed by 30 cycles of 95°C for 3 seconds and 62°C for 30 seconds, and all eight assays were performed simultaneously in a 7500 Sequence Detection System (Applied Biosystems). At the end of the amplification step, PCR products went through a melting curve analysis to determine the specificity of the assay (60–95°C; temperature increment: 0·1°C/seconds). cDNA of A/California/04/2009 virus was used as a positive control.

Results

Robust and specific amplification was achieved in all eight segment-specific real-time RT-PCR reactions.[9] PCR product for each segment of pandemic H1N1/2009 yielded unique melting curve pattern with distinctive melting temperature (Tm), which was not observed in negative and water controls (Figure 1). Reactions with Tm value within 2 SDs of the mean Tm were determined as positive. We evaluated the assays with a number of serologically confirmed human clinical samples. All pandemic H1N1/2009 samples (n = 67) were positive in all eight assays, while all seasonal samples (H1N1 = 38; H3N2 = 66) were negative in all assays, as expected (Figure 1 and data not shown). These results showed that no reassortant of pandemic

Table 1. Primers which target pandemic H1N1/2009 viral gene segments [9]

Segment	Primer*	Sequence
PB2	PB2-1877F	5′ AACTTCTCCCCTTTGCTGCT 3′
	PB2-2062R	5′ GATCTTCAGTCAATGCACCTG 3′
PB1	PB1-825F	5′ ACAGTCTGGGCTCCCAGTA 3′
	PB1-1138R	5′ TTTCTGCTGGTATTTGTGTTCGAA 3′
PA	PA-821F	5′ GCCCCCTCAGATTGCCTG 3′
	PA-1239R	5′ GCTTGCTAGAGATCTGGGC 3′
HA	HA-398F	5′ GAGCTCAGTGTCATCATTTGAA 3′
	HA-570R	5′ TGCTGAGCTTTGGGTATGAA 3′
NP	NP-593F	5′ TGAAAGGAGTTGGAACAATAGCAA 3′
	NP-942R	5′ GACCAGTGAGTACCCTTCCC 3′
NA	NA-163F	5′ CATGCAATCAAAGCGTCATT 3′
	NA-268R	5′ ACGGAAACCACTGACTGTCC 3′
M	M-504F	5′ GGTCTCACAGACAGATGGCT 3′
	M-818R	5′ GATCCCAATGATATTTGCTGCAATG 3′
NS	NS-252F	5′ ACACTTAGAATGACAATTGCATCTGT 3′
	NS-691R	5′ ACTTTTCATTTCTGCTCTGGAGGT 3′

*Number represents nucleotide position of the first base in the target sequence (cRNA sense).
HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; PA, polymerase acidic protein; NS, nonstructural protein; PB2, polymerase basic protein 2.

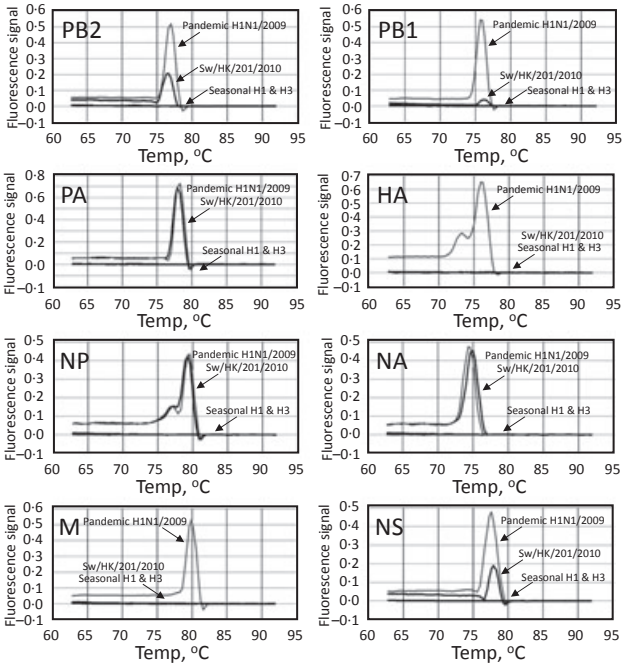

Figure 1. Melting curve analyses of PCR products derived from human seasonal (H1 and H3), pandemic H1N1/2009 and pandemic H1N1/2009 reassortant (Sw/HK/201/2010) viruses.

H1N1 and seasonal viruses was present in the tested human isolates.

We applied these assays to our on-going influenza virus surveillance program in swine. Nasal and tracheal swab samples were collected at an abattoir in Hong Kong and cultured in Madin Darby canine kidney cells or embryonated eggs as described.[12] Positive viral cultures in hemagglutination assays were tested with the established segment-specific real-time RT-PCR assays. Among 59 swine viral isolates collected from 2009 to September 2010, 10 of them were recognized as pandemic H1N1/2009 in all eight segments. They were confirmed to be of pandemic H1N1/2009 origin by subsequent full genome sequencing analyses, showing that there were interspecies transmissions of the virus from humans to pigs.[13,14]

The remaining 49 viruses had one to seven gene segments positive in the segment-specific real-time RT-PCRs. Thirty of them were selected as representative samples for full genome sequencing analyses based on the genotyping data generated in our assays. They were swine H1N1 or H1N2 viruses with their gene segments derived from TR or Eurasian avian-like swine lineages. It should be highlighted that all of their positive gene segments in our assays belonged to the sister groups of pandemic H1N1/2009. Their melting curve patterns were very similar to those derived from segments of pandemic H1N1/2009, except for HA of TR lineage.[9] Our results successfully demonstrated the use of these segment-specific real-time RT-PCRs to recognize gene segments of contemporary TR (PB2, PB1, PA, HA, NP, and NS) and EA (NA and M) swine viruses.[2] The HA-specific assay was able to discriminate pandemic H1N1/2009 from other contemporary swine viruses in the same lineage. Nevertheless, to confirm the identity and to examine all the genetic variations in the viruses of interest, full genome sequencing analyses were necessary.

Discussion

In this study, the biggest obstacles in primer design were sequence similarity and diversity of influenza viruses. We attempted to use degenerated primers, but they were highly non-specific. The finalized non-degenerated primers cross-reacted with genes from pandemic H1N1/2009 and its sister clade TR (PB2, PB1, PA, HA, NP, and NS) and EA (NA and M) swine viruses with some minor sequence mismatches. Three avian (H5N1, H7N7, and H9N2) and 1 classical swine (H1N1) were also tested with our assays. All of these animal viruses were negative, except for NS gene of the classical swine virus.

Our segment-specific real-time RT-PCR assays might be used in high throughput genotyping. They detected pandemic H1N1/2009 viruses and acted as a preliminary screening tool to select virus reassortants of interesting genotypes for further sequencing analyses. In fact, we identified a novel reassortant in January 2010 during the course of this study. This Sw/HK/201/2010 has a previously unidentified viral gene combination as shown in Figure 1. It was confirmed to be a reassortant between pandemic H1N1/2009 and other swine viruses in full genome sequencing characterization. It has a pandemic H1N1-like N1 gene, an EA-like H1, and the other six internal genes derived from TR swine viruses.[13,14]

The eight established real-time RT-PCRs can rapidly reveal the gene-origins of influenza viruses. We are currently using these assays in influenza surveillance in humans and other animals. It is believed that similar strategy might be applied to detect and genotype other influenza viruses and possible reassortants in the future.

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Virus-free ELISA method for the determination of influenza A/H1N1/2009 antibodies in human serum

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Keywords Antibody titers, ELISA, hemagglutination, hemagglutinin receptor binding domain, influenza A/H1N1.

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Abstract

Assays for influenza antibody titers (hemagglutination inhibition and neutralization) require the use of the actual virus, which seriously limits broad implementation. We present and validate a virus-free ELISA method for estimating serum titers against influenza A/H1N1/2009 that uses the recombinant receptor-binding domain of the hemagglutinin A/H1N1/2009 as antigen. Virus ELISA titers were at least 1·5 times higher in sera from convalescent influenza A/H1N1/2009 patients than in non-exposed subjects. Hemagglutination inhibition (HI) assays and ELISAs were performed on 17 samples from convalescent patients. From this set of expected sero-positive subjects, only 58·82% resulted positive by HI, and 100% were diagnosed as positive by ELISA. The specificity of the method was verified by negative results in ferret sera following challenges with different influenza virus strains.

Introduction

Pandemic influenza A/H1N1/2009 infects millions of people around the world. A significant fraction of the world's population may also already have been exposed to the virus and, although asymptomatic, may be at least partially immune to the disease. A precise assessment of the number of people exposed to the influenza A/H1N1/2009 virus is epidemiologically relevant. However, assays typically used to estimate antibody titers against a particular influenza strain, namely HI and neutralization, require use of the actual virus. This seriously limits broad implementation, particularly in regions where high biosafety facilities are unavailable.

Materials and methods

ELISA assay

We developed an ELISA method for the evaluation of presence of specific 2009 H1N1 influenza virus-antibodies in serum samples. Mouse anti-histidine tagged antibodies (100 μl; 5 μg/ml; AbD Serotec®, UK) in PBS (pH 7·2) were dispensed into standard 96-well plates and incubated for 12–16 hour at room temperature. Excess antibody was removed by at least two successive alternate washings with PBS-Tween 0·05% and PBS. Commercial blocking solution (300 μl, Superblock® T20; Pierce®, USA) was added and incubated for at least 2 hour at room temperature. After successive washing steps with PBS-Tween 0·05%, non-glycosylated histidine-tagged recombinant protein (100 μl; 10 μg/ml) was added to each well. This protein consisted of the receptor-binding domain of the hemagglutinin of the Influenza A/H1N1 virus.[1,2] After 1 hour incubation, wells were washed for at least two alternating 5 minutes cycles with PBS-Tween and PBS. A 1:50 dilution of the serum or plasma sample to be assayed (100 μl) was added to each well and incubated at room temperature for 1 hour. After repeated alternating 5 minutes PBS-Tween 0·05% and PBS washes, anti-human IgG antibody solution (100 μl/well; 1:30 000 dilution in PBS-Tween 0·05%) marked with horse radish peroxidase (Pierce®, USA) was added and incubated for 1 hour at room temperature. After repeated alternate washes with PBS-Tween 0·05% and PBS), substrate solution (100 μl; 1-Step Ultra TMB-ELISA; Pierce®) was added to each well. After incubation for 15 minutes at room temperature in darkness, the enzymatic reaction was stopped by addition of 1 m H_2SO_4 (50 μl/well). Yellow color produced by the enzymatic reaction was evaluated by absorbance at 450 nm in a Biotek® microplate reader (USA).

Blank assays using albumin in place of human sera established the ELISA background signal, which was subtracted from sample absorbance signals:

$$Abs_{serum\ sample} = Abs_{serum\ sample\ before\ correction} - Abs_{albumin\ sample}.$$

Absorbance values were normalized based on the average signal of 103 non-exposed subjects (uninfected subjects), and expressed as normalized absorbance (Abs_{norm}):

$$Abs_{norm} = (Abs_{serum\ ample} - Abs_{albumin\ sample})/(Abs_{non\ exposed\ subjects} - Abs_{albumin\ sample}) \quad (1)$$

where $Abs_{serum\ ample}$ is the sample absorbance signal, $Abs_{albumin\ sample}$ is the albumin control absorbance signal, $Abs_{non\ exposed\ subjects}$ is the average absorbance signal of non-exposed subject samples.

For ferret serum samples, the same basic protocol was followed, with minor modifications. An anti-IgG anti-ferret polyclonal antibody preparation was used at a dilution of 1:30 000 in PBS-Tween 0·05%.[2]

Recombinant hemagglutinin

A recombinant receptor-binding domain of the HA of the Influenza A/H1N1/2009 virus, expressed in *Escherichia coli* strains,[2] was used as the ELISA antigen. This 27 kDa protein, designated here as $HA_{63–286}$-RBD, contained amino acids 63–286 of the influenza A/Mexico/InDRE4114/2009(H1N1) hemagglutinin. A sequence coding for a series of six histidines at the N-terminus of the protein was included in the genetic construct to allow purification using Immobilized Metal Affinity Chromatography (IMAC) and attachment to assay surfaces treated with anti-histidine antibodies (or alternatively Co^{+2} or Ni^{+2}).

Human serum samples

This study was conducted according to Declaration of Helsinki principles and was approved by the Institutional Review Board of the Escuela de Biotecnología y Salud del Tecnológico de Monterrey. All volunteer sample donors provided written informed consent.

Samples from 100 children (aged 6–13 years) collected during the year 2008 (prior to onset of the pandemics) were diluted 1:50 in PBS and analyzed by ELISA. Serum samples from 34 convalescent influenza A/H1N1/2009 patients were also collected, diluted 1:50 in PBS, and analyzed. Positive volunteers were recruited from regular patients from the Hospital San José del Tecnológico de Monterrey and Clínica Nova, admitted between April and October 2009. All diagnoses were confirmed using the specific RT-PCR protocol developed by the Center for Prevention and Disease Control (CDC) in Atlanta, GA, USA, and recommended by the World Health Organization (WHO)[3] as the reference method for the detection of the human Influenza A/H1N1/2009 virus. An HI assay, performed by the Department of Infectious Disease, St. Jude Children's Research Hospital, Memphis, TN, USA, was also carried out on 17 positive serum samples using an Influenza A/H1N1/CA/04/2009 virus strain and turkey erythrocytes and dilutions ranging from 1:40 to 1:320. Samples were considered positive when hemagglutination occurred at dilutions equal or higher than 1:40.

Ferret serum samples

A panel of four samples (kindly provided by St. Jude from ferrets exposed to different influenza strains, namely H3N2, H1N1 swine, and H5N1, was also tested by the ELISA method using 1:50 dilutions.

Results

Protein $HA_{63–286}$-RBD specifically and selectively recognizes antibodies from serum samples from convalescent H1N1/2009 influenza subjects. DuBois *et al.*[4] demonstrated that this protein, produced in *E. coli*, folds properly into a 3-D structure practically indistinguishable from the analogous region in the HA of the influenza A/H1N1/2009 virus. $HA_{63–286}$-RBD preserves three of the conformational immunogenic epitopes (Sa, Sb, and Cb) described for influenza A/H1N1 hemagglutinins.[5] The recombinant protein was used as the antigen, attached through histidine tags to microplate surfaces treated with anti-histidine antibodies to discriminate between serum samples from subjects exposed and non-exposed to influenza A/H1N1/2009.

Samples collected before the pandemic onset, and therefore presumed to exhibit low specific antibody titers against influenza A/H1N1/2009, were analyzed by ELISA using the antigen $HA_{63–286}$-RBD. The histogram of normalized absorbance values from this sample set displayed a normal behavior with a standard deviation of 0·57 units. Only 12·5, 9·7, and 3·88% of these samples exhibited normalized absorbance values higher than 1·5, 1·75, and 2·0, respectively. No sample from non-exposed individuals presented an absorbance value higher than 2·5. Variability among samples from non-exposed subjects was much lower than in samples with high specific serum antibody titers from convalescent H1N1/2009 patients. Exposure to the H1N1/2009 influenza virus with this ELISA method can be predicted by absorbance values normalized to those of

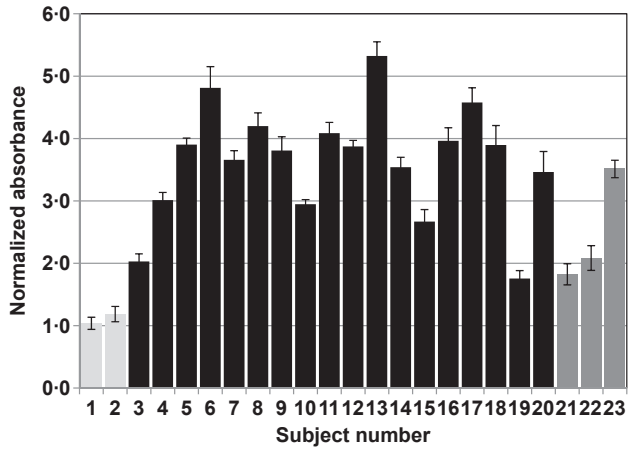

Figure 1. Normalized absorbance values of twenty samples run in the same ELISA microplate. Absorbance values corresponding to negative controls, corresponding to serum samples from non-exposed subjects, did not surpass the threshold value of 1·7 units (light gray bars). Samples from Influenza A/H1N1/2009 convalescent patients, as determined by RT-PCR, exhibited values above 1·7 units (black bars). Positive controls are indicated in dark gray. Error bars indicate the maximum and the minimum value from two replicates.

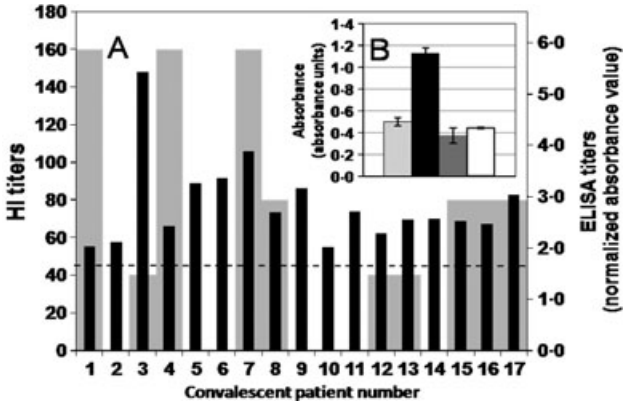

Figure 2. Sensibility and specificity of the HA-RBD ELISA method: (A) Influenza A/H1N1/2009 antibody titers of 17 serum samples from convalescent patients as determined by Hemagglutination inhibition assays (grey bars), and by ELISA using protein HA- (black bars). (B) Serum samples from ferrets exposed to different influenza strains were assayed by the ELISA method presented here (H5N1 in white; H1N1 swine in dark gray; H1N1/2009 in black; H3N2 in light gray). The sample corresponding to Influenza A/H1N1/2009 exhibited twice the absorbance signal than the rest of the samples.

serum from uninfected subjects. Consequently, for reliable results, inclusion of samples from non-exposed subjects on every assay microplate is necessary.

Figure 1 shows the analysis of 20 human serum samples, including 17 samples from convalescent patients with positive diagnosis by RT-PCR. Three positive (dark gray bars) and two negative controls (light gray bars) were included in the same microplate. All serum samples corresponding to convalescent subjects exhibited absorbance values 1·75–4·5 times higher than negative samples (Figure 1). Normalized absorbance values above 1·5 suggested exposure to the virus, although, a more conservative threshold value of 1·75 units is proposed for discrimination between exposed and non-exposed subjects. The ELISA method described here yields adequate reproducibility and a high signal/noise ratio within determinations in the same microplate and among different microplates. Using a normalized absorbance value of 1·75, the method was able to discriminate samples from convalescent patients, preferably after the third week of infection, and at least up to the twenty-fourth week of exposure.

Assay sensibility was further validated against results from HI assays. A previously reported study showed that all members in a pool of fourteen samples diagnosed as positive by HI exhibited normalized absorbance values higher than 1·5, and 85% of them exhibited normalized absorbance values higher than 2·0.[1] In general, high HI titers (>320) were correlated with normalized absorbance values higher than 4·0. Figure 2A shows results using the HA-RBD ELISA method and the HI assay on a pool of seventeen known positive serum samples corresponding to convalescent H1N1/2009 patients. All samples determined as positive by HI (10 samples) were also positive by ELISA. While sensitivity of the HI assay was 10/17 = 58·88%, the ELISA method recognized all samples correctly as positive (100% sensitivity) when a threshold of 1·5 or 1·75 was used. Figure 2B shows that sera from ferrets infected with other influenza strains (H3N2, H1N1 swine, and H5N1) showed no cross-reactivity when analyzed by ELISA.

Discussion

In summary, the HA-RBD ELISA method presented here consistently distinguished influenza A/H1N1/2009 infected and non-infected individuals, particularly after the third week of infection/exposure. Since no actual viral particles are required, this assay can be readily implemented in any basic laboratory. In addition, should sufficient vaccine be unavailable, this ELISA could determine the level of specific antibodies against the virus and presumably the extent of partial protection in a subject. Therefore, the ELISA protocol might allow better administration of vaccination programs during pandemic or seasonal influenza outbreaks.

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A monoclonal antibody-based ELISA for differential diagnosis of 2009 pandemic H1N1

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Abstract

The swine-origin 2009 pandemic H1N1 virus (pdmH1N1) is genetically related to North American swine H1 influenza viruses and unrelated to human seasonal H1 viruses. Currently, specific diagnosis of pdmH1N1 relies on RT-PCR. In order to develop an assay that does not rely in amplification of the viral genome, a conventional sandwich ELISA for detection of the pdmH1N1 was developed. The sandwich ELISA was based on three monoclonal antibodies (3B2, 5H7, and 12F3) against pdmH1N1. 5H7 and 12F3 were selected as capture antibodies and biotin-conjugated 3B2 was subsequently selected as the detection antibody in the ELISA. The results showed the ELISA had high specificity for pdmH1N1 strains and no reaction with other swine H1 viruses, human seasonal H1N1 or H3N2 viruses, or avian influenza viruses. The limit of detection of the ELISA ranged from $3{\cdot}2 \times 10^3$ to $1{\cdot}5 \times 10^4$ $TCID_{50}$/ml. When the ELISA was used to detect viruses in nasal wash samples from infected ferrets, it showed 90·1% sensitivity and 100% specificity compared to the ''gold standard'' – virus isolation. Our studies highlight a convenient assay for specific diagnosis of the 2009 pandemic H1N1-like viruses.

Introduction

In April 2009, a novel H1N1 influenza virus emerged in North America and caused the first influenza pandemic of the 21st century.[1–4] The 2009 pandemic H1N1 (pdmH1N1) has a unique gene constellation that was not previously identified in any species or elsewhere. It is genetically related to the triple reassortant swine H1N1 influenza viruses currently circulating in North America, with the exception of the neuraminidase (NA) and matrix (M) genes, which are derived from a Eurasian swine influenza virus.

Swine H1N1 influenza viruses were first isolated in 1930 and continued to circulate in North America with very little antigenic changes (classical swine H1N1) until 1998. Since 1999, however, the antigenic make up of swine H1 viruses has shown increased diversity due to multiple reassortment events and the introduction of H1N1 genes from human influenza viruses. Currently, four swine H1 clusters (α, β, γ, δ) are found endemic in the North American swine population.[5,6] These swine H1 viruses show substantial antigenic drift compared to the classical swine H1 viruses. Cluster δ swine H1 is derived from current human H1 viruses, and there is a substantial antigenic divergence between classical swine H1 and human seasonal H1 viruses. Epidemiological evidence shows a two-way transmission of influenza viruses between swine and humans, and such events lead to the emergence of the pdmH1N1 virus.[5,7,8] Phylogenetic analysis have suggested that possible ancestors of the eight genes of pdmH1N1 were circulating in the swine population for at least 10 years prior to the emergence of the pdmH1N1 virus in humans, although the

pdmH1N1 virus itself was not isolated from pigs until after the pandemic. Interestingly, pdmH1N1 infections have been reported not only in humans and pigs, but also in other animal species such as turkeys, cats, ferrets, cheetahs, and dogs.[9–11] After the first report of pdmH1N1 infection in swine in Canada, other countries, including Argentina, Australia, Singapore, Northern Ireland, Finland, Iceland, England, United States, Japan, and China reported outbreaks of pdmH1N1 in swine as well.[9,12–14] The ample geographic range of pdmH1N1 outbreaks in swine, its apparent broad host range, and the possibility of two-way transmission between swine and humans poses a tremendous challenge for controlling the virus. Therefore, to differentiate pdmH1N1 from other H1 strains, particularly in swine and human populations, is an important issue to ascertain the magnitude of the disease caused by the pdmH1N1. In this study, we developed an ELISA assay to discriminate pdmH1N1 strains from other swine and human H1 viruses.

Materials and methods

Viruses, cells, and antibodies

Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA, USA) were maintained in modified Eagle's medium (MEM) containing 5% FBS. A/California/04/09/H1N1 virus (Ca/04) was kindly provided by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Other viruses are listed in Table 1. Viruses were propagated in MDCK cells and stored at −70°C until use. Viruses were titrated by the Reed and Muench method to determine the median tissue culture infectious dose ($TCID_{50}$).[15] Three monoclonal antibodies (3B2, 5H7, and 12F3) against HA of 2009 pandemic H1N1 were prepared in our laboratory following previously described methods (Shao and Perez *et al.*, unpublished).

Purification and labeling of mAbs

mAb 3B2, 5H7 and 12F3 were purified on a Protein G–Sepharose affinity column (Upstate Biotechnology, Lake Placid, NY, USA). Biotinylation of the detection antibody in the ELISA was performed using Sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-(biotinamido)hexanoate; Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Sandwich ELISA

Purified 5H7 and 12F3 were selected as the capture antibody, and biotin-conjugated 3B2 was selected as the detection antibody, and HRP-conjugated streptavidin (Abcom, Cambridge, MA, USA) was developed using the TMB substrate system (KPL, Gaithersburg, MD, USA). In brief, the mixture of the purified 5H7 and 12F3 (2·0 and 2·2 μg/ml respectively, in carbonate/bicarbonate buffer, pH 9·6) was coated to 96-well plates (test well, T) for 12 h at 4°C. At the same time, a control antibody was coated to 96-well plates (control well, C). After blocking the plates with 5% (w/v) non-fat milk in PBS for 1 hour at 37°C, the samples were diluted in extract buffer (1%Tween-20, 0·5%BSA in PBS) and added to the wells (100 μl/well, each sample was

Table 1. Specificity assay of the sandwich ELISA

Virus	Result (T/C)
A/Bristane/59/2007(H1N1)	−0·8
A/Fort Monmouth/1/1947(H1N1)	−1·2
A/PR8/34(H1N1)	−1·2
A/NewCaledonia/20/99(H1N1)	−1·1
A/Malaya/302/1954(H1N1)	−1·2
A/WSN/1933(H1N1)	−0·9
A/mallard/New York/6750/78(H2N2)	−1·1
A/Brisbane/10/2007(H3N2)	−1·2
A/duck/Hongkong/3/75(H3N2)	−1·1
A/Viet Nam/1203/2004(H5N1)	−1·0
A/mallard/Alberta/206/96(H6N8)	−0·7
A/chicken/Delaware/VIVA/2004(H7N2)	−1·0
A/mallard/Alberta/194/92(H8N4)	−1·0
A/guinea fowl/Hong Kong/WF10/99(H9N2)	−0·9
A/pintail/Alberta/202/00(H10N7)	−0·8
A/duck/Maryland/2T70/2004(H11N9)	−0·9
A/mallard/Alberta/238/96(H12N5)	−0·8
A/mallard/Alberta/146/2001(H13N6)	−1·1
A/swine/Minnesota/02053/2008(H1N1) (α)	−0·9
A/swine/Minnesota/02093/2008(H1N1) (α)	−1·0
A/swine/North Carolina/02084/200(H1N1) (β)	−0·8
A/swine/Kentucky/02086/2008(H1N1) (β)	−1·2
A/swine/Nebraska/02013/2008(H1N1) (β)	−0·8
A/swine/Ohio/02026/2008(H1N1) (γ)	−0·8
A/swine/Missouri/02060/2008(H1N1) (γ)	−0·7
A/swine/Iowa/02096/2008(H1N1) (γ)	−1·2
A/swine/Ohio/511445/2007(H1N1) (γ)	−0·7
A/swine/North Carolina/02023/2008(H1N1) (γ)	−0·9
A/swine/Texas/01976/2008(H2N2) (δ)	−0·8
A/swine/Iowa/02039/2008(H1N2) (δ)	−0·9
A/swine/Minnesota/02011/2008(H1N2) (δ)	−0·7
A/swine/Iowa/15/30(H1N1) (classical)	−0·8
A/swine/Tennessee/25/77(H1N1) (classical)	−1·1
A/Netherlands/602(H1N1pdm)	+11
A/California//04/2009(H1N1pdm)	+4·2
A/Mexico/4108/2009(H1N1pdm)	+10
Rg-NY/18HA1: 7NL/602(H1N1pdm)*	+12
Rg-D225G-HA-NL/602(H1N1pdm)†	+3·5

*Rg-NY/18HA1:7NL/602, reverse genetic recombinant carrying the HA gene from A/New York/18/2009 (H1N1) and remaining seven genes from A/Netherland/602/2009(H1N1).
†Rg-D225G-HA-NL/602 (kindly provided by Dr Ron A. M. Fouchier), reverse genetic A/Netherland/602/2009(H1N1) virus carrying the HA gene containing D225G mutation.

added to four wells–two for T wells and two for C wells–and the mixture was incubated at 37°C for 1 hour. After four washes, 100 μl biotin-conjugated 3B2 (0·25 μg/ml) in dilution buffer (0·5% BSA in PBS) was added to the wells and the mixture was incubated for 1 h at 37°C. Following three washes, 100 μl diluted HRP-conjugated streptavidin (62·5 ng/ml) in dilution buffer was added to the plates. After incubation for 1 h at 37°C, the plates were washed five times, and the binding developed using the TMB substrate system for 30 minutes. The ratio of the average OD_{650} value of the T wells to that of the C wells (T/C) of individual samples was calculated. T/C values >1·5 were considered positive in the sandwich ELISA.

Results

Specificity of sandwich ELISA for pdmH1N1 detection

We developed three monoclonal antibodies, 3B2, 5H7, and 12F3, against a prototypical pdmH1N1 strain, A/California/04/2009 (H1N1) (Ca/04). These monoclonals were used to develop a rapid sandwich ELISA for specific diagnosis of pdmH1N1 strains. Purified 5H7 and 12F3 were used as capture antibodies, whereas the biotin-conjugated 3B2 was used as detection antibody. The sandwich ELISA showed strong reaction with different pdmH1N1 strains as described in Table 1. The T/C ratios of Ca/04, A/Netherland/602/2009 (H1N1) (NL/602), A/Mexico/4108/2009 (H1N1), and A/New York/18/2009 (H1N1) were 11, 10, 4·2, and 12, respectively, which are higher than the cut off value of 1·5.

In order to evaluate if the sandwich ELISA could distinguish the pdmH1N1 from other swine H1 clusters (α, β, γ, δ), 14 swine influenza strains spanning these clusters were tested. These viruses were first diluted 1:10 in extract buffer, and then added to the coated plates. As shown in Table 1, the T/C ratios of these viruses were <1·5, and therefore showed negative ELISA result. Likewise, testing of human seasonal virus strains A/Brisbane/59/2007 (H1N1), A/FM/1/1947 (H1N1), A/PR8/1934 (H1N1), A/NewCaledonia/20/99 (H1N1), A/Malaya/302/1954(H1N1), A/WSN/1933 (H1N1), and A/Brisbane/10/2007 (H3N2) also showed negative ELISA results. Furthermore, the sandwich ELISA showed no cross reaction with avian influenza viruses, including strains of the H2, H3, H5, H6, H7, H8, H9, H10, H11, H12, and H13 subtypes.

More recently, the mutation D222G in the HA of some pdmH1N1 strains has been associated with exacerbated disease and altered receptor binding.[16–20] To evaluate if such mutant could be detected in our sandwich ELISA, we tested a mutant of A/Netherland/602/2009 (H1N1) carrying the D222G mutation (engineered by reverse genetics). As described in Table 1, our ELISA could still capture the D222G mutant virus and showed a positive reaction, which highlights the specificity of our assay for pdmH1N1 strains, even those with mutations.

Limit of the detection of the pdmH1N1 sandwich ELISA

To evaluate the sensitivity of the ELISA, we used the serially diluted pdmH1N1 viruses to determine the limit of detection (LOD). As shown in Table 2, in our ELISA the highest positive dilutions of NL/602 and Ca/04 were 1:320 and 1:160, respectively. The LOD of the sandwich ELISA by $TCID_{50}$ was $3{\cdot}2 \times 10^3$ and $1{\cdot}5 \times 10^4$ $TCID_{50}$/ml, for NL/602 and Ca/04, respectively. It is important to note that the T/C ratio from NL/602 and Ca/04 viruses showed clearly a dose dependent effect, while the T/C ratio of A/swine/Iowa/30 (H1N1) did not show the same dependence and was always <1·5, corroborating the high specificity of the sandwich ELISA for pdmH1N1 strains. Although we did not compare our ELISA with other current commercial rapid influenza detection kits, the LOD of our ELISA assay is similar to other commercial kits that detect human seasonal influenza virus.[21]

Comparison of the sandwich ELISA with the "gold standard" – virus isolation

In order to further evaluate the feasibility of the application of the ELISA to clinical samples, 70 nasal wash samples

Table 2. Limit of detection (LOD) of the sandwich ELISA

Virus	Titer ($TCID_{50}$/ml)	Result (T/C) at different dilution								LOD (TCID50/ml)
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	
NL/602 (H1N1)	5 × 10^6	+(11)	+(10)	+(5·4)	+(3·8)	+(1·9)	+(1·7)	−(1·4)	−(1·0)	1·5 × 10^4
Ca/04 (H1N1)	5·032 × 10^5	+(8·0)	+(4·8)	+(3·6)	+(2·1)	+(1·6)	−(1·4)	−(1·1)	−(1·1)	3·2 × 10^3
Sw/IA/30 (H1N1)	1·58 × 10^6	−(0·8)	−(0·8)	−(0·9)	−(1·0)	−(0·9)	−(0·8)	−(1·1)	−(0·9)	–

from ferrets, 56 of those previously infected with Ca/04 and shown positive by virus isolation, were tested. The samples were diluted 1:1 in extract buffer and then tested using the sandwich ELISA. Result showed 51 out of 56 positive samples by virus isolation were positive also by the sandwich ELISA (sensitivity 90·1%). The 14 samples tested that were negative by virus isolation were also negative in the ELISA, indicating 100% specificity for our assay. These results show not only that our ELISA has high compatibility with the virus culture method, but also indicates this application can be used for clinical samples.

Discussion

Although real time RT-PCR targeting the HA gene has been used for specific diagnosis of pdmH1N1 with high sensitivity,[22–27] it is a method that requires manipulation of the sample to extract viral RNA, and it is prone to cross-contamination during the PCR steps. In this study, we described a convenient sandwich ELISA based on three mAbs developed against the pdmH1N1 strain. The ELISA not only shows high specificity for pdmH1N1 strain, but also shows great sensitivity. The ELISA could distinguish pdmH1N1 strains from human seasonal H1 and H3 viruses and, more importantly, from other swine H1 viruses. We must note that current rapid diagnostic tests cannot be used to differentiate pdmH1N1 from swine or human H1 viruses.[28,29] More recently, Miyoshi-Akiyama *et al.* and Miao *et al.* developed an immunochromatographic assay and a indirect immunofluorescence assay, respectively, for specific diagnosis of pdmH1N1.[30,31] Such tests can distinguish pdmH1N1 strains from human seasonal influenza H1N1, however, these tests have not been evaluated to test whether they can distinguish among other swine influenza viruses.

It is also worth noting that the sensitivity of commercial rapid antigen-based diagnostic tests for detecting pdmH1N1 is lower than that for human seasonal influenza viruses.[28,29] A study by Kok *et al.*[32] showed that sensitivity of the current rapid antigenic tests for pdmH1N1 is only 53·4%, whereas that for seasonal influenza A is 74·2%. Chen *et al.*[33] developed a dot-ELISA and increased the sensitivity for influenza rapid antigen detection. However, the dot-ELISA developed by Chen cannot distinguish among subtypes. The LOD of our ELISA is between $3{\cdot}2 \times 10^3$ to $1{\cdot}5 \times 10^4$ TCID50/ml, comparable to the LOD of rapid diagnostic tests for human seasonal influenza viruses.[21] Compared to the ''gold standard''–virus isolation–our sandwich ELISA showed 90·1% sensitivity using ferret nasal washes. Our results highlight the potential application of our sandwich ELISA for the specific diagnosis of pdmH1N1 viruses.

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Results of influenza virus detection using different methods

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Introduction

The timely and reliable laboratory evidences are vital factors for field epidemiologists trying to control outbreaks of infectious diseases and for the practicing clinicians to properly manage disease cases. Therefore, analysis of new detection methods in comparison to the routine ''classical'' methods is essential to select new methods to be introduced into health service practices, especially in developing countries. In this study we have compared rt-RT-PCR detection of influenza viruses and direct fluorescent-antibody assay using R-Mix hybrid cells (A549&Mv1Lu) with the ''classical'' cell culture methods in developing country settings.

Materials and methods

Clinical samples

In this study, we analyzed 503 nasopharyngeal swabs collected during 2008–2009 influenza season from ILI patients of the Baganuur district of Ulaanbaatar and Selenge province, Mongolia, and 7000 nasopharyngeal swabs collected in 133 ISSSs during 2009–2010 influenza season.

Table 1. Results of detection by real-time RT-PCR, MDCK and R-Mix hybrid cell culture

				MDCK cell culture			R-Mix hybrid cell culture		
Influenza seasons	**Influenza virus subtype**	**Samples**	**Real time RT-PCR**	**All**	**Positive HA 1:4**	**Negative**	**All**	**Positive**	**Negative**
2008–2009	A(H1N1)	503	129 (25·6%)	129 (100%)	31 (24%)	104 (76%)	17 (13·2%)	8 (47%)	9 (53%)
	A(H3N2)		2 (0·4%)	2 (100%)	0	0	0	0	0
2009–2010	A(H1N1)pdm	7000	1366 (19·5%)	500 (36·6%)	248 (50%)	285 (50%)	107 (7·8%)	47 (44%)	60 (56%)
	B		394 (5·6%)	364 (92·4%)	242 (66·5%)	122 (33·5%)	27 (6·9%)	22 (81·5%)	5 (18·5%)

rt-RT-PCR percentages have been deduced from the selected respiratory samples. MDCK and R-Mix cell cultures have been inoculated only by rt-RT-PCR positive samples.

Specific nucleic acid detection

The detection of influenza H1, H3, B, and pandemic influenza (H1)pdm virus-specific nucleic acids was performed by rt-RT-PCR in ABI7500 Fast Real Time PCR system using primers recommended by CDC, USA, and SuperScript™ III One-Step RT-PCR and Platinum® *Taq* DNA Polymerase kits (Invitrogen). The cycling protocol was: 30 minutes at 50°C, 2 minutes at 95°C, and 45 cycles of 15 seconds at 95°C, 30 seconds at 55°C.[1]

Rapid detection of influenza infected cells has been performed by DFA using the infected hybrid cells of R-Mix within 48 hours after inoculation, according to the manufacturers instruction (Diagnostic Hybrids, Inc., USA).[2]

Cell cultures

The isolation of influenza viruses was performed on MDCK cell culture by the protocol recommended by CDC, USA.[3]

Results

We detected 1891(25·2%) influenza virus-specific nucleic acid fragments from all tested samples by rt-RT-PCR. Among the positive samples, there were 25·6% A(H1N1), 0·4% A(H3N2), 5·6% influenza B, and 19·5% A(H1N1)pdm with different distributions by time series in different age-groups.

Inoculation of the cell lines by rt-RT-PCR positive samples selected randomly has detected influenza virus in 52·3% (521/995) on MDCK cell culture and 51% (77/151) on R-Mix hybrid cell culture with varying distribution for different strains. In other words, MDCK cell culture technique was better for isolation for pandemic influenza viruses and DFA using R-Mix hybrid cell culture technique for detection of seasonal influenza viruses (Table 1).

Average times needed for the final results for different methods were: 4 hours for rt-RT-PCR, 48 hours for DFA on R-Mix and 10 days for MDCK cell culture with two passages at least.

The peak of the seasonal influenza A virus detection occurred in the 7–8th weeks of 2009, however the pandemic influenza detection peak was observed in the 43–44th weeks of 2009 (Figure 1). The outbreaks by seasonal influenza viruses was observed mostly among the children of 0–15 years of age, and pandemic influenza virus outbreak was observed mostly in the adults of 16–64 years of age.

Discussion

The results of this study indicate that rt-RT-PCR is the most suitable method for decision makers in epidemiological and clinical settings by sensitivity and timeliness. The final results show that R-Mix DFA requires 12 times longer, and by MDCK cell culturing, 60 times longer periods, than by rtRT-PCR. MDCK cell culture technique has a higher isolation of pandemic influenza viruses, and R-Mix DFA has a greater detection rate of seasonal influenza viruses by our results.

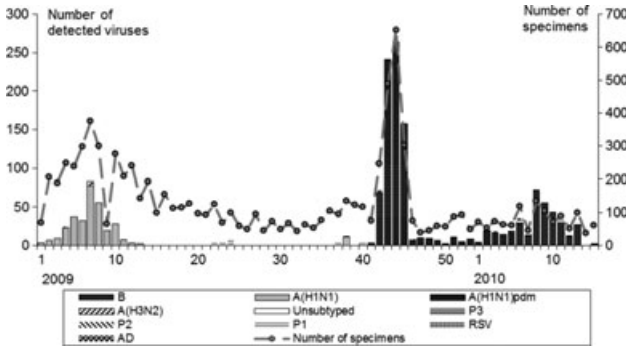

Figure 1. ILI virological surveillance results in Mongolia (2009–2010).

According to our study, with rtRT-PCR, the isolation of positive samples by tissue culture of influenza A viruses was 24% and influenza B viruses was 66·5%, which is lower than in similar Spanish study.[4] However our study illustrates similar results with a Canadian study [5] where the sensitivity of DFA method and tissue culture technique was shown to be lower than rtRT-PCR sensitivity.

As recorded by a study of American researchers,[6] R-Mix hybrid and conventional cell culture techniques have had similar sensitivity, which does not match the results of our study. However, the results of our study match with the results of Italian and American scientists [7,8] where the R-Mix hybrid method for seasonal influenza viruses is higher than MDCK cell culture technique.

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Viral kinetics studies on influenza: when and how many times are nasal samples to be collected?

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Abstract

Background: Viral kinetics is increasingly used to study influenza infectiousness. The choice of the study design, i.e. when and how many times nasal samples are to be collected in individuals depending on the sample size, is crucial to efficiently estimate the viral kinetics (VK) parameters. Material and methods: We performed a model based optimal design analysis in order to determine the minimal number of nasal samples needed to be collected per subject and when to collect them in order to correctly estimate the VK parameters. The model used was a non linear mixed effect model developed with data collected from 44 patients sampled nine times in 9 days (initial design – 504 samples collected), and we used D-optimization for design identification. We also computed the minimal number of participants necessary. Results: Considering that 25% of the influenza-like illness cases are not due to influenza virus, we found that 20 participants with five samples per subject during the two-first days of illness were necessary to optimally perform a VK study in experimental or natural influenza infection. Globally, 100 samples were necessary for these designs, instead of 504 with the initial design. Conclusions: These findings should help design further studies on influenza VK, minimizing patient discomfort and cost.

Introduction

Volunteer challenge studies have been used since the 60's to provide data on virus shedding from the respiratory tract during influenza infection.[1] Recently, VK was studied in naturally acquired influenza infection.[2,3] These data are invaluable to describe the natural history of influenza-infection and to compute natural history parameters such as the latent period, generation time, or

the duration of infectiousness.[1–4] However, among the 56 studies used in a meta-analysis about viral shedding kinetics,[1] the designs varied greatly from one to another. These differences led to variable amount of available information concerning the VK. The lack of adequate sampling leads to imprecise estimates. On the other hand, intensive sampling or over-sampling, while associated with highly informative data, may lead to unnecessary discomfort for the patient and cost to the investigator. Optimal design is increasingly used to conceive studies[5] and provides cost-efficient designs. Here we propose an optimised design to model VK in the case of influenza infection. We defined the number of participants, the number of samples to collect and their allocations. This design allows, at a minimum cost and discomfort, accurate VK curves and allows the natural history parameters to be well described.

Materials and methods

Model

A VK population model was proposed for influenza infection. This model describes with eight parameters the relations between free virus, uninfected target epithelial cells, infected epithelial cells, and early immune response. This model was built on a dataset of 44 volunteers from which nasal samples were collected once a day over 9 days. We call this dataset the ''original dataset''.

Three parameters, the induction of the early immune response, the virus production rate, and the virus clearance, did not show inter-individual variability and were precisely estimated (relative standard error below 10%). We considered them as fixed in this research work. Five parameters were hence considered here: β the infection rate, δ the infected cell mortality rate, ψ the effect of early immune response on virus production rate and V_{init} the initial value of virus titre.

Optimisation of the sample times

In order to correctly estimate these parameters it is crucial to determine a design to collect informative data. Optimal designs maximise the amount of information provided by the study. It involves the determination of the number and allocation of sample times per subject as well as the number of participants.[6] D-optimization is based on the maximization of the determinant of the Fisher information matrix and thus minimizes the variance of the parameters.[7] We used the Fedorov–Wynn algorithm implemented in PFIM 3.0 to maximize this determinant,[8] which implies to pre-define a set of possible sample times. With the hypothesis that the inoculation occurred at 8:00 am, we chose three possible hours (8:00, 14:00, and 20:00) for each day with respect of the sleep-time.[8]

To validate the design, we simulated 100 datasets of 44 volunteers with the optimised design obtained. We then estimated the population parameters using MONOLIX 3.1 for each of the datasets. We compared the estimated parameters obtained with the simulated datasets to the parameters used to build the optimal design. We computed the relative bias as:

$$\text{Bias} = \frac{1}{n}\sum_{i=1}^{n}\frac{\hat{\theta}_i - \theta}{\theta}$$

With n: number of successful estimations among the 100 simulated datasets.

$\hat{\theta}_i$: parameter value obtained with the ith dataset.

θ: parameter value obtained with the original dataset.

We also compared the observed RSE from these simulations with the RSE predicted by PFIM and the RSE obtained with the original dataset.

Population size

The RSE is proportional to $\sqrt{n}$, where n is the population size. We can hence deduce the smallest number of participants necessary to obtain RSE below 50%.

$$n_{\min} = n_{\text{predicted}}\left(\frac{\text{RSE}_{\text{predicted}}}{\text{RSE}_{\min}}\right)^2$$

Where $\text{RSE}_{\text{predicted}}$ is the highest predicted RSE (here RSE for ψ) with 44 participants and $n_{predicted} = 44$ and $\text{RSE}_{\min}$ is equal to 0·5.

Considering that 25% of the influenza-like illness cases are not due to influenza virus, the total number of participants should be multiplied by 1·25.

Results

Sampling times

We found that the best design was when all the participants are sampled five times: three times during the second day post-inoculation at 8:00, 14:00, and 20:00 hours and twice on the third day post-inoculation at 8:00 and 20:00 (Figure 1).

Evaluation

The comparison of the relative bias and RSE predicted by PFIM and those obtained after simulation and re-estimation of the parameters are shown in Figure 2.

V_{init} and δ in a lesser extent present bias. Fixed effect parameters are precisely estimated and accordingly to PFIM except for V_{init}.

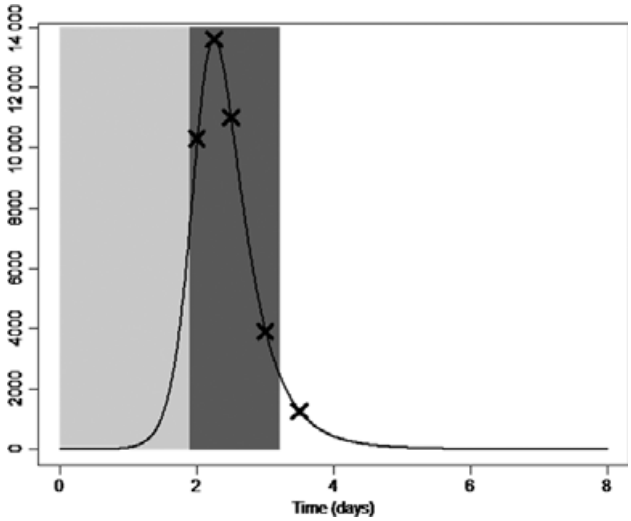

Figure 1. 5-points design (line: population viral titre curve, crosses: optimal sample times, light grey: incubation period, dark grey: systemic symptoms period).

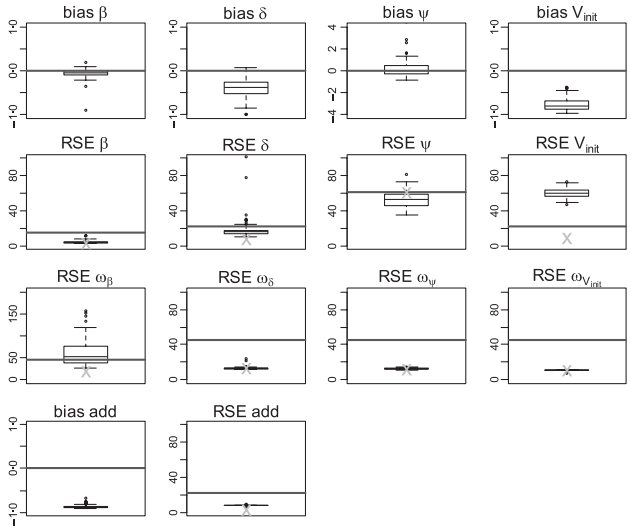

Figure 2. Validation of the model (boxplot: distribution of the values obtained from the 100 simulations, black line: reference value obtained with the original dataset, grey cross: predicted RSE obtained with PFIM).

Population size

We found that 16 participants shedding virus or 20 participants with ILI symptoms are necessary if 25% of them are not infected with influenza virus.

Discussion

We propose an optimised design to accurately study the VK of influenza virus with the minimal number of samples. This design is well balanced between the amount of necessary information and the precision of estimation. We found that 100 samples are necessary to precisely fit the VK curves, which is five times less than the number of samples collected in the original study.[4???] The samples should be collected during the second and third days after inoculation. Yet we showed in a previous work that the incubation period lasted 1·9 days.[4???] Hence, the optimised sample times correspond to the two-first days of symptoms and this design could be applied to naturally acquired infections studies in which the inoculation time is unknown.

An advantage of this design is its practicality and convenience. All samples are collected during the daytime and after the onset of symptoms. It can thus be used for studies with naturally acquired infections. The design was validated with several criteria concerning the accuracy of the estimation with the optimised design. The parameters estimates were generally satisfactory. The parameter describing the effect of the early immune response on the virus production rate was, however, less precisely estimated (predicted RSE = 62%), and the initial value of the viral titre was very different of the one obtained with the original dataset (bias V_{init} on Figure 2). This is probably due to the fact that it was measured at day 2 post inoculation, and that the inter-individual variability is much higher than at day 0. Furthermore, δ (the infected cell mortality rate) seems also to be biased. This may be due to the fact that three parameters were fixed.

The model used was developed from experimentally inoculated healthy volunteers with low serum haemagglutinin antibody titre and with virus inoculation time at 8:00 am. The applicability of the design to naturally acquired infection would depend on the pathogenicity of the virus as well as pre-existing immunity and the relevance of challenge method to natural influenza acquisition.[1] Our design could be directly used to accurately study VK during influenza infections and would reduce the discomfort of patients and the cost of the experimentation.

Acknowledgements

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Usefulness of a self-blown nasal discharge specimen for use with immunochromatography based influenza rapid antigen test

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Keywords Immunochromatography, influenza rapid antigen test, nasopharyngeal swab, self-blown nasal discharge specimen.

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Introduction

Influenza rapid antigen tests (IRAT) have become very popular and are widely used for confirming suspected clinical diagnosis of influenza in Japan.[1] Most of the currently used IRAT that are based on immunochromatography (IC), nasopharyngeal swab, nasopharyngeal aspiration, and throat swab have been approved as specimens for Japanese national health insurance purposes. But the specimen collection by these methods gives patients considerable discomfort, and sometimes appropriate specimens cannot be obtained due to patient resistance, especially by children.

In the present studies, self-blown nasal discharge was used as the specimen for an IRAT, and the results were compared with the results of viral isolation and an identical kit primed with nasopharyngeal swab specimens for seasonal influenza viruses and pandemic (H1N1)2009 virus.

Materials and methods

Patients who visited any of the 10 clinics that belong to the Influenza Study Group of the Japan Physicians Association in the 2006–2007 and the 2009–2010 influenza seasons with influenza-like illnesses exhibiting findings were registered after providing informed consent.

A square plastic sheet of 20 × 20 cm was handed to the patient. Nasal discharge was collected by blowing the nose into the plastic sheet as a specimen for IRAT, i.e. self-blown specimen. Two nasopharyngeal swab specimens were also obtained at the same time for IRAT and virus isolation.

A commercial IRAT, Quick Vue Rapid SP influ (DS Pharma Biomedical Co., Ltd) in the 2006–2007 influenza season and Quicknavi™-Flu (Denka-seiken Co., Ltd) in the 2009–2010, both based on IC were used. Virus isolation was performed by standard methods using Madin-Darby canine kidney cells with one of the nasopharyngeal swab specimens. Influenza A (H3N2), A (H1N1), influenza B, or Pandemic (H1N1) 2009 was determined by haemagglutination inhibition test and/or PCR using specific primer sets.

Results

Self-blown specimens were obtained successfully by 152 (82·2%) of 185 consecutive outpatients in the 2006–2007 season, as seen in Table 1. They comprised 68 males, mean age 23 ± 18 years, and 84 females, mean age 23 ± 17 years. The other specimens from 145 patients were obtained enough to be examined in the 2009–2010 season.

Seasonal Influenza A were isolated from the nasopharyngeal swab specimens of 69 patients, 55 A (H3N2) and 14 A (H1N1) were determined, and B were from 35 patients in the 2006–2007 season. Pandemic (H1N1) 2009 virus was isolated in 100 of 145 patients in the 2009–2010 season.

Comparison of the IC kit results primed with self-blown specimens to the results of viral isolation showed sensitivity to be 87·0% (60/69), specificity 94·6% (80/83), and accuracy 92·1% (140/152) for influenza A. The sensitivity was 85·7% (30/35), specificity 99·1% (116/117), and accuracy 96·1% (146/152) for influenza B. The sensitivity was 88% (88/100), specificity 88·9% (40/45), and accuracy 88·3%

Table 1. Collectable rate of the self-blown nasal discharge specimen compared with age groups in the 2006–2007 influenza season

Age years	Number of registered patients	Number of collectable blown nasal discharge specimens	Percentage of collectable rate (%)
0–6	18	14	77·8
7–15	78	58	74·4
16–65	89	80	89·9
Total	185	152	82·2

Self-blown specimens enough to be examined were obtained 152 from 185 consecutive outpatients, and specimens showed a tendency to be obtained large amount from children rather than the aged.

(124/145) for pandemic (H1N1) 2009. There was no statistically significant difference between the IC kit results primed with self-blown and nasopharyngeal swab specimens for influenza A, influenza B, and pandemic (H1N1) 2009. Correlation between blown nasal discharge specimens and nasopharyngeal swab specimens in the cases of Pandemic H1N1 2009 of the 2009–2010 season is shown in Table 2.

Table 2. Correlation between blown nasal discharge and nasopharyngeal swab specimens in the cases of Pandemic H1N1 2009 of the 2009–2010

		Quicknavi™-Flu		
		Pandemic H1N1 2009 nasopharyngeal swab specimens		
		H1N1pdm	Negative	Total
Quicknavi™-Flu	A positive	78	2	80
Blown nasal discharge specimens	Negative	3	42	45
	Total	81	44	125
Sensitivity		96·3% (78/81)		
Specificity		95·5% (42/44)		
PPV		97·5% (78/80)		
NPV		93·3% (42/45)		
Accuracy		96·0% (120/125)		

There were no statistically significant differences between the IC kit results primed with self-blown discharge and nasopharyngeal swab specimens for influenza A, influenza B and Pandemic (H1N1) 2009.

Discussion

The sensitivity and specificity of various influenza rapid antigen tests have been reported in various settings.[1–4] Direct comparison of the results is difficult because of differences in patient or influenza virus, characteristics such as age, study designs, and other features. In this study of the 2006–2007 influenza season, the sensitivity, specificity, and accuracy of the IC kit primed with nasopharyngeal swab specimens were 87·0%, 94·0%, and 90·8%, respectively. These results were quite comparable to our results of the 2005–2006 season,[3] in which the overall results of other IC kits were 82·9%, 81·0%, and 82·3%, respectively, indicating that the IC kit used is quite reliable.

The sensitivity, specificity, and accuracy of an IC kit will vary by the method of specimen collection. In general, virus titer is considered to be highest with nasopharyngeal aspiration, lower with nasopharyngeal swabs, and lowest with throat swabs. Practically, nasopharyngeal swab is the most popular. The sensitivity, specificity, and accuracy of the IC kit with self-blown discharge specimens compared well with those of an identical IC kit primed with nasopharyngeal swab specimens.

For self-blown specimens, sensitivity and specificity were 87·0% and 94·6% for influenza A, 85·7% and 99·1% for influenza B, 88% and 88·9% for pandemic (H1N1) 2009. Self-blown specimens display sensitivity, specificity, and accuracy comparable to that of conventional nasopharyngeal swab specimens. There was no significant difference in sensitivity, specificity, or accuracy between self-blown specimens and nasopharyngeal swab for influenza A, influenza B, and pandemic (H1N1) 2009. These results suggest that self-blown specimens are as useful as nasal cavity swab specimens for the diagnosis of influenza in the clinical settings.

Nasal discharge, obviously, cannot be collected from infants incapable of blowing their own nose or patients who do not develop a nasal discharge. In this study, self-blown specimens were obtained from 82·2% of the patients. The rate of successful collection was over 70% in the age groups of 0–6 and 7–15 years. These rates would seem to be sufficient for clinical use. The procedure of self-blown specimen collection using a plastic sheet is easy and causes no pain or discomfort. It seems to be more acceptable and safe than the other methods, especially for children. Furthermore, this procedure reduces the risk of influenza transmission from patients to the medical staff members involved in sample collection. Self-blown sample collection may be superior to other sample collection methods in these respects.

We previously reported an inverse correlation between the amount of virus in a specimen and the time to a positive reaction.[4] In this study, there was no significant difference in the mean time to a positive between self-blown

and nasal swab specimens, suggesting that the self-blown specimens contained sufficient viral antigen for the IC kits. The influence of the presence or absence of nasal congestion on the results of the kit was assessed. The sensitivity of self-blown specimens from patients with nasal congestion was significantly lower than that from patients without nasal congestion. It is possible that insufficient capability to blow the nose due to nasal congestion might tend to lead to false negatives. The observation that the time to positive is longer for patients with nasal congestion than for patients without nasal congestion is concordant. Application of self-blown specimen collection only to appropriate patients would increase the sensitivity, which would be important in a clinical setting.

We tested only two commercial antigen detection kit, the Quick Vue Rapid SP influ kit and Quicknavi™-Flu (Denka-seiken Co., Ltd). The resulting sensitivity, specificity, and accuracy of the IC kit primed with self-blown specimens were considered adequate for clinical use. To confirm the usefulness of self-blown nasal discharge specimens, further investigation is necessary using other kits and in different settings.

Conclusion

The usefulness of a self-blown nasal discharge specimen for an influenza rapid antigen test based on immunochromatography was evaluated in the 2006–2007 and 2009–2010 influenza season. Results suggest that self-blown nasal discharge specimens are useful as specimens for influenza rapid antigen tests based on immunochromatography for not only seasonal influenza viruses, but also pandemic (H1N1) 2009 virus.

The specimen collection by the patients themselves will reduce the burden of other collection methods and the risk of infection to the medical staff.

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Development of rapid antigen test for pandemic (H1N1) 2009 virus

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Keywords H1N1, influenza, pandemic, rapid antigen test.

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Introduction

In April 2009, a mixed-origin H1N1 influenza virus was recognized as a new causative agent of influenza-like illnesses (ILI) in humans. Since its emergence, the virus has spread rapidly throughout the world and caused a pandemic. Most commercial rapid antigen tests (RAT) can detect influenza A or B viruses, but cannot specifically distinguish pandemic (H1N1) 2009 virus with seasonal influenza. Recent studies have indicated that the poor performance of the RAT approach and nonspecific detection of the pandemic (H1N1) 2009 virus was the main obstacle to their widespread use in private clinics.[1,2] With the need for a new rapid kit with reasonable sensitivity and specificity for pandemic (H1N1) 2009 virus, we developed a new RAT kit in collaboration with company, Standard Diagnostics, Inc., (Yongin-si, Gyonggi, Korea).

Materials and methods

Monoclonal antibody (mAb) against haemagglutinin (HA) of the pandemic (H1N1) 2009 virus was developed using

Korean isolate and applied to the new kit with the mAb to seasonal influenza virus. We examined the detection limit of the kit using the serial dilution of Korean pandemic virus isolate (A/Korea/01/2009). During December 2009, 432 clinical specimens from patients with ILI were collected at 11 sentinel clinics of six provinces in Korea. The specimens were tested by the new RAT, and the results were compared with those of real-time reverse transcription polymerase chain reaction (rRT-PCR) by US CDC and virus isolation in MDCK cell culture to determine the sensitivity and specificity for the diagnosis of pandemic (H1N1) 2009.

Results

The detection limit of the new kit against HA of A/Korea/01/2009 virus was confirmed to be 10^4 PFU/ml. By contrast, the detection limit against the NP protein was 10^3 PFU. However, when the kit was applied to clinical specimens, no difference between the two targets was found. Using rRT-PCR and viral culture as the references, the performance of the RIDT is shown in Table 1.

Among 432 specimens, 178 were tested positive by rRT-PCR and 186 were tested positive by viral culture. Among the 178 rRT-PCR confirmed cases, 122 were positive, and among the 186 viral culture confirmed cases, 120 were positive with the new RAT. Using rRT-PCR as the reference standard, the overall sensitivity of RAT was 68·5% (95% confidence interval (CI): 61·7–75·3%) and specificity was 98·4% (CI: 96·9–99·9%). With viral culture as the reference, the RAT sensitivity and specificity was 64·5% (CI: 57·6–71·4%) and 97·6% (CI: 95·7–99·5%), respectively. When analyzed by the regions tested, the sensitivity ranged between 57·1% and 95·5% for rRT–PCR and between 53·3% and 87·0% for viral culture as a reference. Among 340 patients who had a record of their symptom onset and sample collection date, 86 (25·3%) visited the clinic on the day of symptom onset, and 158 (46·5%) visited 1 day later. When the RAT performance was evaluated by day of onset, the sensitivity was lower at three or more days after the onset of symptoms; however, the sensitivity was highest at 2 days after onset and reasonable on the day of onset or at 1 day after (Table 2).

Discussion

We found that this new RAT had reasonable sensitivity and high specificity compared with rRT-PCR and viral culture for detecting the pandemic (H1N1) 2009 virus. In one recent study, the sensitivity and specificity of the new RAT kit was 77% and 100%, respectively, and the HA protein for pandemic (H1N1) 2009 was detected more sensitively than the NP protein for influenza A virus.[3] The sensitivity and specificity of our new RAT were lower than those of that study. We found that the test performance varied depending on the clinics in which the tests were performed, and this might be attributable to the persons who collected the specimens. Although the clinicians were trained well for

Table 1. Performance of the new rapid antigen test (RAT) compared with real-time reverse transcription polymerase chain reaction (rRT–PCR) and viral culture for the diagnosis of pandemic (H1N1) 2009 influenza virus

Region	No. of samples tested	Method	Sensitivity, % (95% CI*)	Specificity, % (95% CI)	PPV**, % (95% CI)	NPV***, % (95% CI)
Seoul	82	rRT–PCR	68·8 (52·7–84·8)	100·0 (94·6–100·0)	100·0 (87·6–100·0)	83·3 (73·9–92·8)
		Viral culture	62·9 (46·8–78·9)	100·0 (94·1–100·0)	100·0 (87·4–100·0)	78·3 (67·9–88·8)
Incheon	90	rRT–PCR	64·7 (48·6–80·8)	100·0 (95·0–100·0)	100·0 (87·4–100·0)	82·4 (73·0–91·4)
		Viral culture	62·9 (46·8–78·9)	100·0 (94·9–100·0)	100·0 (87·3–100·0)	80·9 (71·5–90·2)
Daejeon	37	rRT–PCR	81·8 (65·7–97·9)	100·0 (84·8–100·0)	100·0 (87·3–100·0)	78·9 (60·6–97·3)
		Viral culture	70·8 (52·0–89·0)	92·3 (77·8–100·0)	94·4 (89·9–94·4)	63·2 (41·5–84·8)
Gwangju	71	rRT–PCR	95·5 (86·8–100·0)	93·9 (87·2–100·0)	87·5 (74·3–100·0)	97·9 (93·7–100·0)
		Viral culture	87·0 (73·2–100·0)	91·7 (83·8–99·5)	83·3 (68·4–98·2)	93·6 (86·6–100·0)
Jeonbuk	74	rRT–PCR	57·5 (12·2–72·8)	97·1 (91·4–100·0)	95·8 (87·8–100·0)	66·0 (52·9–79·1)
		Viral culture	59·0 (43·5–74·4)	97·1 (91·6–100·0)	95·8 (87·8–100·0)	68·0 (55·1–80·9)
Gangwon	78	rRT–PCR	57·1 (38·8–75·5)	100·0 (94·7–100·0)	100·0 (83·3–100·0)	80·6 (70·8–90·5)
		Viral culture	53·3 (35·5–71·2)	100·0 (94·4–100·0)	100·0 (83·1–100·0)	77·4 (67·0–87·8)
Overall	432	rRT–PCR	68·5 (61·7–75·3)	98·4 (96·9–99·9)	96·8 (93·8–99·9)	81·7 (77·4–86·0)
		Viral culture	64·5 (57·6–71·4)	97·6 (95·7–99·5)	95·2 (91·5–99·0)	78·4 (73·8–83·0)

*CI, confidence interval.
**PPV, positive predictive value.
***NPV, negative predictive value.

Table 2. Performance of the new RAT according to the date of onset and sample collection for detecting the pandemic (H1N1) 2009 influenza virus*

Day after symptom onset	No. of total	Performance against rRT-PCR		Performance against viral culture	
		Sensitivity, %	Specificity, %	Sensitivity, %	Specificity, %
0	86	61·1	96·0	61·8	94·2
1	158	61·3	99·0	55·1	98·9
2	59	92·3	97·0	92·3	97·0
3	22	50·0	100·0	50·0	100·0
4–12	15	37·5	100·0	37·5	100·0
Overall	340	65·0	98·0	62·5	97·4

*Three hundred and forty samples with a known date of onset and sample collection were analyzed.

collecting specimens, there might be some differences in performance. The new RAT kit could detect pandemic (H1N1) 2009 virus specifically. Although the sensitivity was lower than those of rRT-PCR and virus culture, and negative RAT results should be confirmed with more sensitive methods, this kit could be useful in sentinel clinics if used with caution.

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Disclosure

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Potential of the real-time and label-free cell sensor impedance technology to transform cell based infectivity assays for influenza viruses

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Introduction

Determination of infectious virus titres is central to many experiments designed to study the biology of influenza virus. Assays based on the measurements of viral components, whether viral protein or nucleic acid, does not differentiate infectious virus from non-infectious or defective viral particles, which may have no infectivity or biological

activity. Therefore the ''gold standard'' of virus measurement requires bioassays that examine the ability of viral particles to replicate and further infect other cells. Titration on Madin-Darby canine kidney (MDCK) cells in a 96 well plate format is commonly used to measure influenza virus titre. This method is labour intensive, subjective in their read out of cytopathic effect, and takes several days to obtain a result. Microneutralization tests that quantitate neutralizing antibody titres and assays of drugs for antiviral activity also require 96 well based assays of residual virus infectivity. Therefore, technologies that improve on the titration of infectious virus will be of great benefit.

This study utilized the xCelligence system (Roche Applied Science), which adopts microelectronic biosensor technology to monitor dynamic, real-time label free and non-invasive analysis of cellular events.[1] The system measures electronic impedance using an array of microelectrodes located at the bottom of each culture well (E-plate 96). Adherent cells are attached to the sensor surface of electrode arrays, and changes in impedance can be detected and recorded. The xCelligence system can monitor cell events induced by viral infection, such as changes in cell number, adhesion, viability, morphology, and motility. Measured electrode impedance is expressed as dimensionless Cell Index and is graphically represented using software to show the phenotypic changes of a cell population over time. The aim of this study is to demonstrate that using this platform to measure real-time cell index has potential to circumvent many of the limitations of the currently established procedures of end point titration of virus infectivity and for microneutralization assays.

Materials and methods

Cells and viruses

Madin-Darby canine kidney cells were propagated in growth medium consisting of Minimum Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0·6 mg/l penicillin (Invitrogen), and 60 mg/l streptomycin (Invitrogen), with incubation at 37°C in a 5% CO_2 humidified atmosphere.

Influenza A/Hong Kong/54/1998 (H1N1), a seasonal influenza virus from a patient who suffered from a mild febrile illness, was propagated in MDCK cells maintained in virus medium consisting of Minimum Eagle's medium (Invitrogen) supplemented with 0·6 mg/l penicillin (Invitrogen), 60 mg/l streptomycin (Invitrogen), and 2 mg/l N-p-tosyl-L-phenylalaninechloromethyl ketone-treated trypsin (Sigma, St Louis, MO, USA), with incubation at 37°C in a 5% CO_2 humidified atmosphere. Virus stocks were aliquoted and stored at 70°C until use, and the 50% tissue culture infectious dose ($TCID_{50}$) of the virus stock was determined by titration in MDCK cells according to standard procedures,[2] and the $TCID_{50}$ of the stock virus was calculated by the method of Reed and Muench.[3]

Microneutralization assay

To perform a microneutralization assay, MDCK cells seeded at a density of 1000 cells/well in an E-Plate 96 was removed from the xCelligence system after approximately 48 hour; growth medium was then removed, cells washed, and replaced with 100 μl virus-medium.

A human serum, which is known to contain high titre antibody against the H1N1 virus was heat inactivated for 30 min at 56°C, and twofold serial dilutions were performed in virus medium. The diluted serum was mixed with an equal volume of virus medium containing influenza virus at 400 $TCID_{50}$/100 μl. After incubation for 2 h at 37°C in a 5% CO_2 humidified atmosphere, 50 μl of virus-antibody mixture was added to the MDCK cells to give each well an equivalent virus dose of 100 $TCID_{50}$. A back titration of the virus challenge dose was performed, and a cell control (free of virus) was performed in quadruplicates. After incubation at room temperature for 30 minutes, the E-Plate 96 was then placed back onto the xCelligence system in the incubator and maintain at 37°C with 5% CO_2, and the Cell Index values were measured every 15 minutes for at least a further 72 hour.

The same procedures were performed with cells seeded in conventional 96 well cell culture plates for parallel comparison with the currently used standard method. In this case, cells were examined for cytopathic effect under an inverted microscope after 3 days of infection and the lowest virus dilution, which protected the cells from viral induced cytopathic effect taken as the neutralizing end point.

Results

Real time cell index monitoring during influenza virus infection MDCK cells

After 48 hour of seeding MDCK cells at 1000 cells/well, standard microneutralization assay for influenza virus was performed. Integral to this assay, a serial titration of the input virus at 0·5 $\log_{10}$ increments was carried out. Wells infected with the undiluted virus (100 $TCID_{50}$/well), the Cell Index commenced dropping at a steeper gradient than the no-virus cell control after approximately 3 hour of infection (Figure 1). This drop in cell index continues at a consistent slope until it flattened out when approaching zero Cell Index. This steep decrease in Cell Index with constant gradient was also observed for virus dilutions up to and including 2 $\log_{10}$ (100-folds), and the profile shifted with increased time in proportion to the dilution made to the virus. Virus dilutions beyond 2 $\log_{10}$ have Cell Index

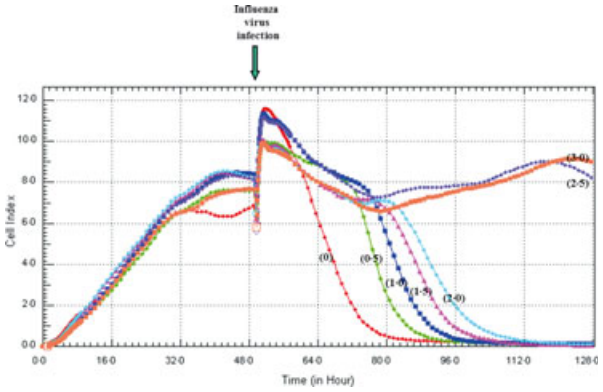

Figure 1. Real time cell index monitoring during influenza virus infection of Madin-Darby canine kidney (MDCK) cells. Cells were infected with serial dilutions of influenza virus commencing at 100 $TCID_{50}$ per well at 0.5 log_{10} increments (dilutions in parenthesis). Data shown is the mean of 4 replicate culture wells.

profiles similar to the no virus input control, and this corresponds to the absence of cytopathic effect as determined by microscopic observation at 72 hour after infection. Hence, there was a correlation between the amount of virus used for infection, the onset of the influenza virus-mediated cytopathic effect, and the steep decline in cell index.

Real-time cell index monitoring of microneutralization assay

A human serum with known microneutralization antibody titre to H1N1 virus was used in this study to investigate the real time Cell Index changes that occur during the assay (Figure 2). Using influenza virus treated with serum dilutions up to and including a dilution of 1:160, the cell index profile remained essentially the same as the no virus cell control, which correlates with the lack of cytopathic effects under microscopic observation at 72 hour of infection. At a serum dilution of 1:320, the steep decrease in Cell Index, which is characteristic of cellular cytopathic effect induced by the virus, became evident at around 50 hour post infection, and this was reduced to 40 hour when serum dilution of 1:940 was used. In contrast, for the virus – no antibody control, the onset time for this steep decrease in Cell Index occurs at approximately 7 hour. For both serum dilutions of 1:320 and 1:940, full cytopathic effect was observed microscopically at 72 hour of infection. From microscopic observation of cytopathic effect, according to the current standard procedures, the neutralizing titre of the human serum used in this study is at 1:160 as it is the last dilution of the serum that prevented cytopathic effect from being detected.

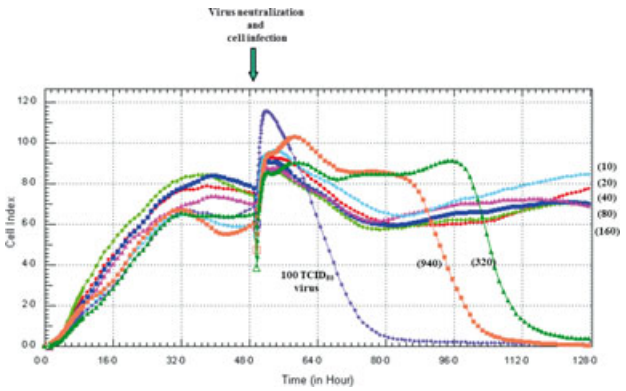

Figure 2. Real time cell index monitoring of microneutralization assay. Human serum at 1:1 dilution increments were used (serum dilution factor in parenthesis) to neutralize H1N1 virus at 100 $TCID_{50}$. Data shown is the mean of 4 replicate culture wells.

Discussion

An essential part of the microneutralization assay is to confirm the titre of the input virus (normally 100 $TCID_{50}$/well) by performing a titration assay with decreasing serial dilutions of the virus. Under normal procedures, cells are examined microscopically after 72 hour of infection for sign of cytopathic effects. In the case of MDCK cells, the cytopathic effect is cell death, which is indicative of the presence of live influenza virus infecting and replicating in the cells. Therefore, the titre of the virus is taken as the last dilution in which cytopathic effect is present. Parallel real-time cell index measurements demonstrated that for wells with cytopathic effects, the profile exhibits a steep gradient linear decrease in cell index after infection with the virus, which can be termed the ''CPE plunge.'' The time in which the CPE plunge became evident appears to be inversely proportional to the amount of virus, therefore the opportunity exists to utilize this aspect to calculate or compare quantitatively different virus concentrations.

For unequivocal assignment of cytopathic effect, it normally requires 2–3 days after infecting the cells, with 3 days after infection being the standard time to read virus titration and microneutralization assays. Using the real-time cell index monitoring, it is found that apparent cytopathic effect can only be observed microscopically when the Cell Index has dropped to near zero. As the time of onset of the ''CPE plunge'' becomes evident many hours prior to observable cytopathic effect, it is possible that the time to results can be drastically reduced after some formulation of the method.

We compared the current standard method in perfoming a microneutralization assay with one utilizing the real-time cell index measurement to investigate whether this approach is able to offer better performance over the existing one. The current standard neutralization assay is the

microscopic observation of antibody mediated protection from virus cytopathic effect in MDCK cells. This study showed that this may also be achieved by examining the profile generated from the real-time measurements of the cell index. Using real-time cell index monitoring, it is possible to detect inhibitory activity at higher dilutions of the anti-serum than can be detected by the standard microscopic observation of cytopathic effect. Therefore, the real-time cell index monitoring could potentially be developed to be a more sensitive method for measuring anti-viral activity. As drug resistant strains of influenza A viruses [4] including the 2009 pandemic H1N1 are being reported, the real-time cell based monitoring system may also have the potential to be developed for use as a diagnostic platform for drug resistance assays.

This study suggests that real-time cell index monitoring has the potential to substantially reduce human resources in reading results, as well as reducing time-to-result of these assays from 3 days to two. The saving could be substantial for work involving bio-hazard level 3/4 pathogens such as H5N1 viruses as personnel working with these organisms are require to be highly trained and experienced. In addition, the reduction in transferring plates to and from the microscope in reading cytopathic effect will substantially reduce the possibility of accidents from occurring. Furthermore, the system provides objective digital data to an otherwise subjective assay method, which can improve standardization, data exchange, and hence collaboration between different laboratories. With more detailed validation and development, real-time cell index monitoring could transform the way we study and diagnose infection with pathogens such as influenza viruses.

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Evaluation of a new protocol for universal detection of human influenza A and B viruses and simultaneous sub-typing of influenza A(H1N1) 2009 virus by multiplex real-time RT-PCRs

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Abstract

The emergence of a novel H1N1 influenza A virus of swine origin, the pandemic A(H1N1) 2009, with transmissibility from human to human in April 2009 posed pandemic concern and required modifications to laboratory testing protocols. A new protocol for universal detection of influenza A and B viruses and simultaneous subtyping of influenza A (H1N1) 2009 virus, composed of two-one-step RT-PCRs, Fast Set InfA/InfB and Fast Set H1N1v (Relab, Italy), was

evaluated and compared to the reference protocol recommended by WHO. Fast Set InfA/InfB was able to detect influenza A and B viruses circulating between 1995 and 2008 belonging to different subtypes and lineages, and no cross reactions were observed by either Fast Set InfA/InfB or Fast Set H1N1v. The WHO assay was found to have a slightly lower end-point detection limit (10^{-5} dilution) in comparison to the new protocol (10^{-6}). Specificity of the assays was 100% as assessed on a panel of stored clinical samples including Adenovirus, Respiratory Syncytial virus, Metapneumovirus, Parainfluenza virus, *S. pneumoniae*, *N. meningitidis*, *H. influenza*, and human influenza viruses. The new assay panel allows the detection, typing, and subtyping of influenza viruses as requested for diagnostic and surveillance purposes. The high sensitivity of the protocol is coupled with capacity to detect viruses presenting significant heterogeneity by Fast Set InfA/InfB and with high discriminatory ability by Fast Set H1N1v.

Introduction

A rapid and sensitive assay for the detection of influenza virus in clinical samples from subjects with ILI or low respiratory tract infections is a fundamental tool for epidemiological and virological surveillance, management of hospitalized patients, and control of virus nosocomial transmission. The emergence, in April 2009, of a novel H1N1 influenza A virus of swine origin, the pandemic (A(H1N1) 2009), with transmissibility from human to human poses pandemic concern and required modifications to the laboratory testing protocols.[1] Molecular diagnosis of influenza is generally achieved through a two-phase process: a screening phase for the detection of virus, and the subsequent strain characterization performed by either sub-type-specific RT-PCR or entire/partial genome sequencing.[2] During a pandemic, simultaneous implementation of both the detection of influenza A and B influenza viruses and identification of the new subtype is useful for clinical and epidemiological reasons. Here, we describe a new protocol including two-one-step RT-PCRs, Fast Set InfA/InfB and Fast Set H1N1v (Relab, Italy) that allows universal detection of all influenza A viruses and, simultaneously, all subtypes that are influenza A(H1N1) 2009.

Materials and methods

Specificity and clinical sensitivity of the two-one-step RT-PCRs (Fast Set InfA/InfB and Fast Set H1N1v; Relab, Italy) were evaluated by testing 306 selected specimens, including:

- Fifty samples collected from nasopharyngeal swabs representative of influenza viruses, belonging to different subtypes and lineages, and other respiratory viruses and bacteria circulating in Italy between 1995 and 2008.
- Six purified A(H5N1), A(H7N7), and A(H9N2) strains, kindly supplied by Alan Hay, WHO Influenza Centre, London, UK.
- Two hundred-fifty influenza positive samples selected according to type, subtype, clade and viral concentration from >2500 specimens received by the Liguria Influenza Reference Laboratory between January 1st and December 31st, 2009.

Since 1995, nasopharyngeal swabs sampled from patients suspected of having contracted the influenza virus have been collected in viral transport medium, and upon arrival into the laboratory, the samples were divided in ≥3 aliquots. Those not immediately processed were stored frozen at −80°C. Stored samples were used for this evaluation, and all specimens were re-extracted for the study. Samples collected between 1995 and 2008 included specimens positive for:

- Human seasonal influenza A(H1N1) viruses ($N = 14$) including A/New Caledonia/20/99(H1N1) and A/Solomon Islands/3/2006 clusters.
- Human seasonal influenza A(H3N2) viruses ($N = 16$) including A/Sydney/5/97(H3N2)-like, A/Fujian/411/2002(H3N2)-like, A/California/7/2004(H3N2)-like, and A/Wisconsin/67/2005 (H3N2)-like viruses.
- Human influenza B viruses ($N = 7$) belonging to both B/Yamagata and B/Victoria lineages.
- Nasopharyngeal swabs positive for other respiratory viruses and bacteria (adenovirus, A and B respiratory syncytial viruses (RSVA and RSVB), metapneumovirus, parainfluenza virus type 1, 2 and 3, *S. pneumoniae*, *N. meningitidis*, *H. influenzae*) ($N = 13$).

Samples collected during 2009 included 150 specimens resulting positive for influenza A(H1N1) 2009 with high (Ct < 32, $N = 60$) or low (Ct 32–37, $N = 90$) title, 36 specimens resulting positive for influenza A(H3N2) with high (Ct < 32, $N = 34$) or low (Ct 32–37, $N = 2$) title, 8 specimens resulting positive for influenza B (Ct 25–32), and 55 negative samples using Fast Set InfA/InfB and Fast Set H1N1v and typing/subtyping assays.

No seasonal A(H1N1) have been detected since January 1st, 2009. Furthermore, 1 weak positive sample using Fast Set InfA/InfB, but negative at block PCR and typing/subtyping assays was tested. The analytical sensitivity of the test under investigation was determined testing ten-fold serial dilutions of seasonal influenza A(H1N1), seasonal influenza A(H3N2), new pandemic influenza A(H1N1) 2009, and B cell culture-grown viruses. The intra-assay reproducibility was measured by testing the same A(H1N1) 2009 positive sample 15 times in the same experiment, while the inter-assay reproducibility was confirmed by

testing the same samples in 3 independent experiments. To evaluate the performance of the protocol, all samples were tested using a block PCR confirmation test (Seeplex® RV12 ACE Detection),[3] and all specimens collected between January 1st and December 31st, 2009 and dilutions were also assayed using the recommended WHO/CDC protocol of real-time RTPCR for influenza A(H1N1). Typing and sub typing were performed using the WHO protocol and/or sequencing.[4] Viral RNA was extracted from swabs using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. Fast Set InfA/InfB and Fast Set H1N1v are two multiplex one-step Real Time PCR assays developed and evaluated by the Liguria Regional Reference Centre for diagnosis and surveillance of influenza in collaboration with Relab Diagnostics. Both assays contain primers and a dual-labelled hydrolysis probe that targets two regions of the matrix gene (Table 1).

Amplification conditions were as follows: reverse-transcription 50°C for 15 minutes, denaturation 94°C for 2 minutes, then 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds. The entire amplification process extended for 110 minutes. An internal control real-time assay was also incorporated in order to detect PCR inhibition, failed extraction/PCR and technical error. The CDC Realtime RTPCR (rRTPCR) Protocol for Detection and Characterization of Swine Influenza includes a panel of oligonucleotide primers and dual-labelled hydrolysis (Taqman®) probes to be used in real-time RTPCR assays for the *in vitro* qualitative detection and characterization of swine influenza viruses in respiratory specimens and viral cultures. This protocol recommends three primer-and-probe sets: InfA, amplifying a conserved region of the matrix gene from all influenza A viruses; SW InfA, designed to specifically detect the nucleoprotein (NP) gene segment from all swine influenza viruses and SW H1, designed to specifically detect the hemagglutinin gene segment from A(H1N1) 2009.[5] The Seeplex® RV12 ACE Detection for auto-capillary electrophoresis is a multiplex block RT-PCR that applies DPO™ (Dual Priming Oligonucleotide) technology and is designed to detect 12 major respiratory viruses, 11 respiratory RNA (influenza A and B virus, Parainfluenza virus type 1, 2 and 3, respiratory Syncytial virus A and B, rhinovirus A/B, Coronavirus OC43 and 229E/NL63) viruses and 1 DNA (adenovirus) virus, from patients' samples including nasopharyngeal aspirates, nasopharyngeal swabs and bronchoalveolar lavage.[3]

Conventional viral culture was performed inoculating 0·1 ml of each specimen into MDCK-SIAT1 seeded into 24-well plates for influenza isolation. Virus detection was performed by the hemagglutination test using 0·75% guinea pig red blood cells (RBC).

Results

Specificity and clinical sensitivity results of the new protocol are reported in Table 2. Fast Set InfA/InfB was able to detect influenza A and B virus circulating between 1995 and 2008 belonging to different subtypes and lineages, and no cross-reactions were observed by either Fast Set InfA/InfB or Fast Set H1N1v. Among specimens collected between January 1st and December 31st, 2009, all 60 Fast Set InfA/InfB and Fast Set H1N1v high titre positive samples resulted positive using the WHO/CDC assay and showing reactivity using InfA and SW InfA primer-and-probe sets. Among 90 low titre A(H1N1) 2009 positive samples at Fast Set InfA/InfB, 28 (31·1%) were not detected by the WHO/CDC assay, but were positive using Seeplex® RV12. The WHO/CDC SW H1 primer-and-probe set works in 96·7% (58/60) and 1·1% (1/90) of high and low titre A(H1N1) 2009 positive samples, respectively. All A(H3N2) strains collected during 2009 and initially detected by Fast Set InfA/InfB were confirmed after RNA re-extraction by Seeplex® RV12 and WHO/CDC assay showing reactivity using the InfA primer-and-probe set. All

Table 1. Primer and probe sequences used for the new protocol

Virus target	Target gene	Sequence Primer	
Influenza A	Matrix protein	Forward	ACAAGACCAATCCTGTCACCTCT
		Reverse	GCATTTTGGACAAAGCGTCTAC
		Probe	CAGTCCTCGCTCACTGGGCAC
Influenza B	Matrix protein	Forward	ACCAGTGGGACAACCAGAC
		Reverse	GCTCTTTCCGGGGATGG
		Probe	ATCATCAGACCAGCAACCCTTGCC
Influenza H1N1 2009	Matrix protein	Forward	CCAAGGAGGTGTCACTAAGC
		Reverse	TTCACAGCATCGGTCTCAC
			GTGAGACCGATGCTGTGAA
		Probe	GCTGCTTTTGGTCTAGTGTGTGCCACTCAC

Table 2. On-field specificity and sensitivity results

Collection period	Fast Set			WHO/CDC			Seeplex			Typing/subtyping	*N* samples (%)
	A	B	H1 2009	InfA	SW InfA	SW H1	A	B	H1 2009		
1995–2008	Pos	Neg	Neg		N.P.		Pos	Neg	N.P.	A(H1N1)	14 (25)
	Pos	Neg	Neg		N.P.		Pos	Neg	N.P.	A(H3N2)	16 (28·6)
	Pos	Neg	Neg		N.P.		Pos	Neg	N.P.	A(H5N1)	2 (3·6)
	Pos	Neg	Neg		N.P.		Pos	Neg	N.P.	A(H7N7)	2 (3·6)
	Pos	Neg	Neg		N.P.		Pos	Neg	N.P.	A(H9N2)	2 (3·6)
	Neg	Pos	Neg		N.P.		Neg	Pos	N.P.	B	7 (12·5)
	Neg	Neg	Neg		N.P.		Neg	Neg	N.P.	Neg	13 (23·2)
											56
2009	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Neg	Pos	A(H1N1) 2009	58 (23·2)
	Pos	Neg	Pos	Pos	Pos	Neg	Pos	Neg	Pos	A(H1N1) 2009	2 (0·8)
	Pos*	Neg	Pos	Pos	Pos	Pos	Pos	Neg	Pos	A(H1N1) 2009	1 (0·4)
	Pos*	Neg	Pos	Pos	Pos	Neg	Pos	Neg	Pos	A(H1N1) 2009	61 (24·4)
	Pos*	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Pos	A(H1N1) 2009	28 (11·2)
	Pos	Neg	Neg	Pos	Neg	Neg	Pos	Neg	Neg	A(H3N2)	34 (13·6)
	Pos*	Neg	Neg	Pos	Neg	Neg	Pos	Neg	Neg	A(H3N2)	2 (0·8)
	Pos*	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	1 (0·4)
	Neg	Pos	Neg		N.P.		Neg	Pos	Neg	B	8 (3·2)
	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	55 (22)
											250

*Ct > 32 using Fast set InfA/Inf B; N.P., not performed; Pos, positive; Neg, negative.

B viruses initially detected by Fast Set InfA/InfB were confirmed after RNA re-extraction by Seeplex® RV12. One influenza A case identified by the WHO/CDC kit (InfA primer-and-probe set, Ct values: 37·5, SW InfA primer-and-probe set: negative) and new protocol (A primer-and-probe set, Ct values: 34·1, A(H1N1) 2009 primer-and-probe set, Ct values: 34·6) was not detected by either Seeplex® RV12 or by WHO subtyping protocol and/or sequencing, suggesting a very low viral load or unspecific results by real time assays. The analysis of serial dilutions of cell culture-grown A(H1N1) 2009 showed that the detection limit of Fast Set InfA/InfB, Fast Set H1N1v, and Seeplex® RV12 was identical (10^{-6}) and $1\log_{10}$ lower than that using the WHO/CDC protocol (10^{-5}). A similar analysis with respect to A(H1N1) and A(H3N2) strains indicated that Fast Set InfA/InfB sensitivity (10^{-6} and 10^{-7}, respectively) was $1\log_{10}$ lower than that showed by Seeplex® RV12 (10^{-5} and 10^{-6}, respectively). In comparison with the new protocol, the WHO/CDC assays, considering InfA primer-and-probe set, was found to have a slightly lower end-point detection, detecting the 10^{-5} A(H1N1) and A(H3N2) dilution. Also in detecting influenza B virus, Fast Set InfA/InfB sensitivity (10^{-6} and 10^{-7}, respectively) was $1\log_{10}$ lower than that showed by Seeplex® RV12 and the WHO/CDC protocol. Data on intra-assay and inter-assay precision, measured as CV% of Ct showed that the dispersion indices observed had values of less than 3%.

Discussion

Since the identification of influenza A(H1N1) 2009 virus in the U.S. and Mexico, and since sustained human-to-human transmission of the virus was clear, a number of assays were developed for the detection of the new strain.[6–14] One possible criticism is the integration of the new assays in the laboratory algorithm for diagnostic and surveillance purposes. In particular, the rapid and sensitive detection of influenza A and B viruses and subtyping of new A(H1N1) 2009 is critical for the determination of the spread and extent of new virus and to define the epidemiological picture of circulating strains. This article describes the performance of a rapid and sensitive real-time PCR-based protocol including three primer-and-probe sets with the same amplification profile: A) amplifying a conserved region of the matrix (M) gene from all influenza A viruses; B) designed to specifically detect the NP gene segment from lineage Yamagata- and Victoria-lineage B viruses, and A(H1N1) 2009, designed to specifically detect the M gene segment from A(H1N1) 2009. In our experience, the new protocol is more sensitive than the WHO/CDC assays, showing 1–2 $\log_{10}$ lower detection limit in detecting A(H1N1), A(H3N2), A(H1N1) 2009, and B viruses. The higher sensitivity of the new protocol was confirmed by clinical sensitivity data: 31·1% out of low titre A(H1N1)

2009 samples were detected using the new protocol that resulted negative using the WHO/CDC assays. The unfortunately low quantity of low titre A(H3N2) samples collected during 2009 did not allow us to highlight differences between assays Fast Set InfA/InfB, and Fast Set H1N1v positivity was always confirmed by Seeplex® RV12, which demonstrated high sensitivity, showing a detection limit comparable or lower when compared with those observed using the WHO/CDC assays. The high analytical sensitivity of Seeplex® RV12 is reported by Kim [3] who observed a detection limit of 10 copies per reaction for each type/subtype of influenza viruses.

The high sensitivity of the new protocol is coupled with its capacity to detect viruses presenting a significant heterogeneity by Fast Set InfA/InfB and high discriminatory ability by Fast Set H1N1v. Fast Set InfA/InfB was able to identify representative influenza viruses of circulating strains during the last decade belonging to different subtypes, lineages, and clusters, and Fast Set H1N1v primer-and-probe set reacted selectively with A(H1N1) 2009 target. A recent report demonstrated that the SW InfA assay is not specific to A(H1N1) 2009 and is able to detect both human and avian (H5N1) influenza A viruses and so there is the potential for misidentification.[15] High titre (Ct 8·5 and 15·6 at Fast Set InfA/InfB) A(H5N1) viruses did not react with Fast Set H1N1v primer-and-probe set (data not shown). Available human A(H5N1) sequences are similar within the H1N1v primer-and-probe regions, but having 4–5 mismatches in the forward primer and, more notably, two of the mismatches occurred within 9 nucleotides of the 3 end, an important determinant for primer specificity. In conclusion, this protocol can be a powerful tool in the diagnostic laboratory setting for specific simultaneous analysis of several samples in minimal time, showing enhanced sensitivity in detecting influenza viruses, and high discriminatory ability in identifying the new pandemic A(H1N1) 2009.

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A university–corporate partnership to enhance vaccination rates among the elderly: an example of a corporate public health care delivery

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Keywords Mass vaccination, university–corporate partnership, vaccine program.

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Abstract

Public health campaigns usually rely on governmental infrastructure and finance for vaccine implementation programs. However, there are many financial and physical barriers which preclude widespread and effective vaccine administration, especially among the elderly. On an international scale, both government agencies and citizen groups have a vested interest in searching for more resourceful methods of attaining significant immunization levels (>75% of the population). In fact, it seems to have become both a grassroots civic and governmental goal, especially among developing countries.

We implemented the unique strategy of enlisting the assistance of a privately-owned food market chain to address the public health issue of mass vaccination for the elderly. In this context, Publix Pharmacy and the University of South Florida (USF) recently developed both a handbook and a training program to facilitate the administration of vaccinations.

Between 2008 and 2009, the Publix–USF partnership resulted in administration of over thirty thousand influenza A (H3N2) vaccinations, 76% of which were given to adults over 55 years of age. Consequently, vaccine administration costs were decreased by using corporate resources and bypassing overly strained municipal resources.

This unique university–corporate partnership successfully delivered H3N2 vaccine to a vulnerable cross-section of society at a lower cost and with minimal side effects and morbidity. It may be safely projected that university-corporate partnerships could result in an effective method for rendering a vital service to an aging and especially vulnerable segment of the population.

Introduction

Government policy and funding are the foundation of immunization programs on an international scale. For example, in the United States, governmental programs account for over 50% of the monetary outlay used for immunization.[1] Until 2006, the Global Alliance for Vaccines and Immunizations (GAVI) acted as a catalyst for implementing vaccine and immunization programs in each targeted country.[2] Under the auspices of GAVI—collaborations between governments, charitable organizations, and multinational health agencies (such as UNCIEF and the WHO)—many countries have increased their spending for vaccination programs. However, development of financially sustainable immunization programs geared toward reaching the majority of the population are still at a nascent level of evolution.[3] The development of more innovative and cost-effective approaches has become imperative in order to reach a greater number of vaccination candidates.

Administering the influenza vaccine only to the subpopulation of over 65 year olds would save an estimated 220 000 quality-adjusted life years in a cohort of approximately half the world's population.[4] Widespread public vaccination programs are made more complex by the continuing development of newer vaccines, concomitant specialized administration costs, and the logistical challenge of conveying recipients to vaccination points of service.[1,5] In spite of the increasing complexity of mass vaccination, cost-benefit analyses clearly favor annual influenza vaccination in the elderly population on an international scale.[4,6]

Recently, in 2008, influenza vaccine administration was reported to reach between 32% and 82% of the elderly population, which denotes varying degrees of success within each particular country.[7,8] However, there was also

a report of a uniform plateau effect at around 75% of the population, beyond which additional vaccination coverage was difficult to achieve.[9] Physical limitations to vaccination seem to be more insurmountable for the elderly. Unfortunately, this is the population segment which could experience the most significant vaccination-associated mortality reduction.[10]

Methods

We employed the unique strategy of involving the resources of Publix supermarkets, a corporate food market chain, to address the public health issue of widespread vaccination for the elderly. We took advantage of recent 2005 changes in the Florida statutes, which expanded the scope of pharmacists' practice to include administration of vaccines. Subsequently, Publix Pharmacy and the University of South Florida (USF) developed a handbook and training program to facilitate and enhance vaccine administration by Publix pharmacists.

By using proprietary pharmacists and more practical supply storage, we were able to decrease the costs of vaccine administration. The consumer was charged $10 for administration costs plus the cost of the injection itself, regardless of insurance or eligibility for governmental subsidy.

Although patients were initially self-selected, they were ultimately excluded if they had demonstrated prior adverse effects to influenza vaccinations or to any of the components of such vaccinations.

Results

Between 2008 and 2009, the Publix–USF partnership vaccinated 30 063 people against influenza A (H3N2), of which 29 404 were Florida residents. The age range was 1–105 years old with a median age of 65 years old. Seventy-six percent of the participants were over 50 years old (see Figure 1).

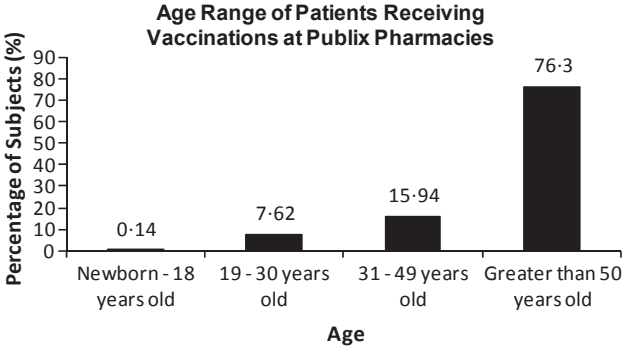

Figure 1. Percent of vaccinated patients in each age range. Three-quarters of the population was >50 years old.

Within the population surveyed, the reported side effects of the vaccine in this study were not serious, but included: vertigo, cold sweats, chills, vomiting, syncope, rash, nausea, stomach pain, elevated blood pressure, injection site reaction, inflamed bursa, and bilateral thigh discomfort.

Participants from all socioeconomic classes were vaccinated. An income-by-zip code analysis revealed 44% of those vaccinated resided in zip code areas where the average household income was <$50 000 per year. Of those remaining, 25% had an average income of $50 000–$70 000 per year, and 31% had an income of >$70 000 per year.

Each person vaccinated was charged ten dollars for administration costs. This represents a decrease in the administration costs ranging from one dollar to ten dollars saved per vaccine.[11,12]

Conclusion

This unique university–corporate partnership successfully delivered H3N2 vaccine to a high-risk population with decreased vaccine administration costs. The influenza vaccine is well-tolerated, with minimal side effects when patients who have a history of adverse reactions are excluded. We can postulate that university-corporate partnerships may indeed be effective at reaching the aging population which is a challenge in most communities.

This delivery model may prove to be another tool for improving the efficiency of mass immunization by facilitating accessibility, which results in wider coverage. This model also enhances delivery of healthcare by decreasing costs of immunization regardless of whether the payer is a government, insurance company, or self-pay consumer. The GAVI initiative stressed three goals for accomplishing sustainability and independence in immunization programs.[3] The goals were to: (i) mobilize additional resources from governmental and non-governmental sources; (ii) improve program efficiency to minimize additional administration resources needed; and (iii) increase the reliability of funding.

Empowering privately owned corporations within the community, such as food markets or pharmacies, to administer vaccines mobilizes additional resources to readily achieve the first goal of GAVI. Mobilizing resources of non-healthcare, corporate vaccination locations enhances accessibility due to travel convenience. In our study, participants came from all socioeconomic classes, suggesting that ease of access is independently hindering mass vaccination, and that people of all incomes are more likely engaged when access issues are eliminated.

The second and third goals were also accomplished by recruiting a corporation's resources for vaccine administration (refrigeration, storage, and employees). This minimizes the money spent from vaccine program funds to support

the infrastructure of immunizations, thus improving financial efficiency and sustainability. Financial efficiency implies that money is spent to safely reach as large a portion of the population as possible. By using corporate storage facilities instead of paying for independent facilities, money can be spent elsewhere. More vaccines can be purchased and more money can be spent on media communications to encourage vaccination. Sustainability requires the ability to fund annual vaccination programs which reach 75% of the population or greater.

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Rapid detection of pandemic influenza in the presence of seasonal influenza

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Keywords Case ratio, cusum, false alarm, pandemic detection, syndromic surveillance.

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Background

Key to the control of pandemic influenza are surveillance systems that raise alarms rapidly and sensitively. In addition, they must minimise false alarms during a normal influenza season. We develop a method that uses historical syndromic influenza data from the existing surveillance system 'SERVIS'[1] monitoring seasonal ILI activities in Scotland.

Materials and methods

We develop an algorithm based on WCR of reported ILI cases to generate an alarm for pandemic influenza. WCR is defined as the ratio of the number of reported cases in a week to the number of cases reported in the previous week. From the seasonal influenza data from 13 Scottish health boards, we estimate the joint probability distribution (Figure 1) of the country-level WCR and the number of health boards (denoted by N_{HB}) showing synchronous increases in reported influenza cases over the previous week. Pandemic cases are sampled with various case reporting rates from simulated pandemic influenza infections (using an individual-based stochastic model of the spread of pandemic cases in Great Britain[2]) and overlaid with seasonal SERVIS data from six influenza seasons 2001–2007. A typical influenza season in a temperate country like Scotland is of 33 weeks. Although pandemic influenza can start any time of the year, in order to overlay simulated pandemic cases with seasonal ILI cases, we consider only these 33 weeks as pandemic starting weeks. Using these combined

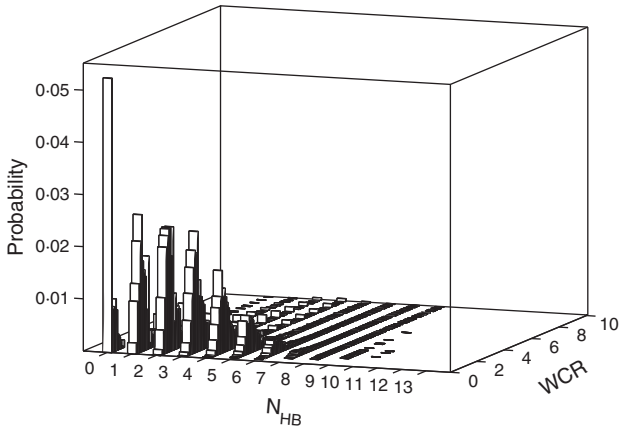

Figure 1. The joint probability distribution of (*WCR*, N_{HB}). The joint probability distribution of WCR and N_{HB} reporting increases in the ILI cases over the previous week. This probability diagram was constructed using SERVIS data from six seasons, 2001–2002 to 2006–2007. WCR is binned with bin size 0.1.

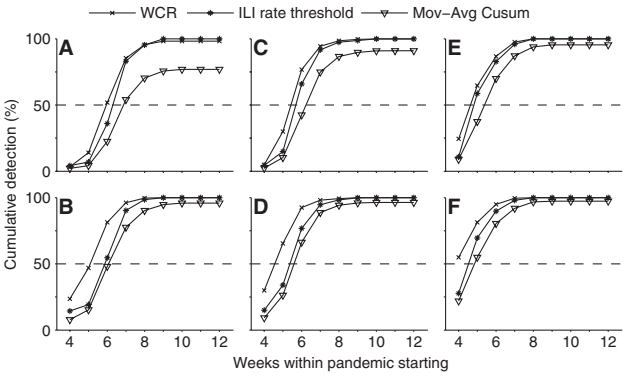

Figure 2. Speed of detection in the early weeks of pandemics. Cumulative probability of detection as a function of times taken in pandemic detection (i.e., weeks within pandemic starting). These plots are for different specificity and pandemic case reporting rate: (A) 99% and 0.5%; (B) 95% and 0.5%; (C) 99% and 1%; (D) 95% and 1%; (E) 99% and 5%; and (F) 95% and 5%. The dashed line represents the 50%-level of pandemic detections.

time series we test our method, as well as the ILI rate threshold method (similar to the UK HPA's ILI baseline activity rate) and the Mov-Avg Cusum method,[3,4] for speed of detection and sensitivity, at pre-set specificity values of 95% and 99%. A total of 59 400 (300 simulated pandemics × 6 influenza seasons × 33 weeks) combined time series were used for the results presented in this paper. Also, the 2008–2009 SERVIS ILI cases were used for testing detection performances of the three methods with a real pandemic data.

Results

We compare our method, based on our simulation study, to the Mov-Avg Cusum and ILI rate threshold methods and find it to be more sensitive and rapid. The WCR method detects pandemics in larger fraction of total runs within the same early weeks of pandemic starting than does any of the other two methods (Figure 2).

As shown in the table, for 1% pandemic case reporting rate and detection specificity of 95%, our method is 100% sensitive and has MDT of 4 weeks, while the Mov-Avg Cusum and ILI rate threshold methods are, respectively, 97% and 100% sensitive with MDT of 5 weeks. At 99% specificity, our method remains 100% sensitive with MDT of 5 weeks. Although the threshold method maintains its sensitivity of 100% with MDT of 5 weeks, sensitivity of Mov-Avg Cusum declines to 92% with increased MDT of 6 weeks. For a two-fold decrease in the case reporting rate (0·5%) and 99% specificity, the WCR and threshold methods, respectively, have MDT of 5 and 6 weeks with both having sensitivity close to 100%, while the Mov-Avg Cusum method can only manage sensitivity of 77% with MDT of 6 weeks.

Retrospective detection of the H1N1 pandemic

The first cases of the 2009 pandemic were reported in Scotland in the 29th week of the season. The WCR algorithm as well as the Mov-Avg Cusum method detects the pandemic 12 weeks later in week 41. The ILI threshold method detects it 1 week later in week 42. Both the WCR and Mov-Avg Cusum methods therefore outperform the ILI threshold method by 1 week in the retrospective detection of the 2009 pandemic in Scotland.

Conclusions

While computationally and statistically very simple to implement, the WCR method is capable of raising alarms rapidly and sensitively for influenza pandemics against a background of seasonal influenza. Although the algorithm has been developed using the SERVIS data, it has the capacity to be used at large scale and for different disease systems where buying some early extra time is critical.

More generally, we suggest that a combination of different statistical methods should be employed in generating alarms for infectious disease outbreaks. Different detection methods would provide cross-checks on one another, boosting confidence in the outputs of the surveillance system as a whole.

Disclosure

Data presented in this manuscript were also published in BMC Public Health (2010), Brajendra K. Singh, Nicholas J. Savill, Neil M. Ferguson, Chris Robertson and Mark E. J. Woolhouse Title, Rapid detection of pandemic influenza in the presence of seasonal influenza. MS ID: 142981135 8419843.

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The first wave of influenza A(H1N1)2009 pandemics in Mongolia

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Keywords Epidemiology, influenza A(H1N1)pdm virus, influenza and influenza-like illness, influenza sentinel surveillance system, influenza virus gene segments, Mongolia, pandemic phases.

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Introduction

Real-time evidence being created worldwide will greatly contribute to the full understanding of influenza pandemics. Here we report the real-time epidemiology and virology findings of the influenza A(H1N1)2009 pandemics in Mongolia.

Materials and methods

The epidemiological and virological data collected through ISSS of NIC, NCCD, Mongolia (real-time information on registered ILI cases and virological laboratory results are available from the weekly updates in the NIC, Mongolia website: http://www.flu.mn/eng/index.php?option=com_content&task=category&sectionid=5&id=36&Itemid=51) were used for analysis in relation to the previous seasonal influenza activities in the country.

Influenza viruses were detected in naso-pharyngeal samples from ILI patients by rt-RT-PCR with Applied Biosystems Fast Real Time PCR System 7500, using primers and instructions supplied by CDC, USA.[1] Influenza viruses were isolated by inoculation of rt-RT-PCR-positive samples of MDCK cell culture according to the standard protocol.[2] Ten representative strains of A(H1N1)pdm viruses were selected for sequencing of different gene segments, namely: A/Ulaanbaatar/5882/2009, A/Ulaanbaatar/6133/2009, A/Ulaanbaatar/6266/2009, A/Ulaanbaatar/6525/2009, A/Zavkhan/8299/2009, A/Umnugovi/9586/2009, A/Dundgovi/9746/2009, A/Ulgii/9911/2009, A/Bayanulgii/9912/2009, and A/Dundgovi/381/2010. Sequencing of influenza virus gene segments was performed in Applied Biosystems 3130xl Genetic Analyzer using primers and instructions supplied by CDC, USA,[3] and bioinformatic analysis was performed with ABI/SeqScape v.2.5 and mega4 programs.

Results

The pandemic alert in Mongolia was announced by the Government on April 28, 2009, just after the WHO announcement of the pandemic alert phase, and planned containment measures were intensified. Despite intensive surveillance, no A(H1N1)pdm virus was detected in Mongolia until the beginning of October 2009. Around 40 suspected cases, mostly arriving from the A(H1N1)pdm epidemic countries, tested zero by rt-RT-PCR for A(H1N1)pdm virus. The first A(H1N1)pdm case detected by the routine surveillance system in Ulaanbaatar City, the Capital of Mongolia, was confirmed by rt-RT-PCR on October 12, 2009 (41st week of 2009). The reported ILI cases escalated rapidly, reached the peak in the 43–44th week of 2009, and gradually decreased thereafter (Figure 1).

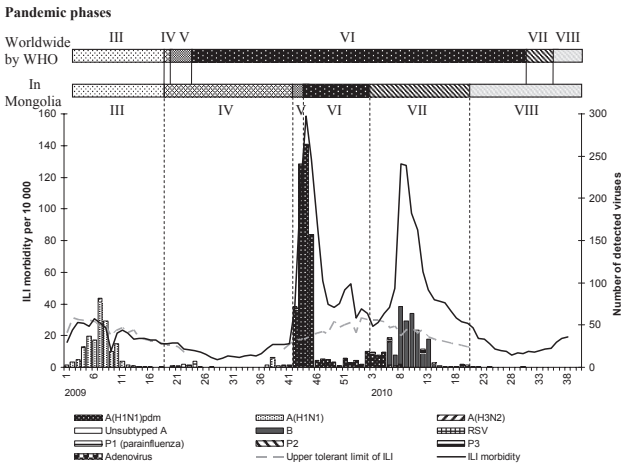

Figure 1. Dynamics of the registered ILI cases and viruses detected in Mongolia in 2009/2010 season.

Influenza viruses detected in October, November and December 2009 were 100% A(H1N1)pdm strains (Table 1), confirming the 1st wave of ILI epidemics in 2009/2010 season was caused by the pandemic virus. The registered ILI cases dropped under the upper tolerant level in the 3rd week of 2010. However, the registered ILI cases increased again from the 5th week of 2010, and peaked at the 8–9th weeks of 2010. The viruses isolated during this 2nd peak were influenza B strains (Figure 1 and Table 1).

For the genetic characterization of the Mongolian pandemic isolates, 3 gene segments I (PB2), 2 gene segments II (PB1), 3 gene segments III (PA), 9 gene segments IV (HA), 3 gene segments V (NP), 4 gene segments VI (NA), 7 gene segments VII (M), and 3 gene segments VIII (NS) of the representative A(H1N1)pdm Mongolian strains were sequenced, and all sequences have been deposited in the GenBank (accession numbers: CY050844, CY0533648, CY050846, CY050845, CY065987, CY065989, CY065990, CY 065988, CY065991, CY065985, CY065984, CY065986, CY052366, CY053365, CY054546, CY054547, CY055169, CY 055170, CY055171, CY056363, CY055308, CY057191, CY057083, CY065995, CY065997, CY065998, CY065996, CY 065999, CY065993, CY065992, CY065994, CY054548, CY054549, CY073448). All 8 genes of Mongolian strains were possessing 99·4–99·9% similarity with the GenBank deposited gene sequences of the original pandemic strain A/California/072009(H1N1).

Discussion

The WHO declared the pandemic alert phase (phase IV) on April 27, 2009,[4] and was prompted to announce the pandemic phase (phase V) two days later.[5] After 43 days, the WHO declared the beginning of the pandemic peak period (phase VI) on June 11, 2009.[6]

However, in Mongolia, the pandemic alert period continued for 168 days. Mongolia was free of the pandemic virus during the whole first wave of the pandemics in the Northern Hemisphere.

With the confirmation of the 1st influenza A(H1N1)pdm case on October 12, 2009 in Ulaanbaatar, Mongolia entered into the pandemic phase (phase V), and after just 2 weeks, the registered ILI cases peaked, confirming Mongolia shifted into the pandemic peak period (phase VI), which

Table 1. Dynamics of the detected and isolated influenza viruses in Mongolia in 2009/2010 season

		rt-RT-PCR			Inoculation of MDCK cell culture			
			Among them				Among them	
Months	Tested samples	Positive (%)	A(H1N1) pdm	B	Tested samples (rt-RT-PCR)	Positive (%)	A(H1N1) pdm	B
2009.X	2805	1154 (41·1%)	1154 (100%)	0 (0%)	339 (29·3%)	86 (25·3%)	86 (100%)	0 (0%)
2009.XI	1449	484 (33·4%)	484 (100%)	0 (0%)	257 (53%)	148 (57·5%)	148 (100%)	0 (0%)
2009.XII	800	86 (10·7%)	85 (98·8%)	0 (0%)	63 (73·2%)	55 (87·3%)	55 (100%)	0 (0%)
2010.I	534	80 (14·9%)	79 (98·8%)	1 (1·2%)	36 (45%)	28 (77·7%)	28 (100%)	0 (0%)
2010.II	684	201 (29·3%)	45 (22·4%)	156 (77·6%)	148 (73·6%)	83 (56%)	3 (3·6%)	80 (96·4%)
2010.III	690	239 (34·6%)	0 (0%)	239 (100·4%)	224 (93·7%)	158 (70·5%)	0 (0%)	158 (100%)
2010.IV	336	10 (2·9%)	0 (0%)	10 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2010.V	221	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2010.VI	176	1 (0·5%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
2010.VII	67	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2010.VIII	95	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Нийт	7857	2255 (28·7%)	1847 (81·9%)	408 (18·1%)	1068 (47·4%)	558 (52·2%)	320 (57·3%)	238 (42·7%)

coincided with the 2nd wave of pandemics in many countries of the Northern Hemisphere (see, picture 1 and Table 1). Despite the relatively milder clinical manifestations, the disease burden for the health service was enormous, while the morbidity per 10 000 population at the peak period was 5–6 times higher above the upper tolerant limit, and 3–4 times higher above the seasonal influenza outbreaks. In contrast to the seasonal influenza outbreaks where over 80% of the registered ILI cases have been in the age group under 15,[2] it has been observed that over 50% of the registered cases in this pandemic peak period were in the age group of 16–60.

On January 18, 2010, we regarded the pandemic had entered into the post-peak period (phase VII) when the registered ILI cases became lower than the upper tolerant limit, during which time Mongolia experienced an influenza B outbreak.

On May 24, 2010 we determined that Mongolia entered the post-pandemic period (phase VIII) as the influenza virus isolations were almost stopped, and after no pandemic virus detected for 3 months. WHO announced pandemic VII and VIII phases much later.[7,8]

This first ever real-time laboratory confirmed influenza pandemics in Mongolia and confirmed some variations of pandemic spread in different parts of the World. The comparison of deduced amino-acid sequence changes have shown that the Mongolian strains belong to the clade 7, according to the classification of A(H1N1)pdm influenza strains suggested by M. Nelson,[9] which has circulated worldwide since July 2009. This is also evidence that the 1st wave of the pandemics did not hit Mongolia.

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Development and implementation of the WHO public health research agenda for influenza

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Abstract

WHO initiated the development and implementation of a *WHO Public Health Research Agenda for Influenza* to facilitate research to respond to public health needs related to influenza. The Agenda is built around five major research ''streams'': (i) reducing the risk of emergence of pandemic influenza; (ii) limiting the spread of pandemic, zoonotic, and seasonal epidemic influenza; (iii) minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza; (iv) optimizing the treatment of patients; and (v) promoting the development and application of modern public health tools. Implementation of the identified research topics is expected to underpin public health decision-making that can save lives and mitigate potential medical, economical, and social disruption due to influenza.

Introduction

The *WHO Public Health Research Agenda for Influenza*[1] is aimed to support the development of evidence needed to strengthen public health guidance and actions essential for limiting the impact of influenza on individuals and populations.

Materials and methods

A review of WHO technical publications and other publications outlining influenza research from other global public health and scientific organizations was conducted. Input from technical experts on critical knowledge gaps in influenza was sought. Proposed research topics were classified into five research streams:

- Stream 1: Reducing the risk of emergence of pandemic influenza
- Stream 2: Limiting the spread of pandemic, zoonotic, and seasonal epidemic influenza
- Stream 3: Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza
- Stream 4: Optimizing the treatment of patients
- Stream 5: Promoting the development and application of modern public health tools

An interim document was developed and served as the basis for the final document *WHO Public Health Research Agenda for Influenza* through a global consultation held in November 2009 at Geneva and to finalize the agenda and facilitate its implementation.

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Results

Each stream-specific group reviewed and discussed the proposed organization, content, rationale, and global health importance of their designated research stream. Specific research recommendations were made for topics within each stream:

Stream 1: Reducing the risk of emergence of pandemic influenza

1.1 Factors associated with the emergence of influenza viruses with zoonotic or pandemic potential
- **1.1.1** Investigate virus-specific factors associated with zoonotic and pandemic potential
- **1.1.2** Assess the animal host-specific factors associated with zoonotic and pandemic potential
- **1.1.3** Study the environmental and animal husbandry-specific factors associated with zoonotic and pandemic potential

1.2 Factors associated with human infection at the human-animal interface
- **1.2.1** Investigate modes of transmission in human infection with animal viruses
- **1.2.2** Study human behavioral factors associated with infection by animal viruses
- **1.2.3** Determine the genetic relationship to human susceptibility to animal viruses

1.3 Surveillance at the human-animal interface
- **1.3.1** Develop joint animal/human surveillance
- **1.3.2** Develop diagnostic tests for joint animal and human surveillance
- **1.3.3** Perform operational research to evaluate joint animal and human surveillance
- **1.3.4** Research on social, political, economic, and legal strategies for animal influenza outbreak reporting

1.4 Preventive measures to reduce the risk of emergence of zoonotic and pandemic influenza viruses
- **1.4.1** Investigate animal intervention strategies
- **1.4.2** Develop human intervention strategies related to the animal–human interface
- **1.4.3** Conduct operational research to integrate animal and human health strategies for prevention
- **1.4.4** Evaluate the public health, social, and other impacts of intervention strategies under different epidemiological conditions

Stream 2: Limiting the spread of pandemic, zoonotic, and seasonal epidemic influenza

2.1 Factors affecting person-to-person transmission
- **2.1.1** Investigate the importance of droplet, contact, and airborne transmission in influenza
- **2.1.2** Study the transmission and infectivity of influenza in different settings and activities
- **2.1.3** Investigate transmission potentials of influenza during infection in humans
- **2.1.4** Examine host factors such as age, pre-existing immunity, and vaccination in modulating influenza transmission
- **2.1.5** Study the stability of influenza on different environmental surfaces and under varying conditions in virus transmission

2.2 Dynamics of virus spread at global and local levels
- **2.2.1** Studies the seasonality of influenza virus infection and its implication in global spread
- **2.2.2** Assess the spread of epidemic and pandemic influenza in different epidemiological settings
- **2.2.3** Study the interaction between influenza strains and other respiratory pathogens, and their effect on transmission and spread
- **2.2.4** Study the response strategies during early spread of human cases of pandemic influenza virus including containment and border control policies

2.3 Public health measures to limit transmission
- **2.3.1** Study the effectiveness, cost-effectiveness, and feasibility of measures such as hand hygiene, masks, and respirators
- **2.3.2** Study the effectiveness, cost-effectiveness, and feasibility of measures at the environmental and community level
- **2.3.3** Research on the selection, timing, and implementation of public health measures
- **2.3.4** Usage of surveillance data in public health interventions in different situations and decision-making regarding cessation of public health interventions

Stream 3: Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza

3.1 Determining disease burden and social impact
- **3.1.1** Conduct disease burden studies and its impact
- **3.1.2** Determine the methods for surveillance and burden studies
- **3.1.3** Evaluate the influenza vaccine preventable disease burden and impact
- **3.1.4** Establish the economic burden of seasonal and pandemic influenza
- **3.1.5** Determine best approaches to inform influenza control programs in competing health priorities
- **3.1.6** Assess social determinants of health under different epidemiological settings and social impact

3.2 Improve immunogenicity, availability, and delivery of influenza vaccines

3.2.1 Improve vaccine strain selection process
3.2.2 Enhance applications of existing vaccines (immunogenicity, safety, dose-sparing, etc.) especially for high-risk groups
3.2.3 Evaluate procedures for rapid response, surge capacity, rapid deployment, and tracking of influenza vaccine usage
3.2.4 Research on animal models for evaluation of vaccines
3.2.5 Develop new vaccine for enhanced safety and immunogenicity, particularly in under-resourced settings
3.2.6 Identify and develop methodologies for correlates of protection and priming for different vaccines
3.2.7 Develop innovative trial methodologies for effectiveness and safety of novel vaccines for pre-licensure and post-licensure studies
3.2.8 Expand studies on pharmacovigilance and reduction of disease burden for post-licensure vaccine evaluation
3.2.9 Examine and develop ways to harmonize the regulatory processes for rapid safety monitoring and standardized evaluation of potency

3.3 Public health policies to reduce the impact of disease
3.3.1 Evaluate strategies to optimize vaccine uptake and acceptability
3.3.2 Develop immunization policies using community-based input
3.3.3 Study social, ethical, and legal standards in public health policy application, particularly in under-resourced populations

Stream 4: Optimizing the treatment of patients

4.1 Factors associated with pathogenesis and clinical severity
4.1.1 Investigate virological factors, innate/adaptive immune, and other host responses in the severity of disease
4.1.2 Define the clinical spectrum of human disease, including risk factors and prognostic markers for severe disease
4.1.3 Assess the incidence, etiology, and pathogenesis of bacterial infections associated with influenza, as well as optimal treatment, prophylactic, and preventive measures
4.1.4 Study the role of pre-existing and co-infections in the severity of influenza disease
4.1.5 Study the role of host genetic factors on susceptibility and severity of influenza infection

4.2 Improve clinical management of patients
4.2.1 Develop reliable and affordable point-of-care diagnostics for influenza
4.2.2 Identify markers and develop point-of-care tools for the management of influenza disease
4.2.3 Optimize antiviral treatments through development of new formulations, delivery routes or systems, and antiviral drug combinations
4.2.4 Develop treatment including adjunctive treatments for low resource settings and ease of use in pediatric and intensive care settings
4.2.5 Optimize management of increased risk groups for severe disease and complications including intensive care practices

4.3 Health care capacity and response
4.3.1 Evaluate the effectiveness of responses to pandemic, epidemic, and zoonotic influenza, and development of new assessment tools
4.3.2 Conduct studies on surge capacity needs, including development of triage schemes in different resource settings, and surge planning to maintain adequate staffing
4.3.3 Research to develop alternative health delivery systems including home care, community facilities other than hospitals, and others
4.3.4 Develop best practices for the protection of health care workers
4.3.5 Identify clinical care pathways and principles which optimize health care delivery in different resource settings
4.3.6 Develop rapid assessment methods and new interventions during health emergencies, including systems for clinical data collation, sharing, and assessment in real time

Stream 5: Promoting the development and application of modern public health tools

5.1 Modern tools for early detection and monitoring of disease
5.1.1 Identify, appraise, and adapt technologies for early detection and application in surveillance at the human-animal interface
5.1.2 Develop, integrate, and continuously evaluate innovative approaches for influenza surveillance and monitoring with other existing disease monitoring systems
5.1.3 Study on efficient mechanisms on sharing data, clinical specimens, and viruses with consideration for local, ethical, legal, and research perspectives
5.1.4 Examine the timeliness and quality of data needs for early detection from local to district, regional, national, and global levels

5.2 Role of modeling in public health decision making

- **5.2.1** Studies to assess the application of modeling to understand epidemiological and evolutionary processes and estimate key parameters for pandemic and seasonal influenza
- **5.2.2** Examine the application of modeling to assess public health impact of influenza and the effectiveness of interventions
- **5.2.3** Evaluate the application of modeling to assist public health policy planning and strategic decision making
- **5.2.4** Conduct studies to improve model accuracy and realism, and incorporation of emergent interdisciplinary advances

5.3 Modern tools for strategic communication

- **5.3.1** Review evidence on crisis communication from behavioral and social sciences to support communication
- **5.3.2** Develop methods that can rapidly assess knowledge, attitudes, beliefs, and practices to guide communication efforts
- **5.3.3** Develop communication tools and approaches in different cultural settings to promote appropriate risk reduction measures
- **5.3.4** Study the dynamics of rumors, myths, etc. through monitoring and analyzing communication and to develop effective ways to respond
- **5.3.5** Study the ethical, social, economic, and political dimensions of communicating in crisis and develop strategies for working within constraints and maximizing opportunities

Conclusions

Implementation of the identified research priorities is expected to underpin public health decision-making that will help to save lives, reduce health costs and economic loss, and mitigate potential social disruption.

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Surveillance and monitoring of pandemic flu in a resource limited environment: a case of Djibouti and a WHO-Copanflu International Study preliminary report

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Keywords Copanflu, Djibouti, multicentre study, pandemic H1N1, resource limited environment, surveillance.

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Abstract

Background: A syndromic surveillance system using non-classical data sources for detection and monitoring evolution of flu and flu-like illness (ILI) in Djibouti is reported here as part of the preliminary report of Djibouti WHO-Copanflu International Study (WCIS)**. Methodology: Clinical reports, over-the-counter drug sales, lab diagnosis report, and health communication trends were obtained for an integrated statistical analysis. Results: transition to winter is concomitant with upsurge of ILI cases and ILI drug sales. In addition, more rural folks manage ILI infections on self medicament than through clinical consultancy. Inefficient and vague data collections were observed. A successful implementation of WCIS will create a platform upon which challenges faced in Djibouti Health Department in routine surveillance will be addressed to achieve a near-real time surveillance of flu pandemic. Conclusion: Innovations, prompt reporting, and instituting open source syndromic surveillance system software's in resource limited environment like Djibouti will enhance early detection and evolution monitoring of pandemic flu.

**WHO-CoPanFlu International Consortium Study is a Multicenter households cohort study on 2009 A(H1N1) virus by French EHESP and partners in France, Bolivia, Djibouti, Laos, and Mali. Aimed at between countries comparisons of the flu pandemic burden, epidemiology, socio-economic impact, immunology and viral risk determinant factors to infection of an individual at household level.

Introduction

The Spanish flu in 1918/1919 infected and killed millions of people, and threatened to wipe humanity off the face of planet. However, the recent scenarios of influenza H1N1 (2009) pandemics' worldwide occurrence fell short of most scientific prediction on its magnitude and intensity. This dampened their confidence; they cannot state precisely as to when, how, where, and which of the Spanish flu-like pandemic will occur in the future. In support of scientific community and governments, the WHO hasn't gone to slumber, but is reminding its member states to up their post pandemic surveillance and monitoring of influenza virus in circulation for advance preparedness in case of an outbreak.[1] Despite all uncertainty around the pandemic flu H1N1 2009, there remains a common knowledge and understanding that this flu has shown a great potential to evolve and cause huge morbidity and mortality. Although its future magnitude may be unpredictable, its recurring events have severe consequences on human health and the economic well being of everyone.[1] And therefore, advance planning and preparedness is critical in protecting any population in the future, especially those located in resource limited environment without universal health cover and generous disaster emergency funds.[2] In respond to this call, National Institute of Public Health NIPH (Ministry of Health) Djibouti in collaboration with the EHESP French School of Public Health Paris France has launched a 350 household longitudinal prospective cohort study in Djibouti, as part of the WHO-Copanflu International Study Consortium. Of which it intends to unravel the determinants of risks to an individual infection to H1N1 virus at household level. Concomitantly, the strength, weakness, opportunities, and threats of existing surveillance system are evaluated. The systems' potential to predict a near-real-time syndromic outbreak for ILI (and other illnesses) will be done and what can be improved or done away with based on local cohort study shall be adjusted.

Materials and methods

Study Area: Djibouti Nation is located at the horn of Africa, bordering Red Sea, Somali, Ethiopia, and Eritrea. Health care systems relay on subsidies from pocket payment for medical services and non subsidized fee for private health facilities services. Population: Estimated population of Djibouti is >818 159, distributed among the six administrative regions of Djibouti City (475 322), Ali Sabieh (86 949), Dikhil (88 948), Tadjourah (86 704), Obock (37 856), and Arta (42 380), the majority of whom dwell in urban centers (70·6%), and the rest adopts ether nomadic (19·7%) or rural sedentary lifestyle (9·7%).[3] Data Collection: Weekly documentation from NIPH Health Information Department was obtained on: Clinical Services, Pharmaco-Surveillance Services, Laboratory Diagnostics services, Vaccination Program and Health Promotion and Hygiene Services. They were from 19 Health facilities in Djibouti city; 15 governments (Balbala1, Balbala2, Hayableh, PK12, Doraleh, Einguella, Ambouli, Arhiba, Ibrahim Balala, Farahad, Khor Bourhan and Djibouti ville- including General Peltier), and three privates (FNP, FAD and Gendarmerie). Five other regional centers, namely Dikhil, Ali Sabieh, Arta, Obock, and Tadjoura data were used, too.[4] Statistics: All cases of influenza and influenza like (ILI) cases, including pneumonia, from each centre, reported since August 2006–August 2010, were obtained in Excel 2007 (Window 2007 software). Two collapsed sets of a weekly and monthly mean data (of four years period) were clustered in five categories of ILI cases, drug sales, lab results, vaccine consumption, and health promotion. This was followed by a descriptive statistics analysis of cumulative weekly and monthly data to establish presence or absence of trend. Time series analysis was not done due to data limitation. Copanflu Program: As at the time of going to press, the cohort study is at the household recruitment and inclusion Phase and the study covers the Djibouti City. It is in our intention to use the cohort study findings to validate or improve the NIPH Ministry of Health Djibouti ILI surveillance effort for better preparedness.

Results

Clinical Service: 83% of all health facilities are in Djibouti city. Of the 39·9% (326 445) of the population that seeks medical care on influenza and influenza like illness each year, 58·2% (187 586) and 11·9% (38 389) of them are attended to at the city's public and private clinics, respectively (Figure 1). The rest are attended from the Regional Health Centers. The majority of ILI incidence sharply rise with the onset of the winter season (October to April), affecting mostly the middle age group (1–24 years).

Pharmaco-Surveillance: 2% (99 350) of total prescriptions were antipyretic and antiflu drugs, 91·4% (90 765) of which were consumed by peripheral regions, the non Dji-

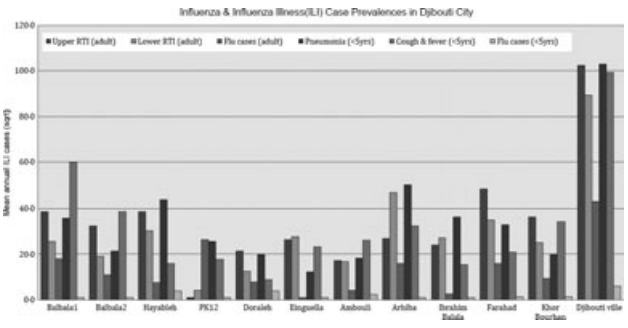

Figure 1. Influenza and LIL case prevalence in Djibouti City.

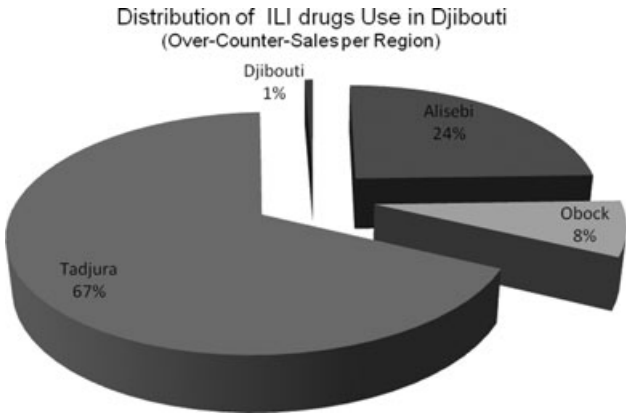

Figure 2. Distribution of ILI drugs used in Djibouti (over-counter-sales per region).

bouti city dwellers (Figure 2). Most ILI drug uptakes in the region were done during the cold seasons of the year. However, the documentation of antivirals use (Tamiflu and acylivir) was lacking in all drug stores of Djibouti.

Lab Diagnostics: The annual ILI lab diagnosis was negligible 0·0072% (250), which can be attributed to less equipped virology laboratories to warrant routine service utility. Documented cases were from previous bouts of avian influenza that had a human incidence from 2006 and 2009.[5] With support of Egypt-based Naval Army Medical Research Unit three (NAMRU 3), clinicians were motivated to sample all ILI patients and submit to collaborating international reference influenza Lab in Cairo, Egypt.

Vaccination: Influenza vaccinations were undocumented, but at least 0·4% (3122) of population sought the service (for yellow fever and meningitis) as mandatory travel advisory or as childhood immunization need. At the time of going to the press, there were at least 80 000 vaccine doses of H1N1 (2009) virus donations yet to be administered.

Health Promotion and Hygiene: Print and audiovisual risk communication remained favorite means of reaching out to urban dwellers (70·6%). While to the rural and nomadic population, person to person communications was the preferable means. To increasing public awareness that will encourage reporting of ILI cases and entrench risk aversion health behavior that limits flu spread, WHO-Co-panflu International Study Djibouti has incorporated basic training on ILI infection and personal hygiene by interviewers during household inclusion.

Discussion

Improving national epidemic surveillance capacity and response under new International Health Regulation[6] is important for any nation, including Djibouti. Our finding indicates the winter season predisposes one to ILI infections; they therefore opt for medical services or self medication depending on their capability and/or understanding. In Djibouti, almost no city dwellers favors self medication over clinical consultation, suggesting the presence of inhibitory factors like distance from the health centers and the cost of accessing consultancy. Common in the absence of universal primary health care setting, it therefore calls for active innovativeness in outbreak detection, disease reporting, and preventive medicine on the part of health authority so as to achieve good population health.[7] In respond to these, NIPH has turned resource limitation to a motivation instead and is working towards institutionalizing a near-real-time syndromic surveillance system as a core functional unit. It capitalizes on three major aspects within its reach: prompt accurate data generation for analysis, EHESP WCIC-study input, and Information Technology use.

Prompt accurate data generation for analysis: Data used in our analysis suffered from un-timeliness (weekly instead of daily basis), incompleteness (vague over-counter drug sales records), entry errors (incidence case reports), and poor collection format (most of data collection forms). Use of satellite handset phones for regional health centers and mobile phones for city sentinel clinics will reduce unnecessary data delivery delays. In addition, creating awareness to data entry personnel on the importance of careful and completeness of entries is important, as is the need to reformat data collection forms to capture exact aspects of surveillance needs for relevant executable analysis.[8] Besides alerting for immediate impending epidemics, these data can also be adopted for projective predictive modeling of annual epidemics, including that for influenza.[9]

EHESP WCIC-study input**:** Djibouti WCIC-study is complementary to the existing syndromic surveillance system, but with emphasis on flu and flu–like illness. Various innovations as suggested above are used in seeking to overcome the prevailing challenges. While every attempt is made to realize its (WCIC-study) objective and for global comparison, lessons learned from successful implementation will form a platform for future refined syndromic surveillance protocol as equally reported elsewhere in Asian countries.[7,10]

Information Technology: National Institute of Public Health Djibouti has an informatics department with sufficient working PCs and personnel to execute efficient data collection and management for epidemiological analysis. However, licensing cost of near-real time syndromic surveillance software is prohibitive, but the open access software with capacity to generate custom graphs, maps, plots, and temporal-spatial analysis output for specific syndromes should make implementation a lot easier. Such output for conditions like flu (or gastroenteritis) will be essential to cause prompt response of the local Public Health Office and International Partners in saving lives and suffering of Djibouti people.[11]

Conclusion

Pandemic flu surveillance and preparedness requires multi-faceted, interdisciplinary, and international approach whose efficiency and efficacy can only be refined over time. Building on the Health Care System's SWOT for preparedness, the EHESP WCIC-study promises to refine surveillance system operation and knowledge on individual's risk determinants to swine flu (H1N1) 2009 virus infection at the household level in Djibouti. These efforts are ultimately creating available control options at the time of need (pandemic occurrence), and at the same time exploring investment in quality data profiling and information technology, which will include syndrome surveillance software systems like essence, ewors, or other open sourced ones.

Acknowledgements

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Evaluation of seasonal influenza vaccination effectiveness based on antibody efficacy among institutionalized elderly in Japan

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Keywords Antibody efficacy, elderly, seroprotection.

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Introduction

The antibody efficacy – which compares the illness frequency between those with and those without a protective level of pre-epidemic HI antibodies (≥1:40) – has been proposed[1]; however, this index has rarely been used due to practical difficulties in confirming the strain-specific disease corresponding to each of the vaccine-induced antibodies.

Materials and methods

We followed 114 elderly individuals residing in a nursing home, whose serum specimens were obtained before and

after undergoing trivalent influenza vaccination, in 2002/2003 influenza season (medium-scale mixed [A/H3N2 and B] epidemic in study area, and A/H3N2 was circulating at the nursing home).[2] The serum antibody titre to each strain of influenza virus was measured by the HI method, using the same antigens as those in the vaccine. All participants' body temperatures, respiratory symptoms, other general symptoms, hospitalization, discharge, and death were recorded daily from 1 November 2002 to 30 April 2003 in a prospective manner. When the participants suffered any influenza-like symptoms, such as sudden fever ≥37·8°C, throat swabs were collected and tested using a rapid diagnosis kit for influenza, which utilizes an immunochromatographic method. The adjusted odds ratios (OR_{adj}) for febrile illness and kit diagnosed influenza were evaluated using multiple logistic regression models adjusting for possible confounders (i.e., age, sex, coexisting conditions, and vaccine strains).

Results

After vaccination, the proportion of subjects achieving an HI antibody titre ≥1:40 (seroprotection level) were 61·4% (51·8–70·4%) for A/H1N1, 79·8% (71·3–86·8%) for A/H3N2, and 26·3% (18·5–35·4%) for B. During the follow-up period, the A/H3N2 strain was isolated therein, and 44 subjects experienced sudden-onset fever (≥37·8°C), and eight subjects were positive for rapid diagnosis kit. Patients with a seroprotection level of the HI antibody titre (≥1:40) had lower incidences of febrile illness (OR_{adj}, 0·35; 95% CI, 0·09–1·28) and rapid kit diagnosed influenza (OR_{adj}, 0·35; 95% CI, 0·03–4·64) than those with a lower titre. Thus antibody efficacy ($1 - OR_{adj}$) against fever related to A/H3N2 and kit diagnosed influenza were both estimated to be 65%.

Discussion

Although statistical significance was not detected due to limited sample size, these results lend support for the usefulness of antibody efficacy.

Disclosure

Some data presented within this manuscript was also published in Hara *et al.*[2]

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Epidemiologic study of human influenza infection in Korea from 1999 to 2007: origin and evolution of A/Fujian/411/2002-like strains

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Keywords Genetic variation, H3N2, hemagglutinin, influenza virus.

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Introduction

Following an outbreak of the A/Fujian/411/2002-like strains in Asia including China, Japan, and Korea in 2002, Australia and New Zealand experienced substantial outbreaks of the same strains in 2003 and worldwide in the 2003–2004 season. There was a continuous circulation of human influenza A/H3N2 viruses in East and Southeast Asia via a regional network from which epidemics in the temperate regions were seeded.[1]

Materials and methods

The virus isolates obtained from nasopharyngeal swab specimens from outpatients were typed and subtyped by the hemagglutination (HA) inhibition assay.[2] 116 HA amino

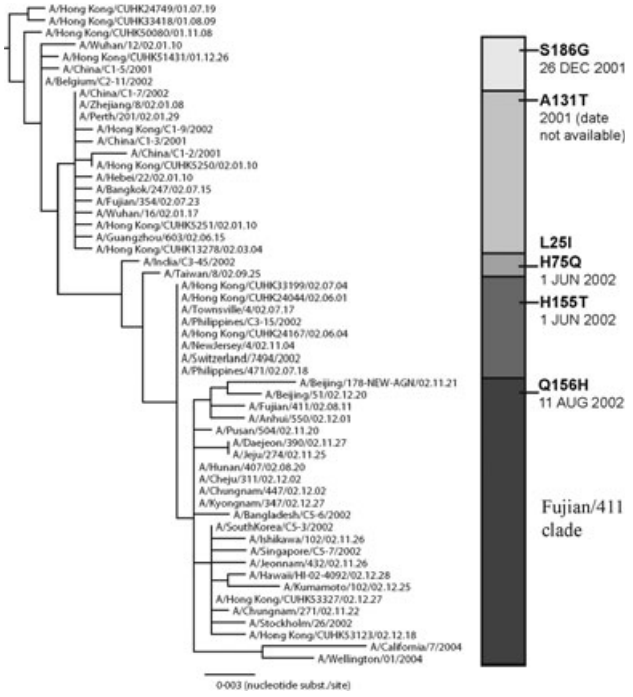

Figure 1. The ML tree of 53 strains around the emergence of the Fujian-411 strain. The grey bar indicates accumulation of amino acid changes and the first reported dates of isolates having the mutations.

acid sequences of Korean strains were included for the reconstruction of a phylogenetic tree. The dataset for ML phylogenetic analysis contained HA1 of H3N2 viruses collected globally from 2001 to 2002. For further technical details of the laboratory methods used, see Kang *et al.*[3]

Results

The emergence of A/Fujian/411/2002 coincided with higher levels of influenza-like illness in Korea than what is typically seen at the peak of a normal season. Most of the intermediates and Fujian-like strains were isolated from Asian countries, and the mutational events associated with the Fujian strains took place in Asia. Closely dated phylogeny from December 26, 2001 to August 11, 2002 showed that the antigenic evolution of the H3N2 Fujian strains had periods of rapid antigenic changes, equivalent to 10 amino acid changes per year (Figure 1).

Discussion

The Fujian-like influenza strains were disseminated with rapid sequence variation across the antigenic sites of the HA1 domain. The antigenic evolution of the Fujian strains was initiated by exceptionally rapid antigenic change that occurred in Asia, which was then followed by relatively modest changes.

Disclosure

Some of the data presented in this manuscript was previously published in Kang *et al.*[3]

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Seroprevalence of antibody to influenza A(H1N1) 2009 in Australian blood donors – before and after the 2009 influenza season, and prior to the 2010 Southern Hemisphere winter

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Keywords Australia, blood donors, H1N1 subtype, human, influenza, influenza A virus, pandemic, seroepidemiologic studies.

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Abstract

Assessment of population exposure to the influenza A(H1N1) 2009 (pH1N1) outbreaks in Australia was made difficult by the generally mild nature of observed disease. We aimed to establish a representative collection of plasma samples from Australian Red Cross Blood Service donors within which to chart the proportion of the population with demonstrated immunity to the novel virus over a series of timepoints, from April 2009 to October 2010. We here present data up to the March/April 2010 timepoint. We compared reactivity to the novel virus strain using haemagglutination inhibition (HI) assays performed on discarded plasma specimens left over from routine testing. Samples were taken from healthy adult blood donors (>16 years) before and after the pH1N1 influenza epidemic that occurred during the Southern Hemisphere winter of 2009, and again prior to onset of the 2010 Southern Hemisphere influenza season. Reactivity to the novel H1N1 2009 strain of influenza was relatively uncommon among the healthy adult population during the first Australian winter wave, rising from a baseline of 12% to 22%. A further increase in the seropositive proportion from 22% to 43% was observed over the summer months, most likely attributable to immunisation. This level of immunity appears to have been sufficient to constrain the 2010 winter epidemic. Together with a final serum collection, planned for late 2010, these data will aid evaluation of the extent and severity of disease in this 'second wave' of pH1N1.

Introduction

Assessment of the extent of disease due to novel influenza A(H1N1) virus (pH1N1) during the 2009 winter outbreaks in Australia was made difficult by the generally mild nature of disease. The epidemic was experienced in a staggered fashion around the country,[1] reflecting the considerable geographical distances between state and territory capital cities (Figure 1). Differences in the intensity of case-finding during the evolving pandemic response and between jurisdictions hindered comparisons of disease burden in distinct geographical regions.[2] Rates of reported hospitalisations and deaths appeared fairly similar across states[1] but, without a consistent exposure denominator, assessment of relative severity was difficult.

We conducted a national serosurvey of antibody to pH1N1 using residual plasma from healthy blood donors collected before and after the 2009 epidemic to estimate pH1N1 exposure.[3] Here we report the findings of that first collection, together with new data on seroprevalence of pH1N1 antibody in specimens gathered in March–April 2010. These latter samples were collected prior to onset of seasonal influenza activity to assess the impact of a national pH1N1 vaccine program conducted in Spring/Summer 2009/10 on the proportion of individuals with antibody titres deemed protective.[4] Findings informed estimates of population susceptibility to pH1N1 prior to the 2010 influenza season and provided a baseline for a subsequent serosurvey that will be collected at the end of 2010 to assess the extent of exposure during the 'second wave.'

Materials and methods

Study populations

Five hundred-one pre-pandemic plasma samples were randomly selected from specimens collected from Cairns and Townsville blood donors in April/May 2009. These samples had been taken at the time of blood donation by the Aus-

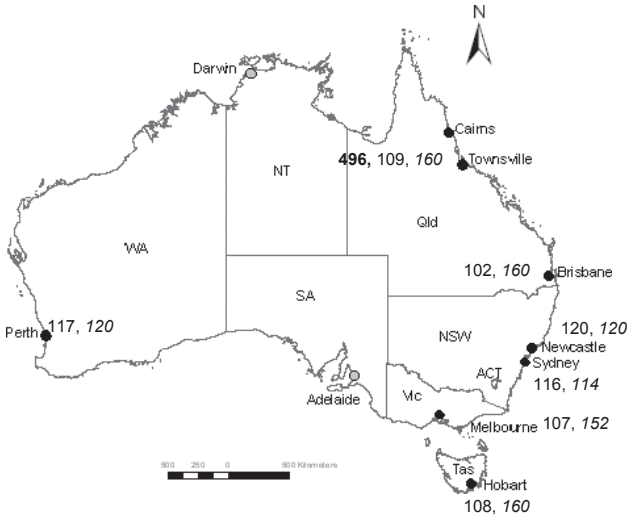

Figure 1. Geographical distribution of specimen collection centres, marked in black. Non-participating state/territory capital cities are shown in grey. *The number of specimens tested from each site is shown for the pre-pandemic collection (Bold), the post-pandemic collection (Normal text) and the pre-influenza season 2010 collection (*Italics*).

tralian Red Cross Blood Service (the Blood Service) for dengue fever surveillance studies. These samples were used to provide a baseline estimate of prevalence of cross-reactive antibody to pH1N1 in the Australian population.

Discarded plasma specimens, taken for virologic testing from healthy adult Blood Service donors, were prospectively collected at two additional timepoints for measurement of antibody to pH1N1. Collection periods were as follows:

1. Late October–early December 2009 (post-pandemic samples).

2. March–early May 2010 (pre-flu season 2010 samples).

Approximately 120 plasma samples were randomly selected from donors in each of Brisbane, Hobart, Melbourne, Newcastle, Perth, Sydney, and Townsville on each occasion. Up to 20 specimens were identified in each of the following age strata: 16–24, 25–34, 35–44, 45–54, 55–64, and >65 years. At the last collection timepoint, there was deliberate over-sampling of the oldest and youngest age strata in which approximately 40 specimens were collected (i.e., up to 160 specimens per site).

Ethical approvals

In accordance with the provisions of the National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research, individual consent was not required for use of these specimens, given the granting of institutional approval by the Blood Service Human Research Ethics Committee.

Laboratory assays

Reactivity of plasma against pH1N1 was measured in haemagglutination inhibition (HI) assays using turkey red blood cells (RBC).[5] Egg-grown A/California/7/2009 virus was purified by sucrose gradient, concentrated and inactivated with β-propiolactone, to create an influenza zonal pool preparation (a gift from CSL Limited). Plasma samples were pretreated with receptor destroying enzyme II (Denka Seiken Co. Ltd), 1:5 (volume/volume) and tested as previously described.[6] Following 1 hour incubation, 25 μl 1% (volume/volume) of RBC was added to each well. HI was read after 30 minutes. Any samples that bound to the RBC in the absence of virus were adsorbed with RBC for 1 hour and reassayed. Samples in which background activity could not be eliminated by these means were excluded from the analysis. Titres were expressed as the reciprocal of the highest dilution of plasma where haemagglutination was prevented.

A panel of control sera and plasma samples was included in all assays. It comprised paired ferret sera pre- and post-infection with the pandemic virus or seasonal influenza A(H1N1), A(H3N2), or influenza B viruses and paired human plasma and sera collected from donors before April 2009 or after known infection with the pandemic virus or after immunisation with the Australian monovalent pandemic 2009 vaccine. All assays were performed by the WHO Collaborating Centre for Reference and Research on Influenza.

Statistical analysis

For each of the three study timepoints and within each age group, the proportion of seropositive individuals (HI titres ≥40) was calculated, with exact (Clopper–Pearson) confidence intervals. The contribution of individual variables (age, gender) and location to seropositive status was assessed in separate multivariate logistic regression models developed to assess the post-pandemic and pre-influenza season 2010 collections. All statistical analyses were conducted in stata 10.

Results

Locations of specimen collection are shown in Figure 1, together with the number of samples tested from each centre. Samples with high background HI titres or discrepancies between assays were excluded at each timepoint as follows: 5 at baseline, 27 from the post-pandemic collection, and 0 in early 2010.

Post-pandemic collection

Exposure to the novel H1N1 2009 strain of influenza was relatively uncommon among the healthy adult population during the Australian winter 2009 outbreak. The difference in seropositivity (HI titre ≥40) assessed by HI assay com-

Table 1. Percentage of donors with HI antibody titres to influenza A (H1N1) 2009 ≥40 by age and timing of specimen collection (with 95% confidence intervals)

Donor age	Pre-pandemic April/May 2009	Post-pandemic October/November 2009	Pre-influenza season March/April 2010
16–24 years	16 (8·3, 23·7) *88**	**37 (28·9, 45·1)**** *138*	49 (42·5, 55·5) *225*
25–34 years	7 (0·4, 13·5) *59*	**22 (15·1, 28·9)** *139*	**41 (32·7, 49·3)** *136*
35–44 years	13 (4·8, 21·2) *64*	15 (8·9, 21·1) *131*	**32 (24·2, 39·8)** *139*
45–54 years	9 (4·1, 14·0) *129*	16 (9·9, 22·1) *138*	**32 (24·3, 39·7)** *140*
55–64 years	13 (7·2, 18·8) *129*	20 (13·2, 26·8) *131*	**45 (37·0, 53·0)** *148*
>65 years	19 (4·2, 33·8) *27*	25 (16·6, 33·4) *102*	**53 (46·0, 60·0)** *198*
All ages	12 (9·1, 14·9) *496*	**22 (19·1, 24·9)** *779*	**43 (39·9, 46·1)** *986*

*Numbers of specimens per group at each timepoint are shown in italics.
**Values highlighted in bold are significantly higher than at the previous timepoint, as shown by non-overlapping 95% confidence intervals.

pared with baseline was 10% overall, rising from 12% to 22% (Table 1). The only jurisdictions in which seropositive proportions were higher in October/November than in the baseline collection were Hobart [31% (95% CI 22·3, 39·7)], Perth [24% (16·3, 31·7)], and Sydney [24% (16·3, 31·7)]. In the multivariate regression model, the only jurisdiction in which exposure appeared somewhat higher than the reference population of Brisbane was Hobart [OR 1·83 (95% CI 0·99, 3·4), $P = 0·06$].

A marked age effect on antibody status was observed at this timepoint, with an increase in the proportion of seropositive individuals in relation to the baseline collection only noted for those aged between 16 and 34 years (Table 1). According to the multivariate model, the youngest and oldest cohorts had similar titres, with all other groups showing significantly lower seropositive proportions than the reference population of 16–24 years [e.g. 45–54 years OR 0·36 (95% CI 0·21, 0·64, $P < 0·0001$)].

Pre-influenza season 2010

An overall increase in the seropositive proportion from 22% to 43% was observed between October 2009 and April 2010, distributed throughout all jurisdictions (Table 1). The proportion protected at this time was lowest in Brisbane [35%, (95% CI 27·6, 42·4)], compared to which immunity was significantly higher in Hobart [49% (41·3, 56·7)], Perth [45%, (36·1, 53·9)], and Townsville [51% (43·3, 58·7)]. The greatest titre difference between the two timepoints was observed in Townsville [32% (21, 43)] compared with the national average [21% (17, 25)].

Antibody titres prior to the 2010 influenza season rose in all age groups, but remained significantly lower among 35–54 year olds than in the youngest age cohort (Table 1). Adjusted ORs for the seropositive proportion in the multivariate model in these age groups were: 35–44 years [OR 0·49 (95% CI 0·31, 0·77)]; 45–54 years [OR 0·48 (0·31, 0·75)]. The relatively low titres observed in these groups reflected small incremental increases in the seropositive proportion across each of the time points studied, suggestive of both low rates of infection and vaccination.

The rise in immunity observed across the population was most likely attributable to immunisation in the majority, given the absence of observed outbreaks and very few notified cases of pH1N1 during the period between the two plasma collections.

Discussion

This study suggests that, while adult exposure to pH1N1 during the 2009 Southern Hemisphere winter was uncommon at around 10%, vaccine uptake in the Australian population over the period November 2009–May 2010 was in the order of 20%. This latter estimate is in keeping with recently published figures for adult pH1N1 vaccine coverage from a national immunisation survey conducted by the Australian Institute of Health and Welfare.[4] In that survey, vaccine coverage was significantly higher in Tasmania than in other states, but mostly in those over 65 years of age, possibly in a subgroup whose health status may have differed from that of the donor population.[4]

No allowance has been made in this analysis for likely waning of natural or vaccine induced immunity, possibly resulting in lower estimates of natural and/or vaccine exposure than may have occurred over the period. Regardless of such intervening processes, the seropositive proportion among Australian adults at the start of the 2010 winter season appeared likely to be sufficient to constrain transmission of infection in the age groups tested. This assertion has been borne out in practice, with only modest levels of influenza reported during the late and protracted 2010 season.[7]

A final serum collection is planned for the end of the 2010 influenza season in Australia from which to assess the

level of exposure in relation to the baseline observed here. The need for epidemiologic studies such as this has been highlighted by groups such as the European Centre for Disease Control to aid evaluation of the extent and severity of the 'second wave,' [8] known to be variable from historical reports of past pandemics in disparate populations.[9]

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Epidemiological diversity analysis upon the incidence of 2009 novel H1N1 flu among school children within and among small regional communities, Japan

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Keywords Agent-based simulation, epidemiological diversity, school children, small regional communities, 2009 novel H1N1 flu.

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Introduction

In 2009–2010, the first wave of the swine-origin novel H1N1 flu (H1N1) pandemic swept across the world, including Japan. To examine the epidemiological nature of this novel infectious disease among school children within and among small regional communities, we have carried out a complete survey on the incidence of H1N1 among school children using absentee reports provided by school health teachers in two small administrative districts (population: about 140 000 in total) in Japan. We then examined the epidemiological diversity on the incidence of H1N1 within and among small regional communities.

Materials and methods

We investigated seventeen elementary and ten junior high schools in Moroyama-town and Sakado-city located in the central part of Saitama Prefecture. Populations are: all ages, 37 015 and 100 634; elementary schools, 1873 and 5389; junior high schools, 1131 and 2568, respectively. The number of school children in each school ranges from 137 to 876. The surveillance system was built on an Apache- and

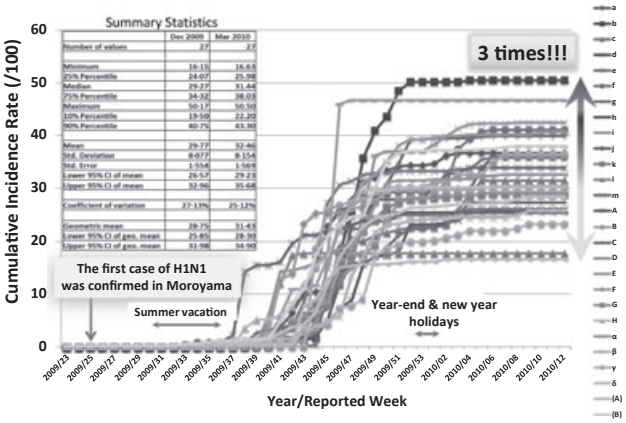

Figure 1. Cumulative incidence in each School. a–m and α–δ, elementary; A–H and (A)–(B), junior high schools in Sakado and Moroyama, respectively. The H1N1 outbreak began immediately after summer vacation (36th week, 2009). The CIs in each school reached 16·2–50·2% and 16·6–50·5% as of the end of December 2009 and March 2010, respectively. The outbreak had almost ceased by the end of Dec 2009. In some schools, however, small recurrences of H1N1 were seen after year-end and New Year vacation (January–March 2010).

MySQL-based WEB server using html, PHP, and JavaScript.[1] School health teachers enter information on children absenteeism due to school infectious diseases via WEB browsers at each school infirmary on a daily basis. In addition to the trend graphs shown on the WEB browser, detailed analyses were reported to the schools and local educational boards weekly. The basic reproduction number (R_0) of H1N1 was estimated according to Becker.[2] Agent-based modeling and simulations were also performed using a multi-paradigm simulator AnyLogic Version 6.5 (XJ Technologies, St. Petersburg, Russia).

Results

By the end of March 2010, cumulative incidence (CI) of H1N1 among school children in Moroyama and Sakado reached 30% and 34%, respectively. The overall R_0 among school children in this area was 1·43. Vaccination rate of children in this area during the surveillance period was reported to be very low (<10%). There was no considerable difference between the epidemic curves in this neighboring town and city. On the other hand, in the individual schools, the CIs as of the end of March 2010 scattered from 16% to 51% (Figure 1) even though the schools are closely located.

To examine the cause of this diversity, we built an agent-based community model[3] consisted of the same numbers of agents as those of children in the actual schools and people in Moroyama and Sakado to simulate the infection. The ratio of probability of infection in schools and the remaining places were assumed to be 1:1 or 10:1. Using a heuristic optimization scheme, we estimated the parameters for the simulations to give the overall CI of 33% (the CI as of the end of March 2010). We then performed simulations repeatedly. The CIs obtained with the repetitive simulations with the assumption of higher probability of infection in schools scattered from 23% to 44%, indicating that the CIs of the small population communities may vary considerably, even though all the agents were assumed to have the same susceptibility to infection at the beginning, and the other conditions were the same.

Discussion

The policies for surveillance/analyses/prevention of communicable diseases in local communities have generally been decided on governmental- and/or each local administrative district-basis (populations: several hundred thousands to several millions) in Japan. We found the considerable variations in the CIs of H1N1 for children among much smaller areas, i.e., the school districts (populations: all ages, several thousands; school children, several hundreds).

We thus conclude that the granularity of surveillance/analyses/prevention should be finer than in the past to achieve the most effective policies against influenza and similar communicable diseases in the local communities. The cause of this diversity can be explained in part by the stochastic nature of infection transmission processes in the small populations shown by the agent-based simulations.

We have already conducted a complete questionnaire survey for the school children and their parents to clarify the relevance of the other issues including differences in environmental factors, preventive policies (e.g., vaccination, school closures), etc., in each school. The detailed analyses will be reported elsewhere.

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Association between pandemic and seasonal influenza vaccination and haemagglutination antibody titers against A/H1N1v: a national representative survey in France, nested in the ''Cohorts for Pandemic Influenza'' (CoPanFlu – France)

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Keywords Antibodies, cross-sectional studies, France, hemagglutination inhibition tests, humans, influenza A virus H1N1 subtype, risk factors.

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Abstract

This study relies on the first available data from the Cohorts for Pandemic Influenza (CoPanFlu)-France project, which is part of the CoPanFlu international consortium, aimed at studying individual and collective determinants of pandemic A/H1N1v influenza across different countries by setting up prospective cohorts of households. This primary analysis studies the association between elevated haemagglutination inhibition (HAI) antibody titers against A/H1N1v and influenza vaccination (seasonal or pandemic) at entry in the cohort. Inclusions began on December 4, 2009 and ended on July 23, 2010. Households were sampled using a random telephonic design in French general population. All household members were eligible to the cohort; however the inclusion of a household required the participation of all members. During the inclusion visits, nurses collected detailed data on subjects as well as blood samples. A standard HAI technique was adapted to the detection and quantification of antibodies to the 2009 A/H1N1v virus. Geometric mean titers (GMTs) were calculated for HAI assays with the use of generalized estimating equations for interval-censored data, and a ''GMT ratio'' (GMTR) was defined as the multiplicative factor applied to the GMT in presence of an explanatory variable.

This preliminary analysis included 1304 subjects belonging to 544 households. The GMT in the population was 35·4 [95% CI: 30·1; 41·8]. This GMT varied among age classes with values of 52·6 [39·2; 70·6], 28·5 [21·2; 38·3], and 38·3 [33·1; 44·5] for subjects below 15, between 15 and 50, and over 50, respectively. The other factors associated to an elevated GMT were previous vaccination by either the pandemic or the seasonal strain and history of influenza-like illness (ILI) since the beginning of the epidemic. In pandemic vaccine recipients, the GMT decreased after

vaccination (GMTR: 0·89 [0·81; 0·99]) per month since pandemic vaccination.

This study confirms previous findings that age, pandemic influenza vaccination, and history of ILI are associated with elevated post-seasonal GMT. This study also shows that seasonal influenza vaccination may have contributed to an increase of the HAI titer, especially in the elderly. Further analyses in this cohort are needed to confirm and explain these first results. The follow-up of subjects involved in the CoPanFlu-France cohort will provide data to study the risk factors for infection by the influenza virus.

Introduction

The first cases of the 2009 A/H1N1v pandemic influenza were reported in Mexico and the United States in April 2009. Given the context of this new influenza virus and considering the likelihood of its pandemic spread, the Cohorts for Pandemic Influenza (CoPanFlu) international consortium was created in order to study individual and collective determinants of pandemic A/H1N1v influenza across different countries by setting up prospective cohorts of households, followed during 2 years.

This study relies on the first available data from the CoPanFlu-France project, which is part of the CoPanFlu international consortium. We studied factors associated with elevated haemagglutination antibody titers against A/H1N1v at entry in the CoPanFlu-France cohort. We focused in this primary analysis on the association between the titers and influenza vaccination (seasonal or pandemic) across age groups.

Materials and methods

Study design

The CoPanFlu-France cohort was set up in fall 2009. Inclusions began on December 4, 2009 and ended on July 23, 2010. Households were sampled using a random telephonic design (Mitofsky–Waksberg method)[1] in a stratified geographical sampling scheme, aimed at including a sample of subjects representative of French general population. All household members were eligible to the cohort, without any age limit. The inclusion of a household required the participation of all members: the refusal of one or more member(s) prevented the inclusion of other members. The protocol was approved by a research ethics committee and written informed consent was obtained for all subjects.

Data collection

This study requires several visits to the households by nurses who collect written data with questionnaires and biological samples. During the inclusion visits, nurses collected from all subjects detailed data regarding medical history, including vaccination and preventive measures against influenza. Blood samples were collected at entry and centralized.

Haemagglutination inhibition assay

A standard HAI technique was adapted to the detection and quantification of antibodies to the 2009 A/H1N1v virus.[2] The titration endpoint was the highest dilution that exhibited complete inhibition of haemagglutination in two independent readings. The lowest read dilution was 1/40.

Statistical analysis

Geometric mean titers were calculated for HAI assays with the use of generalized estimating equations for interval-censored data,[3,4] taking into account a within-household correlation. Multivariate models were derived from this method to identify factors associated with elevated GMTs. We defined the ''GMT ratio'' (GMTR) as the multiplicative factor applied to the GMT in presence of an explanatory variable. For qualitative explanatory variables, a GMTR of N means a predicted N-fold higher GMT for subjects exposed to the considered factor compared to others. For continuous explanatory variable, the same interpretation applies to a unit difference.

Studied factors

The following variables were included in the multivariate models: age, history of pandemic or seasonal influenza vaccination, and history of ILI. Age was categorized in three groups: 0–15 years (reference group), 15–50 years and over 50 years. The definition of ILI was that used by the CDC[5]: fever ≥37·8°C and cough and/or sore throat without another known cause. History of ILI was defined as an ILI reported by the subject between September 12, 2009 (beginning of the influenza epidemic in France) and the date of inclusion.

Results

This preliminary analysis included 1304 subjects belonging to 544 households. Results reported hereafter do not account for missing data.

Description of subjects

Participating households were sized 1–7 subjects, mean size = 2·4. In comparison, the mean size of French households is 2·3 according to the latest national census.[6] The median age of subjects at entry was 40·6 years [IQR: 16·5; 58·6] versus 34·4 [15·2; 52·5] for French population.[7] The proportion of subjects reporting a history of ILI since the beginning of the epidemic varied from 2·9% for subjects over 50 years to 9·1% for subjects below 15 years (Table 1).

Vaccination with the pandemic strain was the highest in subjects below 15 (16%) whereas vaccination with the seasonal strain was the highest in subjects over 50 (44·9%). Detailed data regarding vaccination is given in Table 1.

Factors associated to the GMT

The GMT in the population was 35·4 [95%CI: 30·1; 41·8]. This GMT varied among age classes with values of 51·7 [38·0; 70·5], 29·4 [22·0; 39·4], and 37·4 [32·0; 43·9] for subjects below 15, between 15 and 50, and over 50, respectively (Table 1).

In a multivariate analysis (Table 2), the other factors associated to an elevated GMT were previous vaccination by either the pandemic or the seasonal strain (GMTR: 2·87 [2·28; 3·61] and 1·18 [1·01; 1·39] respectively), and history of ILI (1·41 [1·10; 1·80]). Note that the role of seasonal vaccination was significant only in subjects >50: GMTR: 1·29 [1·09; 1·54] versus 0·99 [0·75; 1·29] for subjects <50, adjusted on pandemic vaccination and history of ILI. In pandemic vaccine recipients, the GMT decreased after vaccination (GMTR: 0·89 [0·81; 0·99]) per month since pandemic vaccination in a multivariate analysis (Table 2).

Table 2. Factors associated to GMT (multivariate analyses)

	GMT ratio [95% CI]	*P*
All subjects (*N* = 1254)		
Age		
0–15 years	1 (ref)	
15–50 years	0·61 [0·50; 0·73]	<0·00001
>50 years	0·60 [0·48; 0·75]	<0·00001
Pandemic vaccine	2·87 [2·28; 3·61]	<0·00001
Seasonal vaccine	1·18 [1·01; 1·39]	<0·04
History of ILI	1·41 [1·10; 1·80]	<0·01
Pandemic vaccine recipients only (*N* = 148)		
Age		
0–15 years	1 (ref)	
15–50 years	0·74 [0·47; 1·16]	0·23
>50 years	0·30 [0·17; 0·52]	<0·00001
Months since pandemic vaccine	0·89 [0·81; 0·99]	<0·04
Seasonal vaccine	1·36 [0·90; 2·05]	0·15
History of ILI	1·65 [1·25; 2·17]	<0·0004

GMT, geometric mean titer; ILI, influenza-like illness.

Discussion

This study confirms previous findings that age, pandemic influenza vaccination, and history of ILI are associated with elevated post-seasonal GMT.[8–13] Among non-vaccinated subjects, elevated GMT in the elderly may be the result of exposure to similar viruses in early life, whereas children and young adults with elevated GMT are likely to have been infected by the 2009 A/H1N1v virus.[13–16]

Interestingly, a significant drop in the HAI titer is observed during the months following vaccination with the pandemic strain.

This study also shows that seasonal influenza vaccination may have contributed to an increase of the HAI titer, especially in the elderly. The reason for this association is not obvious: although we cannot discard the hypothesis of a higher incidence of A/H1N1v infections in seasonal vaccine recipients, as described by several other studies,[17–19] the

Table 1. Description of subjects at inclusion and estimated GMT by age class

Age class	0–15 years *N* = 242	15–50 years *N* = 534	>50 years *N* = 478	All subjects *N* = 1254
Sex = male	104 (50·2%)	230 (45·3%)	218 (50·0%)	552 (47·7%)
History of ILI	19 (9·1%)	40 (7·9%)	13 (2·9%)	78 (6·8%)
Influenza vaccination				
None	202 (81·7%)	406 (80·1%)	231 (53·0%)	828 (70·1%)
Seasonal only	6 (2·4%)	49 (9·7%)	147 (33·7%)	205 (17·4%)
Pandemic only	32 (15·5%)	40 (7·9%)	9 (2·1%)	81 (6·9%)
Both	1 (0·5%)	12 (2·4%)	49 (11·2%)	67 (5·7%)
Median time since pandemic vaccination (days) [IQR]	72 [46; 117]	72 [41; 132]	79 [51; 116]	76 [46; 122]
Estimated GMT [95% CI] according to influenza vaccination				
None	43·8 [31·9; 60·2]	29·0 [21·7; 38·7]	31·0 [23·6; 40·8]	30·8 [25·4; 37·4]
Seasonal only	40·0 [33·9; 47·2]	26·9 [12·7; 57·3]	41·3 [34·6; 49·2]	38·4 [32·2; 45·7]
Pandemic only	132·8 [79·4; 222·1]	102·6 [71·3; 147·5]	40·2 [24·1; 66·9]	99·1 [70·4; 139·5]
Both	–*	149·9 [85·9; 261·5]	76·9 [57·0; 103·7]	86·0 [64·4; 114·8]
All subjects	51·7 [38·0; 70·5]	29·4 [22·0; 39·4]	37·4 [32·0; 43·9]	35·4 [30·1; 41·8]

GMT, geometric mean titer; ILI, influenza-like illness.
*No estimation of GMT (only one subject <15 years having received both vaccines).

main explanation may be a cross-reaction between pandemic and seasonal strains.[16,20,21]

Further analyses in this cohort are needed to confirm and explain these first results. The follow-up of subjects involved in the CoPanFlu-France cohort will provide data to study the risk factors for infection by the influenza virus.

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Behavior of influenza A H1N1 2009 in infants under 5 years old in Tucumán, Argentina

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Abstract

In April 2009, the CDC alerted about the appearance of a new strain of IA H1N1 with unknown virulence. Infants under 5 years old had higher risks of hospitalization, complications, and rate of death for SARI. *Materials and methods*: a cross-sectional study was executed from May to December in 2009. The sources were: mandatory reporting form of the province surveillance system, databases of the hospital management information system, clinical pictures reviews, and telephone daily medical reports. Inclusion criteria: children under 5 years old with diagnostic of ILI or SARI and confirmed cases with epidemiological nexus or laboratory confirmation (rRT-PCR, IFI). The age specific mortality rates were calculated with an estimated population for the province according to the National Statistics and Census Institution. *Results*: the ILI rate in infants under 5 years old was 1295·19/100 000 people (95% CI 1236–1356) being higher in infants of 4 years old (1569/100 000 people of 4 years (95% CI 1426–1722) (Table 1). Infants had less risk of getting sick in relation to the rest of the population (RR 0·40 [95% CI 0·33–0·47]) ($P < 0·05$). The chance of SARI in infants was 2·89 (95% CI 2·46–3·39) compared to the rest of the population. The lethality rate was higher in infants under 1 year old (14/100 000 people [4/28 364]). *Discussion*: The evidence suggests that the infants under 5 years old had lower risk of getting sick than the rest of the population, but had higher risk of SARI if they had some past illness. The highest lethality rate was presented in infants under 1 year old. Non-medical interventions had an important role in the epidemic containment for not having a specific vaccination available. As this age group had high risks of hospitalization, it would be advisable to prioritize their vaccination.

Introduction

In April 2009, the CDC alerted about the appearance of a new strain of IA H1N1 with unknown dissemination and virulence. In June, the World Health Organization declared the pandemic.[1,2]

The ILI often presents an unspecific clinical picture in infants under 5 years old, from mild symptoms to SARI, especially in the newborn babies. Infants under 5 years old have higher risks of hospitalization, complications, and rate of death for SARI.[3,4]

On May 7th, Argentina declared the first imported case of IA H1N1, and by the end of the month, it announced the viral circulation in the country. The epidemiological surveillance system of the province arranged that all the patients with influenza diagnosis made by a doctor must be reported. From April 24th to November 14th, 20 212 suspected cases of ILI in the province of Tucumán were reported. The ILI rate was 1353/100 000 people, and IA H1N1 comprised 40/100 000 people. The lethal rate of SARI IA H1N1 was 306·7/100 000 people (62/20 212).[5] The objective of this research was to determine the epidemiological characteristics of the pandemic IA H1N1 in infants under 5 years old in the province of Tucumán between May and December in 2009.

Location

The province of Tucumán is placed in the center of the northwest of the Republic of Argentina. It has a population of 1 493 488 inhabitants of which 138 821 are infants under 5 years old. The crude birth rate for 2008 was 19·9‰. The infant mortality rate was 13·8‰. Respiratory pathologies in infants under 5 years old were the third cause of death in the province (12%). The public health system of the province is composed by three sectors: public, private, and welfare. With 91 health facilities as a total, the average of available beds is 3‰ per inhabitants and 6‰ per neonates.

Materials and methods

A cross-sectional study was executed from May to December in 2009 in the province of Tucumán, Argentina. The

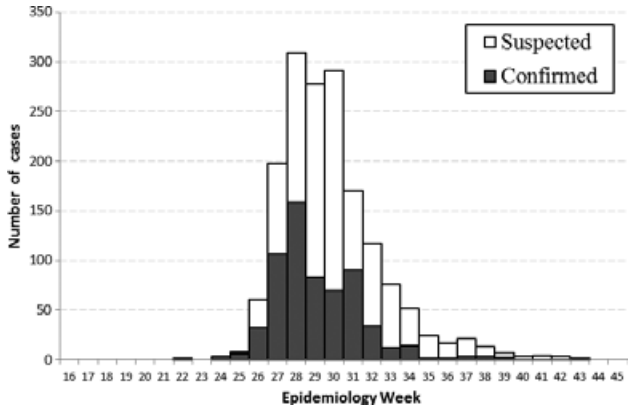

Figure 1. Epidemic curve of IA in infants under 5 years old by 2009 epidemiology week Tucumán, Argentina (n = 1798). Data from the Epidemiology Department Tucuman, Argentina.

following sources were used: mandatory reporting form of the surveillance system of the province filled by a doctor, databases of the hospital management information system, clinical pictures reviews, and telephone daily medical reports (patients with SARI).

Inclusion criteria:

- *Suspected Case of ILI*: Sudden appearance of fever higher than 38°C, cough, or sore throat. It may or may not be accompanied by asthenia, myalgia or prostration, nausea or vomiting, rhinorrhea, conjunctivitis, adenopathy, or diarrhea.
- *Confirmed Case*: Every suspected patient with an epidemiological nexus or laboratory confirmation of IA virus infection. Laboratory diagnosis of influenza was confirmed in respiratory samples (nasopharyngeal and pharyngeal swabs) with rRT-PCR specific primers for pandemic H1N1 influenza (performed at reference laboratories of the Argentina National Health System) and with IFI (in an Influenza Sentinel Surveillance Unit of province of Tucuman).
- *Dismissed Case*: those cases with negative laboratory tests or different clinical evolutions.
- *Comorbidity:* ROBS, cardiovascular diseases, COPD, immunosuppression, human immunodeficiency virus, cancer, nephropathy, obesity, neurological diseases, malnutrition, and aggravated bacterial infection.

Data were analyzed using EPI 2000 software (Epi Info™ CDC Atlanta, EEUU). Central tendency and perceptible measures 25–75 ($P_{25–75}$) were used for the analysis. The odds rations, risk ratio and 95% confidence interval were calculated to compare ambulatory with hospitalized patients, confirmed and dismissed, <5 years old and the rest of the population. It was considered significant a rate of $P < 0{\cdot}05$. The age specific mortality rates were calculated with an estimated population for the province according to the National Statistics and Census Institution.

Results

The epidemiological surveillance system of the province received 21 012 ILI reports, 11·77% (2474/21 012) were infants under 5 years old. Twenty seven percent were dismissed (676/2474), and 34% (623/1798) of suspected cases were confirmed. The first IA H1N1 case was a child of 2 years from the province of Buenos Aires, in 22th epidemiological week, and the last suspected case was reported in October 26, 2009 (Figure 1).

The ILI rate in infants under 5 years old was 1295·19/100 000 people (95% CI 1236–1356), being higher in infants of 4 years old (1569/100 000 people of 4 years, [95%CI 1426–1722]). The higher ILI rates in confirmed

Table 1. Effects of influenza A H1N1 by age. May–December 2009 (n = 1798)

Ages (years)	Population	ILI rate (*N* cases)	Confirmed IA rate (*N* cases)	SARI rate (*N* cases)	SARI IA rate (*N* cases)	% ICU admissions (*N* cases)	Mortality rate IA (*N* cases)	Lethality IA (*N* cases)
<1	28 364	969·53 (263)	384·29 (109)	461·85 (131)	179·80 (51)	8% (4)	14·10 (4)	3·67 (4)
1	26 078	1269·25 (329)	498·50 (130)	256·92 (67)	111·20 (29)	7% (2)	7·67 (2)	1·54 (2)
2	28 862	1261·18 (364)	575·15 (166)	114·34 (33)	51,97 (15)	13% (2)	–	–
3	27 735	1409·77 (391)	573·28 (159)	90·14 (25)	32·45 (9)	33% (3)	–	–
4	27 782	1572·97 (437)	212·37 (59)	79·19 (22)	3·60 (1)	–	–	–
Total	138 821	1295·19 (1798)	448·78 (623)	200·26 (278)	75·64 (105)	10% (11)	4·32 (6)	0·96 (6)

Data from the Epidemiology Department Tucuman, Argentina and from National Statistics and Census Institution.
ILI, influenza-like illness; IA, influenza A; SARI, ILI admitted in hospitals. No cases, number of cases; ILI rate, ILI cases/Population for Same Specified Age Group × 100 000; Confirmed IA rate, confirmed IA cases/Population for Same Specified Age Group × 100 000; SARI rate, SARI/Population for Same Specified Age Group × 100 000; SARI IA rate, SARI confirmed IA cases/Population for Same Specified Age Group × 100 000; % ICU Admissions: Proportion SARI IA admission into intensive care, confirmed IA in ICU/SARI IA for Same Specified Age Group × 100; Confirmed mortality rate, Deaths IA Confirmed/Population for Same Specified Age Group × 100 000; Lethatility IA, Deaths IA confirmed/ILI IA confirmed for Same Specified Age Group × 100.

and suspected cases of IA were reported in the epidemiological week 28 (222·58/100 000 people [309/138 821]). Infants had less risk of getting sick in relation to the rest of the population (RR 0·40 [95% CI 0·33–0·47]) ($P < 0·05$). There was no significant difference between both sexes. The highest proportion of cases resided in the Capital (52% [937/1798]) and Cruz Alta (14% [255/1798]). The symptoms, which were significantly different ($P < 0·05$) in the confirmed cases with the rest of the population, were fever, migraine, myalgia, sore throat and cough, in that order. The highest proportion of comorbidity reported was ROBS (8% [151/1798]). The chance of SARI in infants under 5 years old was 2·89 (95% CI 2·46–3·39) compared to the rest of the population. The highest rate of SARI for IA was observed in infants under 1 year old (180/100 000 people) (Table 1). In infants under 5 years old the chance of SARI for IA was important if they had a previous illness (OR 4·97 [95% CI 3·45–7·20]). The median time of hospitalization was 5 days $P_{25–75}$ (3–8). Ten percent (11/105) of SARI IA went into intensive care and 5% (5/105) required mechanical ventilation. The lethality rate was higher in infants under 1 year old (14/100 000 people under 1 year [4/28 364]) (Table 1). Fifty percent (3/6) of the deceased for SARI IA presented comorbidity (malnutrition, neurological pathology, and Bordetella pertussis co-infection).

Discussion

The pandemic of IA H1N1 (2009) was detected for the first time in the Province of Tucumán. The evidence suggests that infants under 5 years old had lower risk of getting sick than the rest of the population (protective factor), but had higher risk of SARI if they had some past illness. The highest lethality rate was presented in infants under 1 year old. Towns with the highest demographic density had superior proportion of cases.

Non-medical interventions had an important role in the epidemic containment for not having a specific vaccination available. As this age group had high risks of hospitalization, it would be advisable to prioritize their vaccination.

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Outbreak of H1N1 influenza – 2009: behavior of influenza H1N1 in school children in the province of Tucumán, Argentina

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Keywords H1N1 subtype, incidence, influenza viruses, oubreak, school, students, surveillance.

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Abstract

Objective: To describe the behavior of Influenza H1N1 2009 epidemic in school aged children population in Tucumán, Argentina. *Materials and methods*: Cross-sectional study. Data were gathered through mandatory reporting forms. *Inclusion Criteria*: Patients with symptoms compatible with Influenza A; school age (5–17 years). *Exclusion*

Criteria: Patients treated with antiviral medication for prophylaxis, respiratory pathologies which did not justify specific medication, and incomplete forms. *Results*: From all notifications, 6342 were cases of ILI in the group aged 5–17 years old; 53% were males. The incidence rate in this group was 17·4 per thousands of inhabitants. The 13% of laboratory samples were Influenza A H1N1, 34% were confirmed as unspecific Influenza, and 49% were dismissed. The school aged children group had a high risks of getting sick (R.R. 1·78 [95% C.I. 1·55–2·04]), especially males. It appeared that school aged children had a protective factor for presenting SARI (OR 0·66 [95% C.I. 0·66–0·89], $P < 0·05$). The lethality rate in this group was 9·32/10 thousands. Headaches, myalgia, coryza, and sore throat were very common and significantly different ($P < 0·05$) than the rest of the population. It was reported a decrease in the EW 28 coinciding with winter holidays (EW 27). The epidemic curve was different in males compared to females during the winter holidays. *Discussion*: School aged children got sick more than the rest of the population, although they presented less proportions of SARI. However, comorbidities were decisive in order to present SARI or death. The epidemic curve was different in males compared to females. Through its analysis, the beneficial effect of school closure was observed, as long as children meet the recommendation to stay home.

Introduction

In April 2009, different countries reported cases of Influenza A H1N1; Mexico reported a high mortality rate associates with this disease.[1] The World Health Organization (WHO) declared the Phase 6 Influenza Pandemic Alert on June 11.[2]

Several reports from different countries describe the behavior of the Pandemic in school aged children. This group plays an important role in the transmission of Influenza. In Germany, during the summer peak, pandemic hardly spread within this group. This might be explained by the timing of the summer school holidays, which started between EW 27 and 31. Since mid October, after the autumn holidays, the school-aged children began to be more affected, and the proportion increased from 16% in the initiation period to 43·8% in the acceleration period.[3] In Australia, 55% of H1N1 cases were school aged children (5–17 years), with a median age of 16 years (29% of cases were aged 13–17 years and, and 26% between 5–12 years).[4] In Canada, the infection rate was highest in this group.[5] In Chile, the incidence rate was 4500/100 000 inhabitants, although in general they had mild desease.[6]

School closure can operate as a proactive measure, aimed at reducing transmission in the school and spread into the wider community, or reactive, when the high levels of absenteeism among students and staff make it impractical to continue classes. The main health benefit of proactive school closure comes from slowing down the spread of an outbreak within a given area and, thus, flattening the peak of infections. This benefit becomes especially important when the number of people requiring medical care threatens to saturate health care capacity. It has its greatest benefits when schools are closed very early in an outbreak, before 1% of the population falls ill. School closure can reduce the demand for health care by an estimated 30–50% at the peak of the pandemic under ideal conditions, but too late in the course of a community-wide outbreak, the resulting reduction in transmission is likely to be very limited. Policies for school closure need to include measures that limit contact among students when they are not in school.[7]

Location

Tucumán is placed in northwest Argentina and has a total area of 22 524 km^2. The population (2001 Census, projection 2009) was 1 493 488 inhabitants; of wich 421 638 were 5–19 years old. The health system of the province is composed of 3 sectors: public, private, and welfare. It has a total of 91 health facilities with internement available and an average of 3 ‰ inhabitants.

Influenza-like illness (ILI) has seasonal and endemic behavior in this province, as evidenced by past records from the National Health Surveillance System and Influenza Sentinel Surveillance Unit of the province. An increase of ILI was reported in 2009, with a peak in the EW 28.

The objectives were:

General objective

To describe the behavior of the Influenza A H1N1 2009 epidemic in school aged children from the province of Tucumán, Argentina.

Specific objectives

- To explore the response to preventive measures by school aged population.
- To assess the effect of the suspension of classes in this group.
- To estimate the magnitude and severity of the disease.
- To observe the effect of co-morbidities in this group.

Materials and methods

A Cross-sectional study was executed from May to December 2010. Data were gathered through mandatory reporting forms, wich were collected from all public and private health centers. Inclusion Criteria: Patients with compatible symptoms with Influenza A; school aged children 5–17 years old. Exclusion Criteria: Patients treated with antiv-

iral medication as prophylaxis, respiratory pathologies which did not justify specific antiviral medication, and incomplete forms.

Definitions

- Suspected case of ILI: cases considered by clinical criteria (Fever higher than 38°C, cough or sore throat. It may or may not be accompanied by asthenia, myalgia or prostration, nauseas or vomiting, rhinorrhea, conjunctivitis, adenopathy, or diarrhea).
- Confirmed case: person with positive laboratory results for Influenza A H1N1 or unspecificed Influenza A (by laboratory results through rRT- PCR or immunofluorescence techniques).
- Dismissed case: by negative or different laboratory results, or different clinical evolution.
- Comorbidities: Chronic illnesses like arterial hypertension, diabetes, asthma, recurrent obstructive bronchial syndrome (ROBS), smoking, chronic obstructive pulmonary disease (COPD), immunosuppression, HIV/AIDS, cancer, nephropathy, obesity; pregnancy was also considered.

Data were analyzed using Epi2000 software (Epi Info™ CDC, Atlanta, EEUU). Rates were calculated and RR was estimated with their respective confidence interval (CI). Population data were taken from 2001 National Census projections. An estimation based on the same census was used for the group between 5 and 17 years old. To observe the effects of other co-variables, the OR and their CI were calculated. Logistic regression was used to evaluate the influence of the comorbidities. X^2 was used to compare proportions.

Laboratory diagnosis of influenza

Respiratory samples (nasopharyngeal and faryngeal swabs) were obtained. They were analyzed at Influenza Sentinel Surveillance Unit of Tucumán, and/or sent to National Reference Laboratory Dr. C. Malbrán (RT-PCR).

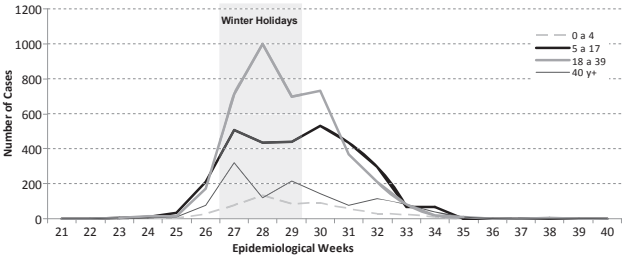
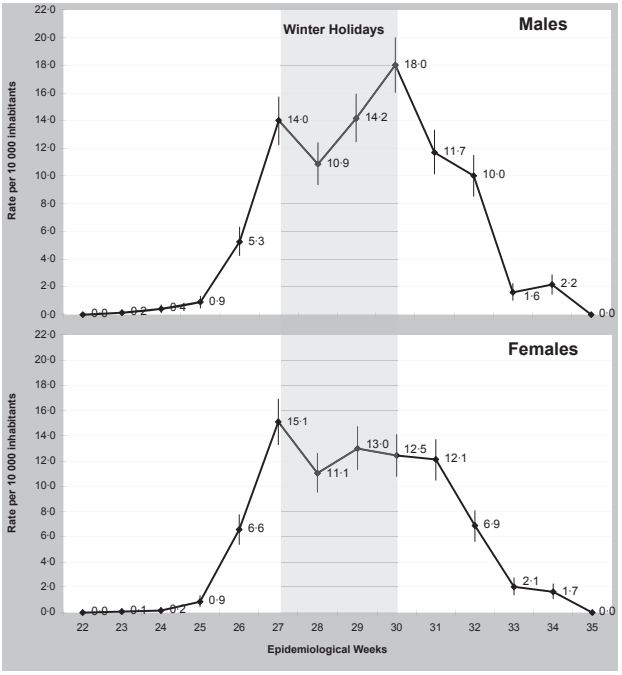


Figure 1. Epidemic curve of Flu per age group and epidemiological week. Tucumán, Argentina. May–December 2009. Source: Epidemiology Department, Tucumán.



Source: Epidemiology Department. Tucumán

Figure 2. Epidemic curve of Flu rates (CI 95%) per sex and epidemiological week in the group 5–17 years old. Tucumán, Argentina. May–December 2009. Source: Epidemiology Department, Tucumán.

Results

From all notifications (20 212), 6342 were cases of ILI in the group aged between 5 and 17 years old, 53% (3340/6342) of which were males. The incidence rate was 17·4, and it differed according to the sexes: 18·0 males and 16·7 females per thousands of inhabitants ($P < 0{\cdot}05$).

Of all Laboratory samples (370) 13% were confirmed as Influenza H1N1, 34% were confirmed as unspecificied Influenza, and 49% were dismissed. The remaining percentage corresponded to the isolation of other viruses (Parainfluenza, Respiratory Syncytial Virus, and Adenovirus).

The school aged group had higher risk of getting sick, in relation to the rest of the population (RR 1·78 [95% CI 1·55–2·04]), especially males (RR 1·97) compared with females (RR 1·66). The highest attack rate was observed in the capital of Tucumán (42/1000 inhabitants).

According to the rest of the population, it looked like being school aged children meant a protective factor for presenting SARI (Severe Acute Respiratory Infection) (OR 0·66 [95% CI 0·66–0·89], $P < 0{\cdot}05$). The lethality rate was 9·32/10 thousand. The risk of dying was low compared to other ages. Persons with comorbidities had significantly higher risk of presenting SARI (OR 1·8 [95% CI 1·35–2·58], $P < 0{\cdot}05$) and of dying (OR 8·1 [95% CI 19·6–3·34], $P < 0{\cdot}05$). Respiratory comorbidities were the most fre-

quent: asthma 2·5% (162/6342) and 2% RORS (122/6342). The symptoms headaches, myalgia, coryza, and sore throat were very common and significantly different ($P < 0·05$) than the rest of the population.

If we compared the group aged 5–17 years with 18–39 years old, the epidemic curve of the first group showed a decrease in the EW 28, coinciding with winter holidays (EW 27) (Figure 1). There was a slight increase in the tendency when classes began, but it showed a clear declination afterwards.

The analysis of rates in school aged children by EW showed a reduction of 22·2% in males and 26·6% in females ($P < 0·05$) at EW 28. However, after the first week of winter holidays, the curve in males had a significant increased to 65·6% compared to EW 27, reaching the highest weekly rate of the epidemic (18/10 000 inhabitants). The reopening of classes coincided with a significant decrease of the rate (34·9%), from 18 to 11·7/10 000 inhabitants in EW 31 ($P < 0·05$). In females, the school closure coincided with a plateau-shaped curve, and the reopening with a significant decrease of 43·4% of the rate, from 12·1 to 6·9 in EW 32 (Figure 2).

Discussion

The school children got sick a lot more than the rest of the population, although they presented less proportions of SARI. However, comorbidities were determined in order to present SARI or death. Symptoms like headache, myalgia, coryza, and sore throat were considered more conducting for the definition of cases in this population in Tucumán.

The epidemic curve was different in males compared to females during the winter holidays. The beneficial effect of school closure was observed as long as persons met the recommendations. The difference between males compared to females during winter holidays could mean that women would have carried out social distance recommendations much better, for example, remained at home. The significant reduction after the opening of classes is a factor to be considered as an effective intervention in the declining stage of the curve.

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A serial cross-sectional serologic survey of 2009 Pandemic (H1N1) in Hong Kong: implications for future pandemic influenza surveillance

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Introduction

Data on antibody levels to 2009 pandemic influenza A/H1N1 (pdmH1N1) virus from a population can be used to estimate the number of infections in the community. In June 2009, we established a comprehensive serologic survey of pdmH1N1 in Hong Kong in which blood samples were collected from the community on a weekly basis. Facilitated by enhanced local laboratory capacity developed since the 2003 epidemic of Severe Acute Respiratory Syndrome, Hong Kong used extensive laboratory testing for pdmH1N1 for all hospitalizations with respiratory illness throughout the 2009 influenza pandemic. Here, we report pdmH1N1 infection attack rate (IAR) during the first wave of the pandemic. We used our IAR estimates to infer the severity of the pandemic strain, including the age-specific proportion of infections that led to laboratory confirmation, hospitalization, intensive care unit (ICU) admission, and death.[1–4] Part of these results are now available in Ref.[5]

Materials and methods

Subjects

Blood donors, 16–65 years old

Starting from 12 June 2009, blood donors from the Hong Kong Red Cross Blood Transfusion Service (BTS) were invited to participate in our serologic survey. Eligible donors were healthy adults aged between 16 and 65 years old. A total of 12 217 serum samples collected between 12 June and 31 December 2009 were tested. Blood donors do not receive any remuneration or compensation.

Hospital outpatients, 5–59 years old

Between 2 September and 31 December 2009, we invited patients visiting the Pediatric and Adolescent Medicine outpatient clinic and the Medicine outpatient clinic at Queen Mary Hospital to participate in our serologic survey. Patients with acute respiratory infections or immunosuppression (including patients on chemotherapy for various malignancy, post-transplant or cirrhotic patients or any patients on systemic immunosuppressants) at recruitment were excluded from participation. A total of 2520 serum samples collected between 2 September and 31 December 2009 were tested.

Subjects of a community study, 5–14 years old

Between 1 November 2008 and 31 October 2009, we conducted a cohort study of pediatric seasonal influenza vaccination and household transmission of influenza. One hundred fifty-one children aged 5–14 were recruited and provided baseline sera in November and December 2008. Between September and December 2009 a further 766 children aged 5–14 were recruited and provided baseline sera for the second phase of the study. For this serologic survey, we tested the 151 sera collected before the first wave and the 766 sera collected after the first pandemic wave.

Informed consent

Written informed consent was obtained from all participants. Parental consent was obtained for participants aged 15 or younger, and children between the ages of 8 and 15

gave written assent. All study protocols were approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

Data on hospitalization, intensive care admission and death

Age-stratified data on virologically confirmed outpatient consultations, hospitalizations, ICU admissions, and deaths associated with pdmH1N1 from 29 April 2009 to 15 November 2009 were provided by the Hong Kong Hospital Authority (the e-flu database).[6] Since May 2009, patients admitted with acute respiratory illnesses routinely underwent laboratory testing for pdmH1N1 virus by molecular methods.

Laboratory methods

Sera were tested for antibody responses to A/California/4/2009 by viral microneutralization (MN).[7]

Outcomes

Most individuals infected with influenza develop antibody titers ≥1:40 by viral microneutralization after recovery.[8] We defined the pdmH1N1 seroprevalence rate as the proportion of individuals who had antibody titers ≥1:40. While MN antibody titers of ≥40 are not by themselves conclusive evidence for pdmH1N1 infection, we have assumed that the increase in cross-sectional seroprevalence between the pre-and post-first wave time periods are evidence of recent pdmHN1 infection. The IAR was defined as the proportion of individuals infected by pdmH1N1 during the first wave. The case-confirmation rate (CCR), case-hospitalization rate (CHR), case-ICU-admission rate (CIR), and case-fatality rate (CFR) were defined as the proportion of pdmH1N1 infections that led to laboratory-confirmation, hospitalization, ICU admission, and death. Due to containment efforts until June 29, 2009 all laboratory-confirmed cases were required to be hospitalized for isolation regardless of disease severity. As such, only surveillance data from June 30 onwards were used to estimate severity measures.

Statistical methods

We estimated the IAR as the difference between the pre-first-wave and post-first-wave seroprevalence rate. We used the estimated IAR as the denominator for calculating the CCR, CHR, CIR, and CFR. We used an age-structured SIR model with 5 age classes (0–12, 13–19, 20–29, 30–59, and ≥60) to describe the transmission dynamics of pdmH1N1 in Hong Kong between 10 June and 15 November 2009. We assumed that the mean generation time was 2·3 days. Using the age-structured transmission model, we estimated the following transmission parameters from the serial cross-sectional serologic and hospitalization data: (i) R_o, the basic reproductive number; (ii) π_1 and π_2, the reduction in within-age-group transmission for 0–12 and 13–19 years old during summer vacation (compared to school days during September–December 2009); (iii) D_R, the average time for neutralization antibodies titer to reach ≥1:40 after recovering from infection; (iv) h_a, the age-specific relative susceptibility with 20–29 years old adults as the reference group. We assumed non-informative priors for all parameters and used Monte Carlo Markov Chain methods to obtain posterior distributions of the parameters.

Table 1. Estimated proportion of the population with antibody titers ≥1:40 by viral microneutralization against pdmH1N1 before and after the first wave, and the estimated infection attack rate using the serial cross-sectional method

	Before the first wave			After the first wave			Infection attack rate
Age Groups	No. positive/ No. specimens	% +ve (95% CI)	Source	No. positive/ No. specimens	% +ve (95% CI)	Source	Posterior mode (95% credible interval)
5–14	0/151	0·0 (0·0–2·4)	[1]	183/422	43·4 (38·6–48·2)	[3]	43·4 (37·9–47·6)
15–19	3/97	3·1 (0·6–8·8)	[2]	34/180	18·9 (13·5–25·4)	[4]	15·8 (8·2–22·1)
20–29	12/336	3·6 (1·9–6·2)	[2]	135/879	15·4 (13·0–17·9)	[4]	11·8 (8·4–14·7)
30–39	17/302	5·6 (3·3–8·9)	[2]	67/676	9·9 (7·8–12·4)	[4]	4·3 (0·9–7·5)
40–49	10/238	4·2 (2·0–7·6)	[2]	45/509	8·8 (6·5–11·7)	[4]	4·6 (1·0–7·9)
50–59	6/352	1·7 (0·6–3·7)	[2]	14/247	5·7 (3·1–9·3)	[4]	4·0 (1·1–7·5)
Overall 5–59	3·3 (2·8–4·7)			14 (13·0–15·4)			10·7 (9·0–12·0)

Sources of specimens:
[1] Pediatric cohort study (2–29 April 2009).
[2] Hong Kong Red Cross Blood Transfusion Service (15–22 June 2009).
[3] Pediatric cohort study (6 November to 19 December 2009).
[4] Hong Kong Red Cross Blood Transfusion Service (1 November to 6 December 2009).

Table 2. Estimated age-specific proportions of individuals with pdmH1N1 infections that were laboratory-confirmed, were hospitalized, were admitted to ICU, and died. Case-ICU and case-fatality rates are expressed as number of episodes per 100 000 infections

Age group	Case-confirmation (%)		Case-hospitalization (%)		Case-ICU (per 100 000 infections)		Case-fatality (per 100 000 infections)	
	Counts (% of all)	Posterior mode (95% cred. int.)	Counts (% of all)	Posterior mode (95% cred. int.)	Counts (% of all)	Posterior mode (95% cred. int.)	Counts (% of all)	Posterior mode (95% cred. int.)
5–14	10 060 (44·1)	4·0 (3·6–4·6)	2133 (50·2)	0·84 (0·76–0·97)	20 (19·4)	7·9 (5·2–12·6)	1 (3·8)	0·4 (0·1–2·3)
15–19	3673 (16·1)	5·4 (3·9–10·5)	522 (12·3)	0·77 (0·53–1·50)	9 (8·7)	13·3 (7·0–34·4)	2 (7·7)	3 (0·9–13·2)
20–29	4314 (18·9)	3·8 (3·0–5·3)	532 (12·5)	0·47 (0·37–0·66)	7 (6·8)	6·1 (3·0–13·9)	1 (3·8)	0·9 (0·2–5·2)
30–39	1868 (8·2)	4·0 (2·2–18·2)	378 (8·9)	0·80 (0·45–3·66)	12 (11·7)	25·4 (10·7–130·5)	4 (15·4)	8·5 (3·2–53·1)
40–49	1615 (7·1)	2·8 (1·6–12·5)	293 (6·9)	0·51 (0·30–2·26)	21 (20·4)	36·4 (18·8–171·5)	6 (23·1)	10·4 (4·4–57·2)
50–59	1264 (5·5)	2·8 (1·4–10·1)	395 (9·3)	0·87 (0·45–3·16)	34 (33·0)	75 (32·7–281·3)	12 (46·2)	26·5 (10·4–108·9)
Overall 5–59	22794 (100·0)	3·9 (3·5–6·2)	4253 (100·0)	0·73 (0·66–1·22)	103 (100·0)	17·6 (13·3–50·1)	26 (100·0)	4·4 (3·2–17)

Results

Virological surveillance data suggested that the first wave of pdmH1N1 in Hong Kong occurred from August to October 2009. Most of the laboratory-confirmed infections in this first wave occurred in individuals aged below 25 years old accounting for >72% of the lab-confirmed cases and hospitalizations, 32% of ICU admissions, and 6% of deaths. Taking into account a delay of 2–3 weeks for antibody titers to appear during convalescence,[8] we found that these virological surveillance data were consistent with our serial cross-sectional seroprevalence data, which indicated a sharp rise in seroprevalence among the 5–25 years old from September to November and a plateau thereafter (data not shown).

Among individuals aged 5–14 years, the seroprevalence rates were similar across time between pediatric outpatient subjects and pediatric cohort study subjects (data not shown). Similarly, for older age groups, the seroprevalence rates were largely similar between blood donor subjects and hospital outpatient subjects (except for the 20–29 years old in November–December). This provided some evidence that despite biases in our convenience sampling scheme, the resulting serologic data provided a reasonably representative description of seroprevalence in the community.

The estimated pre- and post-first-wave seroprevalence rates and the corresponding IAR estimates are shown in Table 1. The severity estimates (CCR, CHR, CIR, and CFR) are shown in Table 2. In summary, we estimated the IAR was 43·4% among 5–14 years old, 15·8% among 15–19 years old, 11·8% among 20–29 years old, 4·3% among 30–39 years old, 4·6% among 40–49 years old, and 4·0% among 50–59 years old. Overall, we estimated a population-weighted IAR of 10·7% (9–12%) among individuals aged 5–59 years through the first wave in Hong Kong. CCR were around 2·8–5·4% among the 5–59 years old. CHR were around 0·47–0·87% among the 5–59 years old. CIR increased from 7·9 (5·2–12·6) per 100 000 infections in 5–14 years old to 75 (32·7–281) per 100 000 infections in 50–59 years old. CFR followed a similar trend with 0·4 (0·1–2·3) death per 100 000 infections in 5–14 years old to 26·5 (10·4–109) deaths per 100 000 infections in 50–59 years old. Compared to children aged 5–14, adults aged 50–59 were 9·5 and 66 times more likely to be admitted to ICU and die if infected.

The best-fit age-structured transmission model gave the following parameter estimates:

1. The basic reproductive number was 1·38 (95%CI, 1·36–1·41).
2. It took an average of 14 (9–21) days for recovered individuals to develop neutralization antibody titer ≥1:40.

3. Compared to 20–29 years old, 0–12 years old children and 13–19 teenagers were 3·7 (3·2–4·5) and 1·6 (1·3–2) times more susceptible to pdmH1N1 infection, respectively.
4. Compared to 20–29 years old, 30–59 years old older adults and 60–79 years old elderly were only 0·42 (0·3–0·6) and 0·33 (0·18–1·5) times as susceptible as the 20–29 years old, respectively.
5. Compared to the school period during September–December 2009, summer vacation reduced within-age-group transmission by 61% (53–72%) among 0–12 years old, but only 12% (3–18%) among 13–19 years old.

Using computer simulations, we estimated that if pre-existing seroprevalence is zero, real-time serologic monitoring with about 1000 specimens per week would allow accurate estimates of IAR and severity as soon as the true IAR has reached 2% (data not shown).

Discussion

We estimated that during the first wave in Hong Kong, 43·4% of school-age children and 10·7% of individuals aged 5–59 were infected by pdmH1N1. A serologic survey in England found similar IARs in London and the West Midlands.[8] Both studies highlight the importance of including serologic surveys in pandemic surveillance. The geographically compact and well-mixed population in the urban environment of Hong Kong permits some degree of confidence in the validity of our IAR and severity estimates. The completeness of the pdmH1N1 surveillance system, well-defined population denominator, and our large-scale serologic survey provide accurate numerators and denominators for the severity measures.

We based severity estimates for pdmH1N1 on the IAR as the denominator. In most previous studies of pdmH1N1 severity, the denominator was clinical illness attack rate, which depends on the probability of symptoms as well as medical care seeking behavior of the population.[2,9] Our estimated CIRs and CFRs are broadly consistent with Presanis *et al.*'s[2] 'Approach 2' severity estimates, but around 7–9 times lower than their 'Approach 1' estimates. Our estimates of CHR are 2–10 times higher than their Approach 2 estimates of symptomatic CHR. However, the hospitalization-death ratio was $4253/27 = 164$ as of November 15 in Hong Kong, but $996/53 = 19$ as of June 14 in New York,[2] suggesting that the clinical threshold for admission in terms of disease severity at presentation may have been lower in Hong Kong.

Our study has a number of limitations. First, we have used antibody titers of ≥1:40 by viral microneutralization as an indicator of recent infection, correcting for pre-existing seroprevalence levels, but this may lead to underestimation of the IAR if some infections led to antibody titers <1:40, or if some individuals with baseline titers ≥1:40 were infected. Second, our estimates of the IAR would be biased upwards if infection with other circulating influenza viruses led to cross-reactive antibody responses resulting in antibody titers ≥1:40. However between August and October 2009, 83% of influenza A viruses detected in Hong Kong were pdmH1N1, and only 3% of isolated viruses were seasonal H1N1 viruses.[10] Third, a minority of severe illnesses associated with pdmH1N1 infection might not be identified by molecular detection methods, for example if admission occurred after viral shedding from the primary infection has ceased, in which case we may have underestimated the disease burden of pdmH1N1. Finally, our analyses are primarily based on seroprevalence among blood donors to the Hong Kong Red Cross, who may not be representative of the whole population. We do not have detailed data on donors to compare their risk of infection with the general population, but we did observe very similar seroprevalence rates across the three groups of subjects in our study, i.e., blood donors, hospital outpatients and participants in a community cohort (data not shown).

In conclusion, around 10·7% of the population aged 5–59 and half of all school-age children in Hong Kong were infected during the first wave of pandemic H1N1. Compared to school-children aged 5–14, older adults aged 50–59, though less likely to acquire infection, had 9·5 and 66 times higher risk of ICU-admission and death if infected. Thus, although the IAR of pdmH1N1 is similar to that of a seasonal epidemic, the apparently low morbidity and mortality of 2009 pandemic influenza (H1N1) appears to be due to low infection rates in older adults who had a much greater risk of severe illness if infected. The reasons why older adults appear relatively resistant to pdmH1N1 infection even though they appear to lack neutralizing antibody remains unclear. If antigenic drift or other adaptation of the pdmH1N1 virus allows these older age groups to be infected more efficiently, the morbidity and mortality of subsequent waves of the pandemic could yet become substantial.

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Disclosure

Some data presented in this manuscript were previously published in Wu *et al.*[5]

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Virus-specific CD4$^+$ and CD8$^+$ memory T-cells in young volunteers after immunization with pandemic live attenuated reassortant influenza vaccines A (H5N2) and A (H1N1)

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Background

It is well known that a primary goal of vaccination is to generate immunological memory against the targeted antigen to prevent disease in a vaccinated person. This ensures an accelerated immune response in the event of future contact with the pathogenic agent, such as a virus. Therefore, it is very important to develop criteria for the assessment of vaccine immunogenicity by measuring both T and B memory cell levels from the vaccinated host. In contrast to inactivated influenza vaccines, live attenuated influenza vaccines (LAIVs) have been shown to provide primarily cellular and local immune responses.[1–3] To date, however, the hemagglutination-inhibition (HAI) test (i.e. detection of serum antibodies) remains the method widely accepted for evaluation of an influenza vaccine's immunogenicity. Improved understanding of the role of cellular and mucosal immunity and their contribution to protecting against severe illness caused by influenza infection has emphasized the need to reconsider methodologies used to evaluate the immunogenic impact of various influenza vaccines. Such new assays need to include methods to measure local antibodies and virus-specific lymphocytes, especially in the case of live attenuated influenza vaccines, because of their potential to induce such broad-based immune responses.[4]

The aim of this study was to assess the ability of new Russian pandemic LAIVs A/17/Duck/Potsdam/86/92 (H5N2) ('Ultragrivak,' registered 25·03·2009) and A/17/California/2009/38 (H1N1) ('Influvir,' registered 13·10·2009) to induce memory T-cells in naïve human subjects and to compare results to levels of HAI antibodies from each subject.

Materials and methods

Vaccines

A/17/Duck/Potsdam/86/92 (H5N2) LAIV was generated by 7:1 genetic reassortment of low-pathogenic avian influenza virus A/Duck/Potsdam/1406-86(H5N2) and master donor strain A/Leningrad/134/17/57 (H2N2).[5,6] The vaccine strain contains HA gene from avian virus, as well as NA and internal genes from the master donor virus. A/17/California/2009/38 (H1N1) LAIV was generated by classical (6:2) reassortment of A/California/07/2009 (H1N1) with the master donor virus.[7] The vaccine strain contains HA and NA genes from a 'wild-type' H1N1 strain and internal genes from the master donor virus.

Volunteers and vaccination

Participants were aged 18 to 20 years and were without contra-indication of LAIV vaccination. Immunogenicity of A/17/Duck/Potsdam/86/92 (H5N2) LAIV was assessed in ten vaccinated persons and ten volunteers inoculated with a placebo (sterile physiological saline solution). Immunogenicity of A/17/California/2009/38 (H1N1) LAIV was estimated in 16 vaccinated volunteers and nine volunteers inoculated with placebo. Viruses or placebo were administered intranasally twice with an interval period of 21 days at a dosage of 0·25 ml per nostril for each vaccination. Physical examination, venous blood and nasal swab samples were collected at four time points during the study: (i) before vaccination (day 0); (ii) 21 days after first vaccination (day 21); (iii) 21 days after the second vaccination (day 42); and (iv) 6 weeks after the second vaccination (day 63).

Serum HAI antibodies were measured by standard HAI assay[8] using 1% human red blood cells. Test antigens for the assay were A/17/Duck/Potsdam/86/92 (H5N2) or A/17/California/2009/38 (H1N1) to match the appropriate vaccine antigen.

Local IgA antibodies in nasal swabs were evaluated by ELISA[9] using whole purified A/17/Duck/Potsdam/86/92 (H5N2) or A/17/California/2009/38 (H1N1) viruses at 16 HAU per 0·05 ml for absorption to ELISA plates. Endpoint ELISA titers were expressed as the highest dilution of sera that gave an optical density (OD) greater than twice the mean OD of six negative controls in the same assay.

Percentages of virus-specific CD3$^+$ CD8$^+$IFN-γ^+ and CD3$^+$ CD4$^+$IFN-γ^+ peripheral blood memory cells were determined using a flow cytometry ICCS assay performed by the published method.[3] PBMCs were prepared with standard Histopaque-1077 gradient centrifugation from heparinized whole blood.

Statistics

Wilcoxon matched pair test, Mann–Whitney *U* test and the Students *t*-test were used for statistical data analysis.

Results

Prior to the first vaccination (day 0), GMTs of HAI antibodies to A/17/Duck/Potsdam/86/92 (H5N2) and A/17/California/2009/38 (H1N1) LAIVs were 1/5·4 and 1/5·7, respectively. In addition, GMTs of sIgA against these specific antigens from nasal swabs were 1/5·7 and 1/9·1, respectively. No HAI antibody titers greater than 1:40 were observed prior to vaccination. Background levels of virus-specific T-cells varied significantly within groups. Mean levels of virus-specific CD8$^+$IFNγ^+ cells were 0·151% to A/17/Duck/Potsdam/86/92 (H5N2) and 0·173% to A/17/California/2009/38 (H1N1). For CD4$^+$IFNγ^+ cells, initial levels were 0·240% and 0·336%, respectively.

Thus, background levels of virus-specific antibodies were low, but prior vaccination or virus exposure in some volunteers produced some pre-existing levels of T cells, thus they were not absolutely immunologically naïve in this sense. Preexistence of H5N1-crossreactive antibodies and T-cells has been observed previously.[10–12]

Effect of vaccination

Antibody immune responses

Both influenza A (H5N2) and influenza A (H1N1) LAIVs stimulated production of serum HAI antibodies and local IgA antibodies in nasal swabs. Following the first vaccination with influenza A/17/Duck/Potsdam/86/92 (H5N2) LAIV, 20% percent of volunteers exhibited seroconversion of HAI antibodies; after the second vaccination, 30% of volunteers exhibited seroconversion. After the first vaccination, a 10% conversion rate of sIgA was observed; after the second vaccination, 60% showed conversions in levels of sIGA. The first vaccination with A/17/California/2009/38 (H1N1) LAIV showed 6·3% of HAI antibodies seroconversions vaccination, and 50% seroconversion after second vaccination. For local sIgA, those results were 18·8% and 31·3% following the first and second inoculation, respectively.

Cellular immune responses

Figure 1 summarizes cellular immune responses observed in the vaccinated versus the placebo group. After the influenza A (H5N2) LAIV inoculation, significant differences in both CD4 and CD8 IFNγ-producing T-cells were observed at day 42 after the second vaccination (d63). These data indicate that healthy young people who never received such avian influenza vaccines and were not exposed to H5N1 wild-type viruses were able to respond to the live attenuated H5N2 influenza vaccine.

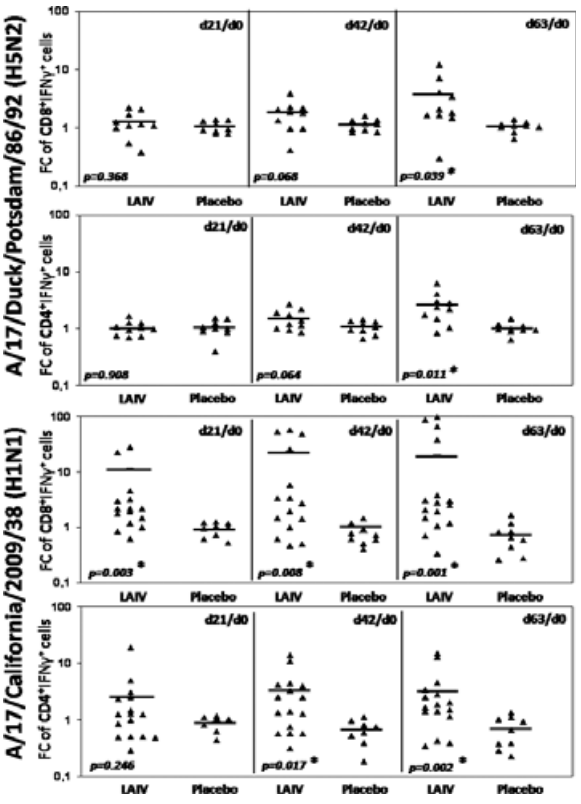

Figure 1. Differences in LAIV-induced fold changes (FC) of CD3+CD8+IFNγ+ and CD3+CD4+IFNγ+ T-cells versus Placebo FC = % of cells after vaccination/% of cells before vaccination (Day 0). d21/d0 – 21 days after first vaccination; d42/d0 – 21 days after the second vaccination; d63/d0 – 6 weeks after the second vaccination. Triangles – individual FC data. Bars – mean FC levels. * – statistically significant data.

Table 1. FCs of CD4 and CD8 T-cells after homological and heterological stimulation of PBMC from volunteers vaccinated with A (H5N2) live attenuated influenza vaccines

Cells	Group	Day	Mean FC after homological or heterological stimulation (mean and dispersion)	
			A/17/Duck/Pots-dam/86/92 (H5N2)	A/17/Solomon Islands/06/9(H1N1)
$CD8^+$ $IFN\gamma^+$	Vaccine (n = 10)	d21/d0	1·24 (0·38 – 2·22)	1·35 (0·38 – 3·81)
		d42/d0	1·77 (0·41 – 3·89)	1·13 (0·26 – 2·46)
		d63/d0	3·56 (0·30 – 12·09)	2·49 (0·09 – 8·51)
	Placebo (n = 10)	d21/d0	1·05 (0·77 – 1·37)	1·04 (0·43 – 2·00)
		d42/d0	1·12 (0·83 – 1·59)	1·01 (0·67 – 1·53)
		d63/d0	1·05 (0·64 – 1·39)	1·12 (0·24 – 3·12)
$CD4^+$ $IFN\gamma^+$	Vaccine (n = 16)	d21/d0	1·04 (0·70 – 1·65)	1·59 (0·25 – 5·80)
		d42/d0	1·51 (0·86 – 2·70)	1·15 (0·17 – 4·25)
		d63/d0	2·60 (0·84 – 6·35)	1·71 (0·08 – 3·75)
	Placebo (n = 9)	d21/d0	1·06 (0·40 – 1·53)	0·98 (0·36 – 2·10)
		d42/d0	1·07 (0·66 – 1·44)	0·90 (0·44 – 1·53)
		d63/d0	1·03 (0·64 – 1·52)	0·76 (0·18 – 1·24)

After the first influenza A (H1N1) LAIV vaccination, reliable increases were observed in $CD8^+$ cells only. After the second vaccination, increases in both $CD4^+$ and $CD8^+$ fold changes were significantly higher in vaccinated volunteers compared to the placebo group. It is noteworthy that cellular immune responses ($CD4^+$ and $CD8^+$ cells) were more marked in the A/17/California/2009/38 (H1N1). Considering the long-term circulation of H1-subtype viruses among humans in contrast to the novelty of H5-viruses, such a result would be expected.

Similar data were also observed following vaccination with the H5N2 LAIV. After first vaccination, the percent of people with notable increases in virus-specific $CD4^+$ and $CD8^+$ T-cells was 20% and 10% to H5N2 and 38% and 75% to H1N1, respectively. After the second vaccination, these results were 40% and 30% to H5N2 and 69% and 69% to H1N1, respectively. Importantly, a significant number of vaccinated volunteers without remarkable increases (≥4-fold) in HAI antibodies had notable increases in $CD4^+$ and/or $CD8^+$ memory cells. The percent of people with notable increases in virus-specific T cells after the second vaccination among HAI(−) volunteers was 40% and 75% to H5N2 and H1N1, respectively.

These results indicate that LAIVs were able to induce broadly responsive, key antiviral immune responses that would not have been detected by the HAI assay alone. Thus, it can be deduced that HAI data alone fails to reveal important broad and specific immune responses to LAIV. Consequently, the HAI test alone is not suitable for assessment of LAIV immunogenicity. Furthermore, vaccination with H5N2 LAIV was able to induce cross-reactive memory T-cells to a seasonal vaccine strain, A/17/Solomon Islands/06/9 (H1N1) (Table 1).

Reliable increases to A (H1N1) were observed in up to 20% of volunteers. There was an inverse dependence between levels of memory T cells before and after vaccination.

Conclusions

1 There were observed crossreactive memory T-cells to A/17/Duck/Potsdam/86/92 (H5N2) and A/17/California/2009/38 (H1N1) in young human subjects prior to vaccination.
2 Immunization of young adults with new pandemic live attenuated reassortant influenza vaccines A/17/Duck/Potsdam/86/92 (H5N2) and A/17/California/2009/38 (H1N1) resulted in induction of antibody and cellular immune responses. The HAI assay data did not fully reflect the breadth or specificity of immune responses to LAIV. Therefore, the HAI assay alone was not sufficient for assessment of LAIV immunogenicity.
3 Cellular immune responses ($CD4^+$ and $CD8^+$ cells) was more marked to A/17/California/2009/38 (H1N1) than to A/17/Duck/Potsdam/86/92 (H5N2).
4 Vaccination with A/17/Duck/Potsdam/86/92 (H5N2) LAIV caused the induction of crossreactive T-cells to the seasonal strain A/17/Solomon Islands/06/9 (H1N1).

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Impact of primary influenza infection on the immune response to secondary bacterial infection in aged mice

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Abstract

Background: Increased susceptibility of older populations to secondary bacterial pneumonia-like infections following influenza infection has been well documented.[1] Recent evidence in mouse models suggests that this increased risk from secondary bacterial infection occurs through a desensitization of the innate immune response.[2] This recent finding, however, does not account for potential differences in immune responsiveness due to age. Materials and methods: To address this parameter, we used three age groups (aged, adult, and young mice) to evaluate the role of age in influenza-mediated vulnerability to secondary bacterial challenge with *Pseudomonas aeruginosa*. All mice were evaluated for multiple parameters including: (i) survival; (ii) lung bacterial load; (iii) total lung protein content; (iv) immune cell infiltration; (v) cytokine/chemokine expression; and (vi) toll-like receptor (TLR) RNA expression profiles. Results: Prior challenge with influenza contributed to aberrant cytokine/chemokine profiles and increased lung cellular infiltrate in response to secondary bacterial infection across all age groups, supporting a critical role for influenza infection in the alteration of immune responses to other pathogens. Also similar to human influenza, these changes were exacerbated by age in mice as demonstrated by increased bacterial load, mortality, and total lung protein content (an indicator of lung damage) after *P. aeruginosa* challenge. Conclusions: These data support a potential role for virus-mediated and age-mediated alteration of innate immune effectors in the pathogenesis of influenza and the increased susceptibility of influenza virus infected mice to secondary bacterial infection. The understanding of the complex interaction of host and pathogen – and the role of age – in human influenza is critical in the development of novel therapeutics and improved vaccine approaches for influenza. Our results support further examination of influenza-mediated alterations in innate immune responses in aged and non-aged animals to

allow elucidation of the molecular mechanisms of influenza pathogenesis in humans.

Introduction

There is considerable evidence in the clinical literature to support the role of influenza infections with an enhanced risk for secondary bacterial pneumonias.[3–5] Given the increased pneumonia-related morbidity and mortality in both the young and elderly populations, there is rationale for gaining a deeper understanding as to the systemic changes in the pulmonary microenvironment. Although there are some recent reports that account for some of the molecular mechanisms at work in this disease process,[2] there is a paucity of experimental evidence that considers the potential effects of age. Developmental changes in the immune system that occur in the aged environment have been well documented with regard to senescence of the adaptive immunity, global changes in myeloid cell function, and the establishment of a general pro-inflammatory state.[6,7] The aim of this work was to provide evidence for the contribution of the aged immune environment to the pathology of influenza mediated secondary bacterial infections.

Materials and methods

Animals used in this study were housed under conditions approved by Tulane University's Institutional Animal Use and Care Committee. Female Balb/C mice used in these studies were divided into three age groups: aged (18 months old), adult (6 months old), and young (2 months old). Each age group was subdivided into two groups: influenza infected and naïve (control). Mice were infected by the intranasal route with 4×10^5 PFU of mouse-adapted Influenza A/PR/8/34. Clinical disease was measured by body weight changes over a 6 week period post influenza challenge, and recovery was determined as return to pre-infection weight. All mice were subsequently challenged intransally with1×10^7 CFU *Pseudomonas aeruginosa* strain PAO1.

Twenty-four hours post-*Pseudomonas* challenge, BAL with sterile PBS was performed on all mice in all groups. Total RNA from the cellular fraction was pooled from three experimental animals from each group. TLR mRNA was detected by qRT-PCR, where expression levels were determined as relative to β-actin mRNA levels. cDNA was synthesized from total cellular RNA from BAL samples using iScript cDNA synthesis kit (Biorad). PCR reactions were composed of 0·1 μg cDNA forward and reverse primers according to optimized conditions and 12·5 μl of 2 × Syber Green icycler supermix (Biorad), in a total volume of 25 μl and were run using a Biorad iCycler utilizing melting point determination. Primers and concentrations used in this study included: MUS_TLR2F: TGCTTTCCTGCTGGAGATTT-600 nm, MUS_TLR2R: TGTAACGCAACAGCTTCAGG-900 nm, MUS_TLR3F: ATATGCGCTTCAATCCGTTC-300 nm, MUS_TLR3R: CAGGAGCATACTGGTGCTGA-600 nm, MUS_TLR4F: GGCAGCAGGTGGAATTGTAT-600 nm, MUS_TLR4R: AGGCCCCAGAGTTTTGTTCT-900 nm, MUS_TLR5F: CTGGGGACCCAGTATGCTAA-600 nm, MUS_TLR5R: ACAGCCGAAGTTCCAAGAGA-900 nm, MUS_TLR7F: GGAGCTCTGTCCTTGAGTGG-900 nm, MUS_TLR7R: CAAGGCATGTCCTAGGTGGT-600 nm, MUS_B-ACTINF: AGCCATGTACGTAGCCATCC-600 nm, MUS_B-ACTINR: CTCTCAGCTGTGGTGGTGAA-900 nm. As a measure of protein leakage into the alveolar space, total protein content in each BAL was measured by BCA assay of each supernatant fraction according to manufacturer's instructions (Pierce). Cytokine and chemokines levels were measured by multiplexed bead array (Bioplex, BioRad). Immune cell characterization of BAL was estimated by flow cytometry. Lymphocyte populations were gated by forward versus side scatter and characterized as B cells ($F4/80^-$, $CD19^+$) or T cells ($CD11b^-$, $CD4^+$). The myeloid population that is composed of macrophages, neutrophils, dendritic cells, and natural killer cells was enumerated by gating all but those found in the lymphocyte gate using forward versus side scatter plots. Flow cytometry data was analyzed using FloJo software (TreeStar). Statistical analysis, where appropriate, was performed using a two-way analysis of variance (age versus influenza infection status) supported by Bonferonni's correction for multiple comparisons.[8]

Results and discussion

A recent finding by Didierlaurent, *et al.*,[2] described an influenza mediated desensitization of TLR function as a primary contributor to an increase in bacterial burden when challenged after resolution of the primary influenza infection. This finding, however, was obtained using animals that were 6–8 weeks of age, where our study included two cohorts of older mice (6 months and 18 months). Using whole protein content of the BAL as an estimate of protein leakage into the lumen of the lung, we found elevated protein content in aged mice as compared to young and adult mice. In aged mice, a slightly lower total lung protein when comparing influenza infected to protein in the BAL from influenza naïve mice challenged with *P. aeruginosa* (Table 1).

Supporting previously published studies showing a generalized pro-inflammatory cytokine environment in the aged immune system, we provide evidence for significantly

Table 1. Summary of experimental findings

		Control			Influenza		
Endpoint	**Age**	**Mean**	**SD**	***N***	**Mean**	**SD**	***N***
BAL total protein (μg/ml)	Young	2·02E+03	1·8E+02	15	1·99E+03	8·0E+01	14
	Adult	2·04E+03	1·4E+02	4	1·81E+03	7·4E+01	6
	Aged	3·14E+03	1·6E+02	6	2·49E+03	3·1E+02	9
Pseudomonas aeruginosa	Young	4·17E+06	6·6E+06	15	2·57E+06	3·8E+06	13
bacterial load	Adult	1·51E+06	1·4E+06	4	3·40E+07	4·7E+07	2
	Aged	1·76E+07	1·2E+07	6	4·24E+07	4·8E+07	8
Cytokine/chemokines (pg/ml)							
GM-CSF	Young	9·31E+02	7·6E+02	4	7·18E+02	2·3E+02	4
	Adult	3·59E+02	3·3E+02	4	1·26E+02	1·9E+02	4
	Aged	2·58E+03	7·3E+02	4	2·18E+03	8·8E+02	4
TNF-α	Young	2·05E+03	7·2E+02	4	2·17E+03	8·5E+02	4
	Adult	1·57E+03	1·0E+03	4	1·16E+03	8·0E+02	4
	Aged	5·66E+03	2·3E+03	4	5·34E+03	3·1E+03	4
IFN-γ	Young	1·00E−05	9·5E-13	4	9·08E-01	2·1E+00	4
	Adult	1·25E+00	2·8E+00	4	1·01E+00	2·5E+00	4
	Aged	1·42E+01	1·7E+01	4	4·53E+01	4·7E+01	4
IL-5	Young	3·96E+01	9·7E+00	4	4·04E+01	1·8E+01	4
	Adult	1·04E+01	6·3E+00	4	2·01E+01	1·3E+01	4
	Aged	8·02E+01	5·7E+01	4	9·31E+01	5·5E+01	4
IL-10	Young	8·76E+01	7·2E+01	4	6·25E+01	2·5E+01	4
	Adult	4·74E+01	4·3E+01	4	3·29E+01	4·7E+01	4
	Aged	3·54E+02	1·0E+02	4	1·60E+02	7·1E+01	4
Inflammatory cell (% of BAL cells)							
B cells	Young	8·85E−01	3·7E-01	2	3·61E+00	3·7E+00	2
	Adult	1·09E+00	0·0E+00	1	2·17E+00	2·1E+00	2
	Aged	7·68E+00	9·1E-01	2	4·31E+00	2·3E+00	2
$CD4^+$ T cells	Young	1·25E+01	3·5E-01	2	1·57E+01	6·4E-01	2
	Adult	2·95E+00	1·8E+00	2	1·05E+01	9·7E+00	2
	Aged	1·06E+01	9·2E-01	2	1·27E+01	4·5E+00	2
Myeloid	Young	9·20E+01	1·4E+00	2	8·95E+01	7·1E-01	2
	Adult	9·15E+01	2·1E+00	2	6·85E+01	7·8E+00	2
	Aged	8·60E+01	2·8E+00	2	8·00E+01	5·7E+00	2
TLR mRNA expression (relative expression to β-actin)							
TLR2	Young	4·23E+01		3	2·57E+01		3
	Adult	1·68E+01		3	2·11E+00		3
	Aged	1·23E+02		3	8·60E+01		3
TLR3	Young	1·63E+03		3	2·23E+04		3
	Adult	1·14E+04		3	8·04E+03		3
	Aged	1·21E+04		3	1·86E+03		3
TLR4	Young	1·70E+02		3	1·69E+02		3
	Adult	9·40E+01		3	1·72E+01		3
	Aged	2·57E+02		3	1·30E+02		3
TLR5	Young	2·39E+03		3	3·55E+02		3
	Adult	5·24E+03		3	1·75E+03		3
	Aged	4·71E+04		3	6·23E+03		3
TLR7	Young	3·53E+02		3	1·23E+02		3
	Adult	2·00E+02		3	8·04E+01		3
	Aged	9·83E+02		3	3·75E+02		3

elevated cytokines/chemokines (TNFα, GM-CSF, IL-5, and IFNγ) in aged mice ($P < 0{\cdot}0001$). Previously reported[2] elevations in TNF-α expression in influenza infected animals measured at earlier time points post challenge suggest an earlier temporal expression of this cytokine not observed in our later post-challenge evaluation. There is also a significant difference in animals that have resolved influenza infection where a decreased amount of GM-CSF

($P = 0{\cdot}0465$) and an increase in IFNγ ($P = 0{\cdot}0323$) was detected. The decrease in GM-CSF correlates well with a previous report that GM-CSF is less prevalent in influenza resolved animals (Table 1).[2]

We also report a noticeable change in the immune cell populations with respect to B-cells, $CD4^+$ T-cells, and the myeloid cell populations. There is a trend of increased prevalence in CD4 T-cells in the post-influenza environment across all ages. B-cell numbers also trend toward increase in influenza treated animals in young and adult animals; however, there is a noticeable decrease in the B-cells in aged animals. Across all age groups, there is a general decrease in frequency of cells that would normally make up the myeloid cellular fraction of the BAL (macrophages, neutrophils, dendritic cells, and natural killer cells) (Table 1).

Our study also shows, as cited by others, that toll-like receptor (TLR) gene expression in the post-influenza environment is decreased in cells found in the BAL[2] after both influenza and *Pseudomonas* infection. Our data support the previous finding of a reduced expression of TLR mRNA in influenza-cleared mice when we measured TLR 2, 3, 5, and 7. Only TLR4 showed differences with respect to age with young mice showing little or no detectable change in TLR4 mRNA expression. Our results show an increase in the expression across all TLRs examined in the aged mice group (Table 1) irrespective of influenza infection status. These data support earlier studies performed with adult mice that showed reduced TLR mRNA expression in the post-influenza environment. This study also expands the current understanding of the potential role of age in influenza mediated bacterial infection-induced mortality.

The impact of these alterations in the immune microenvironment across age groups and infection status is highlighted by the ability of bacterially challenged animals to clear infection. Assessment of bacterial load in the lungs of *P. aeruginosa* challenged mice indicated a difference in young and adult mice if previously infected with influenza virus. In aged mice, both influenza challenged and influenza-naïve mice had higher bacterial loads and less variability when comparing within the age group, supporting the risk of age alone in susceptibility to bacterial pneumonia (Table 1, Figure 1).

Taken together, these data support the potential role for both virus-mediated and age-mediated alteration of innate immune effectors in the pathogenesis of influenza and increased the susceptibility to secondary bacterial infection that results from influenza infection in mice. These findings highlight distinct differences in the immune environment between age groups and thus reveal necessity for further examination as to the mechanisms of immunity across age with respect to current infection status. Garnering a clearer understanding as to the complex interaction of host and pathogen with respect to age in influenza infections is central to the development of increased efficacy in vaccine and therapeutic strategies.

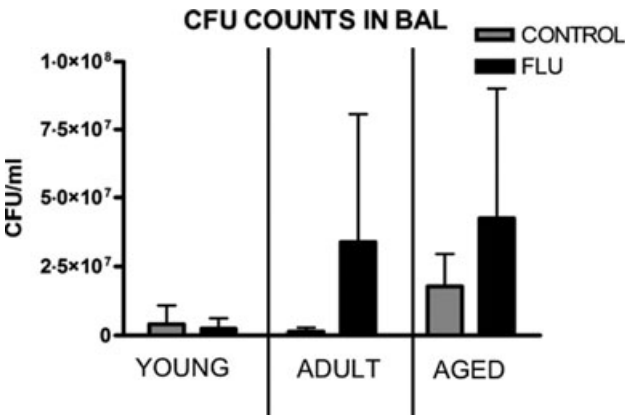

Figure 1. CFU Counts of *Pseudomonas aeruginosa* 24 h PI in BAL.

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Prospective estimation of the effective reproduction number of pandemic influenza in Hong Kong

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Keywords Hospitalisation, influenza, mathematical model, pandemic, transmissibility.

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Background

Pandemic influenza A (H1N1) virus (pH1N1) emerged in early 2009 and rapidly spread to every continent. An urgent priority for international and national public health authorities was to estimate the transmissibility of the pandemic strain for situational awareness and to permit calibration of mitigation strategies. The basic reproductive number, R_0, is defined as the average number of secondary cases that 1 index case generates in a completely susceptible population, and is a common measure of transmissibility. However, it is difficult to estimate R_0 without an understanding of the degree of any pre-existing immunity in the population. The effective reproductive number, R, is defined as the average number of secondary cases that 1 index case generates, and can be estimated over time (i.e. R_t). Wallinga and Teunis[1] described a method to estimate R_t based on illness onset dates of the cases while assuming that all secondary cases would have been detected, and Cauchemez *et al.*[2] extended the method to permit prospective estimation by adjusting for secondary cases that have not yet experienced illness onset at the time of analysis. We describe how the method can further be extended to account for reporting delays, allowing true real-time estimation of R_t during an epidemic, and we illustrate the methodology on notifications of pH1N1 and associated hospitalizations in Hong Kong.

Methods

Sources of data

We obtained data on all laboratory-confirmed pH1N1 infections ('cases') reported between May 1 and November 15, 2009 to the Hospital Authority and Center for Health Protection in Hong Kong collated in the eflu database. A subset of the cases was hospitalised. The database also included information on age, sex, illness onset date, laboratory confirmation date, and contact history (for the early cases). Laboratory-confirmed pH1N1 infection was a notifiable condition throughout our study period.

Statistical analysis

We extended existing methods for estimating R_t over time to allow for reporting delays between illness onset and notification, and between illness onset, notification, and hospitalisation for those cases that were hospitalised, where the reporting delay distribution were estimated empirically from the data.[3] We further extended the methodology to allow for imported cases (infected outside Hong Kong) contributing to the estimation of R_t as infectors but not infectees. We used multiple imputation to allow for missing data on some symptom onset dates to make best use of all available data.[4] We used a serial interval with mean (standard deviation) of 3·2 (1·3) days,[5] and in sensitivity analyses, we used serial intervals with mean 2·6 days[6] and 3·6 days.[7] Statistical analyses were performed in R version 2.9.2 (R Development Core Team, Vienna, Austria).

Results

In late April 2009 following the WHO global alert, Hong Kong initiated containment protocols to attempt to delay local transmission of pH1N1 for as long as possible. These measures included screening at ports, airports, and border crossings, and enhanced surveillance for people with influenza-like illness, particularly for those who had recently returned from abroad. Laboratory testing capacity was substantial due to heavy investment in local infrastructure following previous experiences with avian influenza A/H5N1

in 1997 and severe acute respiratory syndrome in 2003. Laboratory-confirmed pH1N1 cases were isolated until recovery, and their close contacts were placed under quarantine for 7 days. Imported cases were identified sporadically through May and early June 2009.

The first case of pH1N1 not traceable to importation (i.e. a local case) was identified on June 11 and triggered a change to mitigation phase measures. Some containment measures, including isolation of cases, were continued until the end of June to allow a soft transition between containment and mitigation phases. As an immediate measure to try to reduce community transmission of pH1N1, all childcare centres, kindergartens, and primary schools were proactively closed for 14 days (subsequently extended for another 7–14 days to summer vacation in early July).[8] Any secondary schools in which one or more confirmed pH1N1 case was identified were reactively closed for 7 days. On June 13 the government opened eight designated flu clinics across the territory to provide free medical consultation for outpatients with influenza-like illness and free laboratory testing for pH1N1. These clinics resumed regular chronic disease services in mid-August, and laboratory testing and antiviral treatment was restricted to high risk groups in September. The various interventions are highlighted in Figure 1(A), superimposed on the epidemic curve of laboratory-confirmed pH1N1 cases and pH1N1-associated hospitalizations. Around 15% of the cases were hospitalised, and this proportion increased somewhat towards the end of the epidemic.[3]

Figure 1(B) shows the estimates of R_t based on laboratory-confirmed pH1N1 cases. The estimated R_t peaked at 1·5 on June 12, and fell below 1 between 20 June and 3 July (which was within the school closure period). R_t fluctuated between 0·8 and 1·3 through the school summer vacations in July and August, it subsequently increased to around 1·2–1·3 after schools reopened in September until the epidemic peaked in late September, and then fluctuated below 1 as the epidemic declined. The trends in R_t based on H1N1-associated hospitalizations were similar, although with wider confidence intervals due to the smaller number of events (Figure 1C). The extension of the methods to allow for reporting delays avoided substantial bias in real-time estimates of R during the epidemic for the most recent 7 days, and closely tracked the final estimates of R_t.[3]

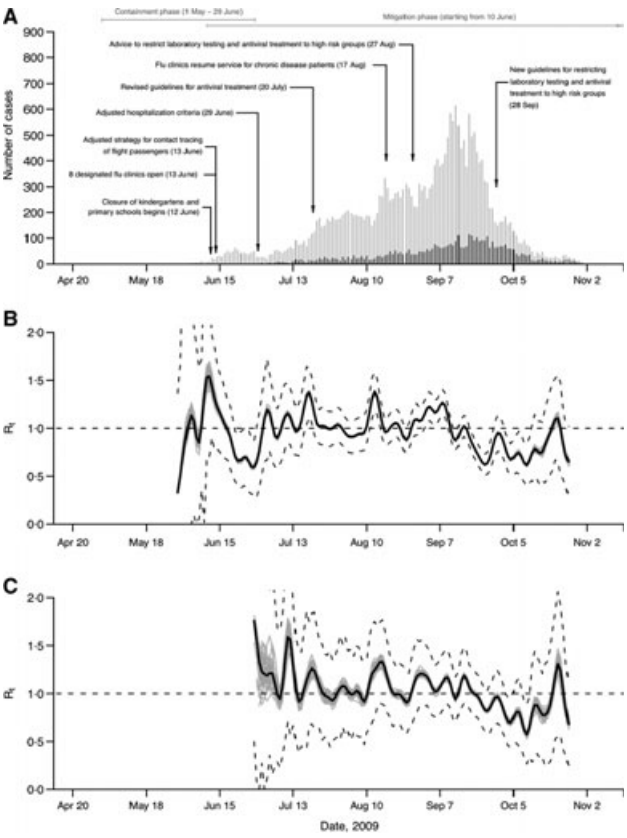

Figure 1. (A) Number of cases of laboratory-confirmed cases of pandemic influenza A (H1N1) virus infection (grey) and hospitalizations (black) by date of illness onset and dates of important control measures, Hong Kong, from April through October 2009. (B) Daily estimates of the effective reproduction number R_t based on pandemic H1N1 notifications with 95% confidence intervals, where the dashed line represents the threshold of $R_t = 1$. (C) Daily estimates of the effective reproduction number R_t based on pandemic H1N1-associated hospitalizations with 95% confidence intervals, where the dashed line represents the threshold of $R_t = 1$.

Discussion

Our results suggest that pH1N1 may have had slightly lower transmissibility in Hong Kong than elsewhere. For example, estimates of R_t were around 1·5–2·0 in New Zealand[9] and Australia.[10] Lower transmissibility in Hong Kong has been associated with school closures in June and July followed by summer vacations from July through August.[8] Furthermore, in Hong Kong the influenza virus usually does not circulate after August,[11] and therefore seasonality could also be a cause for the lower R_t. On the other hand, the interventions applied during the mitigation phase, such as the widespread use of antiviral treatment in Hong Kong and the pre-existing immunity in the ageing population in Hong Kong, may also be associated with lower transmissibility.

There are some limitations to our work. First, we only used aggregated data, and we did not consider the heterogeneity among the cases in terms of sex and age or other factors. Therefore our estimates can only provide a snapshot of the overall trend, but limited information for any specific subset of population. Secondly, we did not consider the possibility that cases might be infected in Hong Kong

and exported to other countries, which could lead to slight underestimation of the transmissibility. One has to be careful in translating the estimated R_t to the effectiveness of any specific interventions, as interventions may not be the only factor influencing the transmissibility; for example, a depletion of the susceptible population during an epidemic can also be a factor for the decline in R_t.[12]

In conclusion, real-time monitoring of the effective reproduction number is feasible and can provide useful information to public health authorities for situational awareness and planning. In affected regions, laboratory capacity was typically focused on more severe cases, and changes in laboratory testing and notification rates meant that that case counts may not necessarily reflect the underlying epidemic. A useful alternative to case-based surveillance is surveillance of the subset of severe infections, for example hospital admissions, or ICU admissions,[13] and our results show that it was feasible to monitor pH1N1-associated admissions in real-time to estimate transmissibility.

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Concepts and applications for influenza antigenic cartography

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Keywords Antigenic cartography, influenza, Matrix Completion, Multidimensional Scaling, temporal model.

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Abstract

Influenza antigenic cartography projects influenza antigens into a two or three dimensional map based on immunological datasets, such as hemagglutination inhibition and microneutralization assays. A robust antigenic cartography can facilitate influenza vaccine strain selection since the antigenic map can simplify data interpretation through intuitive antigenic map. However, antigenic cartography construction is not trivial due to the challenging features embedded in the immunological data, such as data incompleteness, high noises, and low reactors. To overcome these challenges, we developed a computational method, temporal Matrix Completion-Multidimensional Scaling (MC-MDS), by adapting the low rank MC concept from the movie recommendation system in Netflix and the MDS method from geographic cartography construction. The application on H3N2 and 2009 pandemic H1N1 influenza A viruses demonstrates that temporal MC-MDS is effective and efficient in constructing influenza antigenic cartography. The web sever is available at http://sysbio.cvm.msstate.edu/AntigenMap.

Introduction

As a segmented, negative stranded RNA virus, influenza virus is notorious for rapid mutations and reassortments. The mutations on the surface glycoproteins (HA and NA) of influenza viruses are called antigenic drifts, and these antigenic drift events allow the virus to evade the accumulating immunity from previous infection or vaccination and lead to seasonal influenza epidemics. A reassortment event with a novel influenza antigen may result in antigenic shift and cause influenza pandemic. For instance, the 2009 H1N1 pandemic virus is a reassortant with a swine origin HA antigen.

Vaccination is the primary option for reducing the effect of influenza, and identification of the right vaccine strains is the key to development of an effective vaccination program. The antigenicity of an optimal vaccine strain should match that of the epidemic strain. In influenza surveillance program, the influenza antigenic variants are generally identified by the immunological tests, such as hemagglutination inhibition (HI) assay, microneutralization (MN) assay, or ELISA. These immunological assays measure the antigenic diversity between influenza viruses by comparing the reaction titers among the test antigens and reference antisera. However, data interpretation of the data from these assays is not trivial due to the embedded challenges such as data incompleteness, high noises, and low reactors.

By mimicking geographic cartography, influenza antigenic cartography projects influenza antigens into a two or three dimensional map using immunological datasets.[1] Antigenic cartography can simplify the data interpretation, and thus, facilitate influenza antigenic variant identification. Recently, we developed a novel computational method, temporal Matrix Completion-Multidimensional Scaling (MC-MDS), in antigenic cartography construction.[2] In this paper, we described the details of temporal MC-MDS, especially the original concepts introduced in this method, and how they can achieve the robustness in antigenic cartography construction.

Methods

Our method included two integrative steps: it first reconstructs the HI matrices using low rank MC method, and then generates antigenic cartography using MDS with a temporal regularization. The MC concept was adapted from the movie recommendation system in Netflix and the cartography concept from geographic cartography.

MC and Netflix

In 2006, Netflix, an online DVD and Blu-ray Disc rental-by-mail and video streaming company, held a 3-year Netflix Prize contest (http://www.netflixprize.com/) on computational methods for improving its recommendation system.[3] In its recommendation system, Netflix collected the rating data from the individuals. Based on his or her renting history and the ratings in the systems (e.g., from evaluators and other renters), Netflix recommendation system suggests certain movies to a renter. Apparently, no individuals would be feasible to provide ratings for all of the movies, as it will take hundreds of years for a single person to rate over 50 000 movies available from Netflix. Thus, the resulting rating data is an incomplete matrix, and it can be as sparse as less as 1%.[4] The challenge in Netflix recommendation system is a classic MC problem.[4–8] As the inspiration of Netflix Prize contest, many efficient low rank MC algorithms were developed, for instance, OptSpace,[7] SVT,[5] CF,[9] BellKor,[10] PF,[11] and FWLS.[12] Eventually, the team BellKor's Pragmatic Chaos won this contest. Their methods combines nonlinear probe blending and linear quiz blending to come up with a predictor BigChaos.[13]

Matrix completion estimates the unobserved values based on the observed values. The users can refill the missing data without repeating the experiments. Furthermore, MC will help reduce the noises in the data, for instance, those biases by different individuals performing experiments.

In influenza antigenic characterization, HI assay is a commonly used assay for antigenic analysis, since HI assay is relatively economic and easy to perform. However, HI is labor intensive, and it is almost impossible for any individual lab to complete the HI assays for all pairs of antigens and antisera during influenza surveillance. In addition, both testing antigens and the reference antisera are dynamic. For instance, in seasonal influenza surveillance, generally only contemporary antisera are used in experiments. Thus, we will have to integrate multiple HI tables in order to evaluate the overall antigenic changes for influenza vaccine strain selection. The resulting HI tables will be incomplete, and the observed entries in the integrated HI data can be as less as 3%. The completion of this matrix can be formulated as a typical MC.

Briefly, given the combination of HI matrix with m antigens and n antisera, the HI matrix can be represented as $M_{m\times n} = (m_{ij})_{m\times n}$, where m_{ij} denotes the HI values from the reaction between testing antigen i and antiserum j. The low rank MC assumes that both antigen and antiserum can be embedded into a low rank space. To be specific, the low rank MC method is to seek matrix $U_{m\times r}$, $V_{n\times r}$ and a diagonal matrix $\Sigma_{r\times r}$, where $M = U_{m\times r}\Sigma_{r\times r}(V_{n\times r})^T$. In order to achieve this goal, the optimization formulation has been employed, which can be represent as following,

$$\min_X \frac{1}{2}\sum_{i=1}^{m}\sum_{j=1}^{n}(M_{ij}^E - X_{ij}^E)^2 + \lambda g(X), \quad \text{s.t. } X = U_{m\times r}\Sigma_{r\times r}(V_{n\times r})^T \tag{1}$$

where E denotes the observed entries in HI matrix and $g(X)$ is a regularization function. The Eqn (1) is the standard format of a low rank MC formulation.

MDS and geographic cartography construction

The geographic cartography is a common technique to display the cities and their geographic distances in a map. This cartography can be generated using MDS based on a geographic distance matrix. Figure 1(A) shows the antigenic cartography generated using a distance matrix with seven cities, and Figure 1(B) is a map for comparison.

As an analog of geographic cartography, the influenza antigenic cartography maps the influenza antigens into a two or three dimensional map based on the distance matrix generated using immunological data. This incomplete matrix can be filled through MC algorithm discussed in section MC and Netflix.

Low reactors, non-random date incompleteness, and temporal model

Generally, three types of data are present in a combined HI matrix: high reactor, low reactor, and missing values. Among these three data types, high reactors are the most reliable data points. The low reactors are those values present in the HI matrix as "equal to or less than a threshold θ", where θ can be 5, 10, 20, or 40. Low reactors have similar values in the affinity dataset but could be from different binding settings. These low reactors are present due to the detection limits of biotechnology, and they are not reliable. Both these missing values and low reactors make it very difficult to analyze and interpret antigenic correlations amongst tested antigens and reference antigens. To our best knowledge, none of the existing MC method can handle the threshold values.

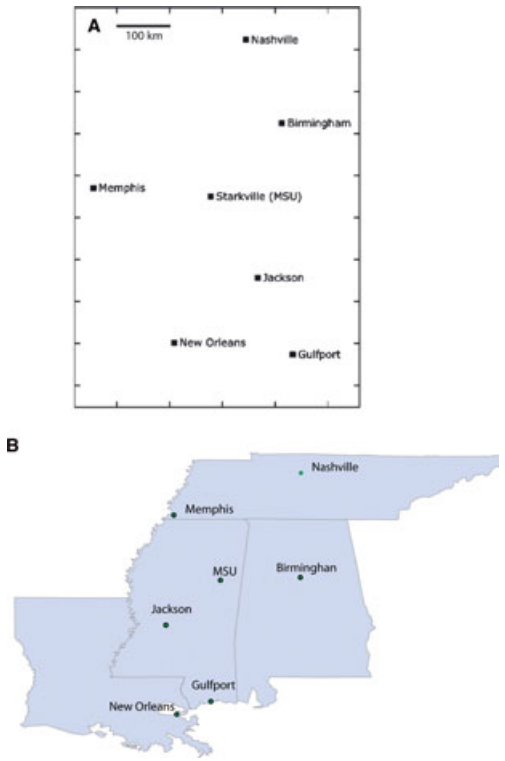

Figure 1. Comparison of the map and geographic cartography generated solely based on seven cities. (A) The cartography constructed by using Multidimensional Scaling; (B) the geographic map of this area generated using ArcGIS 9.3 (Esri company, Redlands CA, USA).

In addition, the non-random incompleteness of influenza immunological datasets generates an additional challenge in traditional MC methods, which are based on the assumption that the observed values are randomly distributed among the matrix. In a typical combined antigenic HI data, most of the off-diagonal entries are missing values or low reactor values.[1]

In order to overcome the above issues, we incorporated a regularization function into the Eqn (1),

$$\min_X \frac{1}{2}\sum_{i=1}^{m}\sum_{j=1}^{n}(M_{ij}^E - X_{ij}^E)^2 I(X_{ij}^E \geq \theta_{ij}) + \lambda g(X), \quad \text{s.t. } X = U_{m\times r}\Sigma_{r\times r}(V_{n\times r})^T \tag{2}$$

where $I(X_{ij}^E \geq \theta_{ij}) = 1$ if $X_{ij}^E \geq \theta_{ij}$ and $I(X_{ij}^E \geq \theta_{ij}) = 0$ otherwise.

This indicator function is only valid for those entries with low reactor values. An alternating gradient decent method is applied to solve the optimization problem in Eqn (2). In addition, a temporal MDS method is proposed to project the antigens into a 2 or 3 dimensional map.

$$\sum_{0\leq t_i - t_j \leq w}(D_{ij} - d_{ij})^2 + \lambda_1 \sum_{a\in G_i} d_{ac_i}^2 + \lambda_2 \sum_{p_1 \leq j-i=k-j\leq p_2}(d_{c_ic_j} + d_{c_jc_k} - d_{c_ic_k})^2 \tag{3}$$

where D_{ij} is the average distance between virus I and virus j, t_i is the isolation year of virus i, d_{ij} is the distance between virus i and virus j in cartography, $d_{ac_i}$is the distance between virus a and center of group i, and $d_{c_ic_j}$ is the distance between the centers of group I and group j. All the parameters are tuned by cross validation. We named this method as temporal MC-MDS.

Results

By applying temporal MC-MDS method in an H3N2 dataset,[2] which includes 4215 (19·56%) observed entries from the reactions among 273 H3N2 testing antigens and 79 reference antisera, 937 of 4215 observed values (22·2%) are

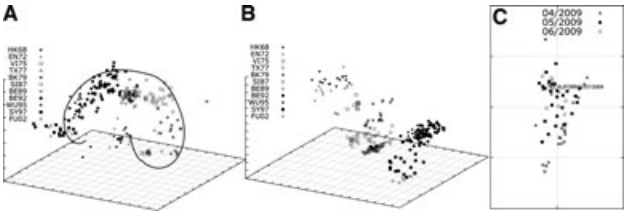

Figure 2. Antigenic cartography with and without temporal model. (A) The three dimensional antigenic cartography for A/H3N2 seasonal influenza virus (1968–2002) without temporal model, and the antigenic clusters were defined in Ref. [2]; (B) the three dimensional antigenic cartography for A/H3N2 seasonal influenza virus (1968–2002) with temporal model; (C) the two dimensional antigenic cartography for 2009 A/H1N1 pandemic influenza without temporal model, and these viruses were labeled in shape by the corresponding month for them to be detected. One grid is corresponding to a twofold change in hemagglutination inhibition experiment.

low reactors. Figure 2(A) is a three-dimensional influenza antigenic map based on this data by using MC-MDS method. The reported 11 clusters (HK68, EN72, VI75, TX77, BK79, SI87, BE89, BE92, WU95, SY97, and FU02) were displayed in the core of a spiral S-shape, and BK79 and BE92 are located at the turning point of this S-shape. However, the antigenic distances between some viruses are incorrect. For example, the distance between HK68 and FU02 in the projection is 7·1223 units, which is close to the distance between HK68 and BK79 (6·5113 units). The main reason leading to those inaccurate distances is the unique distribution of HI datasets described in section 2.3. In comparison, with the temporal model, not only the viruses in 11 clusters have been clearly separated, but also the antigenic distances between each cluster are proportional to their isolation time interval. In this updated cartography (Figure 2B), the antigenic distance between HK68 and FU02 is 15·0633 units, where the distance between HK68 and FU02 is 6·3984 units. This result suggested that the temporal information is critical for antigenic cartography construction for immunological datasets spanning a long time period. The HI data from seasonal influenza surveillance belong to this category.

For seasonal influenza virus/pandemic influenza viruses within a short time span, the temporal model is probably not necessary, as there is lack of long-term immunological pressure present in the population. Figure 2(C) is an antigenic cartography generated using a HI dataset with 2009 H1N1 influenza viruses spanning from April of 2009 to June of 2009. This map demonstrates that there is lack of antigenic drifts during the first wave of this pandemic influenza as all of these viruses are mixed altogether.

Our limited studies on H5 and H7 avian influenza viruses suggested the temporal model is not needed for avian influenza viruses. However, extensive studies are required to investigate whether there is any special data structure present in this type of data.

Conclusion

In this study, we described in details the concepts and applications of new computational method, temporal MC-MDS for influenza antigenic cartography construction. We formulate the influenza cartography as two integrative steps: low rank MC problem from the concept of Netflix movie recommendation system and MDS from geographic cartography construction. In order to handle two additional challenges, including low reactor and non random distribution of antigenic data, a temporal model is incorporated into MC-MDS as temporal MC-MDS. Our applications demonstrated that temporal MC-MDS is effective in constructing influenza antigenic cartography.

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Exploring alternate immune hypotheses in dynamical models of the 1918–1919 influenza pandemic

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Keywords Mathematical modelling, parameter estimation, reproduction number, Spanish flu.

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Introduction

The mechanisms driving the three waves of infection and mortality in the UK in 1918–1919 are uncertain. Although the circulation of three distinct viruses could have generated three waves of infection,[1] the virological evidence required to prove or disprove this hypothesis is lacking. Social distancing, an alternate mechanism for generating fluctuations in the effective susceptible pool and therefore explaining multiple waves of infection,[2,3] was not generally imposed in the UK as it was in the US and Australia. We are therefore motivated to explore the possible role of continual population-level changes in the average protective response against the circulating virus in generating a multi-wave pandemic, within a biologically motivated deterministic model for influenza transmission.

The nature and duration of protection against further infection following recovery from influenza is uncertain and depends on the mode and tempo of viral evolution, as well as the response of the cellular and humoral arms of the adaptive immune system.[4] For a given seasonal/pandemic strain, memory B-cells may generate a specific antibody response in a portion of the adult/elderly population, depending on the exposure to related antigenic sub-types.[5] However neutralising antibodies are unlikely to be a widespread immunological response to a novel (pandemic) strain. Memory T-cells which recognise conserved internal viral proteins may be a more common mechanism for protection; the generation of very high levels of cytotoxic $CD8^+$ T-cells potentially facilitates rapid viral clearance,[6,7] and lower levels of $CD8^+$ T-cells perhaps provide partial protection.[8] In this work we explore key drivers of multi-wave pandemics within phenomenological models that incorporate different immune response mechanisms building on existing models[9,10] incorporating the role of evolving population-level protection in multi-wave pandemics.

Materials and methods

Data and parameter fitting

We use weekly reports of influenza mortality rates[11] for five administrative units in the UK (Blackburn, Leicester, Newcastle, Manchester and Wigan) where records from block censuses instigated by local medical officers to record the cumulative incidence of reported symptoms in each wave in a sample of 1000 or more households are also available.[10] The symptom reporting data allows us to estimate the case fatality rate and thus use the mortality time series to constrain our transmission model. Furthermore, the incidence of individuals reporting symptoms in multiple waves provides information about the acquisition and loss of immunity. We extract the death rate and symptomatic (re)infection rates predicted by our model prevalence for a given set of parameters and estimate a likelihood-based on a comparison to all the death and cumulative reported incidence data assuming a negative binomial error distribution. We utilise Monte Carlo Markov Chain (MCMC) methods with parallel tempering algorithms to maximise this likelihood and obtain parameter estimates. Parallel tempering – which concurrently searches for maximal likelihood parameter solutions on a set of scaled likelihood surfaces – allows for relatively rapid exploration of the parameter space. We use Bayesian information criteria (combined with qualitative assessment of biological plausibility) to aid model selection.

Deterministic compartmental dynamical model for influenza transmission

We have implemented a deterministic compartmental transmission model, which allows for a variety of phenomenological modes of protection against the pandemic virus. To facilitate this, we stratify the population into two groups; the 'experienced' population (stratum 1) who have had been exposed to an influenza virus and the 'naive'

population (stratum 2) who have not. In each stratum, i hosts may be classified as either susceptible S_i, exposed $E1_i$ and $E2_i$, having (recovered from) a symptomatic I_i (R_i), or asymptomatic A_i (RA_i) infection. Note that the states TQ_i, $TQ2_i$, $E2_i$, T_i, and $T2_i$ are included so that the hosts move between the key epidemiological states with a peaked (rather than exponential) distribution of waiting times. Hosts in the experienced stratum may exhibit reduced susceptibility, infectiousness, and symptomatic proportion compared to naive hosts, parameterised by ε_I, ε_S, and ε_α, respectively; however note that depending on the model parameters, there may be fully susceptible hosts within the experienced stratum. In addition, we assume homogeneous population mixing and a constant basic reproduction number R_0 with the force of infection:

$$\beta_{ij} = \frac{R_0 v}{\alpha N[x_E(\varepsilon_s\varepsilon_\alpha\varepsilon_I) + (1 - x_E)]} \begin{pmatrix} \varepsilon_s\varepsilon_\alpha\varepsilon_I & \varepsilon_s\varepsilon_\alpha \\ \varepsilon_I & 1 \end{pmatrix},$$

modulated by a sinusoidal seasonal term with amplitude b_1 with phase chosen to maximise transmission in the winter season. Here N is the total population size, and x_E is the initial fraction in the experienced strata. The proportion of symptomatic cases α and the case fatality rate μ are permitted to vary from wave to wave (and given indices 1, 2 or 3 accordingly). The transmission dynamics is described by the following set of coupled ordinary differential equations.

$$\frac{dS_i}{dt} = -\sum_{j=1}^{2} \beta_{ij} I_j S_i + \phi_Q TQ2_i + S_{in,i},$$

$$\frac{dE1_i}{dt} = \sum_{j=1}^{2} \beta_{ij} I_j S_i - \gamma E I_i,$$

$$\frac{dE2_i}{dt} = \gamma E1_i - \gamma E2_i,$$

$$\frac{dI_i}{dt} = \alpha\gamma E2_i - \nu I_i,$$

$$\frac{dA_i}{dt} = (1 - \alpha)\gamma E2_i - \nu I_i,$$

$$\frac{dR_i}{dt} = \nu I_i - \phi R_i,$$

$$\frac{dT_i}{dt} = (1 - \rho_i)\phi R_i - \phi T_i,$$

$$\frac{dP_i}{dt} = \rho_i\phi R_i + \rho_i\phi RA_i,$$

$$\frac{dQ_i}{dt} = -\phi_Q Q_i,$$

$$\frac{dRA_i}{dt} = \nu A_i - \phi RA_i,$$

$$\frac{dTA_i}{dt} = (1 - \rho_i)\phi RA_i - \phi TA_i,$$

$$\frac{dTQ_i}{dt} = \phi_Q Q_i - \phi_Q TQ_i,$$

$$\frac{dT2_i}{dt} = \phi T_i - \phi T2_i,$$

$$\frac{dTA2_i}{dt} = \phi TA_i - \phi TA2_i,$$

$$\frac{dTQ2_i}{dt} = \phi_Q TQ_i - \phi_Q TQ2_i,$$

where $S_{\text{in},1} = \sum \varphi TQ2_i$ and $S_{\text{in},2} = 0$ in order to divert recovered infectious hosts from the naive stratum into the experienced stratum. The probabilities of gaining permanent protection are $\rho_1 = \rho$ and $\rho_2 = 0$. The latent exposed period is fixed to be $\gamma = 1/1{\cdot}3$ days, and the rate of recovery is parameterised by $\nu = 1/T_{\text{inf}}$, where T_{inf} is the infectious period. Hosts with prior sterilising protection begin in Q_1 and move into S_1 at rate $\varphi_Q = 3/T_{wQ}$. Recovered hosts (R_i) migrate back to S_1 at a rate $\varphi = 3/T_w$. The state P_1 contains hosts with permanent protection. The modes of protection captured in this model are:

- **i.** permanent prior protection (beginning in state P_1),
- **ii.** waning prior protection (beginning in state Q_1),
- **iii.** permanent acquired protection with probability ρ (moving into state P_1),
- **iv.** waning acquired protection with probability $1 - \rho$, and,
- **v.** partial prior protection (beginning in state S_1) resulting in reduced infectiousness (ε_I), susceptibility (ε_S), and symptomatic proportion (ε_α).

In the context of this model, 'permanent' protection refers to protection which lasts for the duration of the epidemic.

Results

Here we explore the results of parameter fitting to two models which differ in the nature of the assumed pre-existing protection in the community at the beginning of the pandemic. Protection hypothesis 1 assumes that the prior protection is sterilising but temporary, whilst protection hypothesis 2 assumes that the prior protection is partial but permanent and may act on susceptibility, infectiousness,

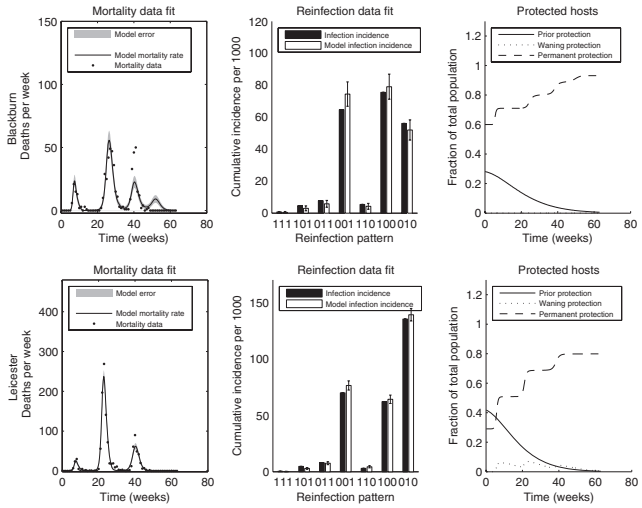

Figure 1. Current best fit for Blackburn (above) and Leicester (below). The left panel shows model fit (black line) to the mortality data (black points) and the Poisson error on the model (grey shaded area). The middle panel shows the re-infection data (black) and the model prediction (white) with Poisson error bars. The right panel shows the time evolution in the number of people in the temporary waning (dotted line), prior (solid line), and permanent (dashed line) protection states.

and/or asymptomatic proportion. Each model allows waning acquired protection and for a proportion ρ of the experienced population to gain permanent protection following infection. Fitted parameters common to each model are T_{inf}, b_1, ρ, T_w, α, μ and the proportion beginning in P x_I.

Prior protection hypothesis 1: sterilising, waning prior protection

We fix $x_E = 1$ and fit for $Q_1(\text{t} = 0)/N$ and T_{wQ} so that protective modes i, ii, iii, and iv are enabled (Figure 1).

It is important to note that due to the slow convergence of the MCMC chains, we cannot guarantee that our parameter estimates correspond to the global minimum. Furthermore, parameter estimates can only be meaningfully interpreted for good fits to the data. Due to the prediction of a fourth (unobserved) wave for the model fit to Blackburn, we do not report these parameter estimates here. The fits to the Leicester data are generated with the parameter set $R_0 = 5{\cdot}7$, $\alpha_1 = 0{\cdot}25$, $\alpha_2 = 0{\cdot}65$, $\alpha_3 = 0{\cdot}65$, $T_w = 0{\cdot}28$ -years, $T_{wQ} = 0{\cdot}45$ years, $S_1(\text{t} = 0)/N = 0{\cdot}27$, $P_1(\text{t} = 0)/N = 0{\cdot}29$, $Q_1(\text{t} = 0)/N = 0{\cdot}44$, $b_1 = 0{\cdot}005$, $T_{\text{inf}} = 0{\cdot}8$ days, and $\rho = 0{\cdot}72$ ($0{\cdot}77$).

Protection hypothesis 2: partial, permanent prior protection

We fix $Q_1(\text{t} = 0)/N = 0$ and fit for x_E, ε_α, ε_I, and ε_S so that protective modes i, iii, iv, and v are enabled (Figure 2).

The parameters corresponding to the fit in Figure 2 for Leicester are $R_0 = 7{\cdot}4$, $\alpha_1 = 0{\cdot}09$, $\alpha_2 = 0{\cdot}50$, $\alpha_3 = 0{\cdot}61$, $T_w = 0{\cdot}22$ years, $P_1(\text{t} = 0)/N = 0{\cdot}01$, $S_2(\text{t} = 0)/N = 0{\cdot}48$, $b_1 = 0{\cdot}021$, $T_{\text{inf}} = 0{\cdot}94$ days, $\rho = 0{\cdot}561$, $\varepsilon_\alpha = 0{\cdot}9966$, $\varepsilon_I = 0{\cdot}946$, and $\varepsilon_S = 0{\cdot}594$.

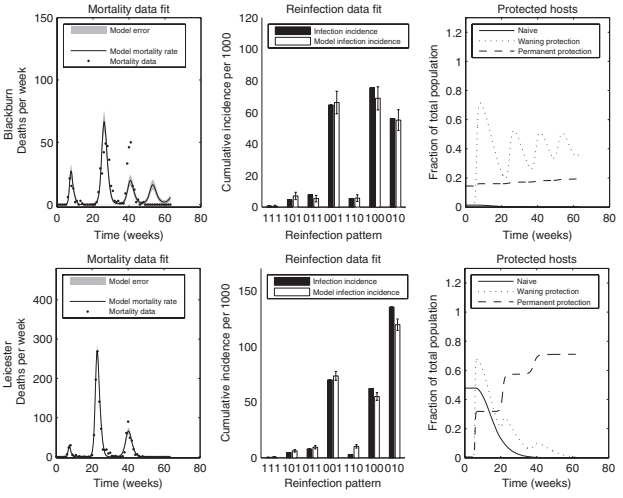

Figure 2. As for Figure 1 with for the model with protection hypothesis 2. The solid black line in the third panel now shows the evolution of the proportion of the population in the naive stratum.

Discussion

Our model with protection hypothesis 1 – which, similarly to the model discussed in Ref. [9], assumes that a sub-population has waning sterilising prior protection – is able to generate multiple waves of infection via the continual replenishment of S_1 from an initially large proportion (over 40%) of hosts with prior protection in Q combined with the waning of acquired immunity in around 23% of cases on a time-scale of 3 months. Disease severity as measured by symptomatic proportion increases from 25% in the first

wave to above 60% for the second and third waves. Over a quarter of the population are initially permanently immune, and a large R_0 value of 5·7 drives transmission in the remaining population.

Protection hypothesis 2 – which assumes that prior protection offers partial susceptibility and/or reduced infectiousness or symptomatic disease – performs slightly more poorly; the fit to the Leicester data has an inferior likelihood (although the mortality data only likelihood is a little larger), despite the higher dimensionality of the model. Nevertheless, the model fit still mirrors many characteristics of the data, particularly for Leicester. We note that for this model, α_1 is very near the lower limit, corresponding to ubiquitous exposure in the first wave. In this scenario, refuelling of the susceptible pool to generate secondary and tertiary waves is still possible due to a shorter waning time of acquired protection (well within 3 months) and a lower probability of gaining permanent protection following infection, when compared with the parameter estimate for hypothesis 1. The parameter estimates suggest that approximately 50% of the population initially experiences reduced disease severity ($\varepsilon_\alpha \sim 0{\cdot}66$), but similar susceptibility and infectiousness. A larger value for $R_0 \sim 7{\cdot}4$ is required to drive transmission despite low numbers beginning in P_1, due to the large number of hosts who acquire temporary or permanent immunity early on in the pandemic.

It is clear that, at least mathematically and perhaps biologically, there are multiple possibilities for the structure of population-level protection which are compatible with the generation of multiple pandemic waves. However, whilst the models considered here are able to explain the observed mortality and reinfection data for some patterns of infection and mortality (e.g. Leicester), they are not consistently able to reproduce a pandemic which dies out after three waves across the connected populations we are studying (e.g. for Blackburn). It is challenging to construct a deterministic model for the spread of disease within multiple locations in the UK in 1918, which assumes homogeneous mixing without modulation of the transmission rate by social distancing. An improved model working with these assumptions likely requires a richer structure for the host protection response than the structures we have explored thus far. We are currently seeking improved fits to the data by implementing a number of biologically defensible extensions to our model, including incremental immunity whereby T_w increases by a factor χ after each exposure to the pandemic flu, and incremental loss of prior protection whereby α increases as hosts lose their sterilising prior protection.

It is important to note that the mechanism(s) generating differences in the pandemic experience recorded in geographically connected locations is an open question; true differences in demography, varying degrees of reactive social distancing, inhomogeneities in the circulation (or circulation history, i.e. prior immunity) of viral strains, stochastic variations, and/or unique socio-cultural/behavioural conditions may all contribute to this effect.

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A decision support tool for evaluating the impact of a diagnostic-capacity and antiviral-delivery constrained intervention strategy on an influenza pandemic

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Keywords Antiviral drugs, delivery capacity, mathematical model, pandemic preparedness.

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The 2009 H1N1 experience in Australia and elsewhere highlighted the difficulties faced by public health authorities in diagnosing infections and delivering antiviral agents (e.g. oseltamivir) as treatment for cases and prophylaxis for contacts in a timely manner. Consequently, forecasts from mathematical models of the possible benefits of widespread antiviral interventions were largely unmet. We summarise results from a recently developed model that includes real-world constraints, such as finite diagnostic and antiviral distribution capacities. We find that use of antiviral agents might be capable of containing or substantially mitigating an epidemic in only a small proportion of epidemic scenarios given Australia's existing public health capacities. We then introduce a statistical model that, based on just three characteristics of a hypothetical outbreak [(i) the basic reproduction number, (ii) the reduction in infectiousness of cases when provided with antiviral agents as treatment, and (iii) the proportion of cases that present for medical attention], accurately predicts whether or not an antiviral intervention strategy will be successful. The model highlights the importance of having data collection tools in place prior to a pandemic outbreak, so as to make accurate and timely estimates of key epidemiological parameters unique (in both time and place) to any particular epidemic.

Introduction

Governments and public health agencies worldwide, spurred by outbreaks of SARS and H5N1, have developed preparedness strategies to mitigate the impact of emerging infectious diseases, including pandemic influenza. Pandemic response plans are presently being revised in light of the 2009 H1N1 experience.[1–3] Many developed countries amassed large stockpiles of neuraminidase inhibitors (NAIs) with the expectation that they could be used to not only treat the most severely ill, but curb transmission in the community.

Without relevant field experience indicating how NAIs should be distributed, mathematical and computational modelling has been used to inform optimal deployment policy in a pandemic scenario.[4–10] Models of population transmission were used to infer likely effects on epidemic dynamics, using data from human and animal studies of experimental infection and NAI efficacy trials. In the Australian (and wider) context, models indicated the potential for substantial benefit at the population level if NAIs were distributed in a liberal manner, targeting close contacts of indentified cases.[11] Furthermore, results indicated that use of limited NAI resources in this way may improve the impact of case treatment due to the effects on epidemic dynamics.[11]

However, these models did not take into account logistic and other real-world constraints, such as finite diagnostic and antiviral distribution capacities, which were identified as limiting factors during the Australian 2009 H1N1 pandemic response.[12–14] In particular, if using positive PCR diagnosis as a 'decision to treat' test, delays to confirmation of diagnosis, particularly once total laboratory capacity was exceeded, prevented timely delivery of NAIs to both cases and contacts of cases.[12]

In previous work,[15] we have extended our existing models to examine how diagnostic strategies [e.g. using PCR confirmation versus syndromic influenza-like illness (ILI) presentation as a decision to treat], diagnostic-capacity, and NAI distribution capacity each impact on the ability to deliver an effective intervention. The model uses case severity (the proportion of infections deemed severe) to determine the overall presentation proportion, and so the ability to identify individuals eligible for NAI treatment and contact prophylaxis.

Figure 1(A) shows a key result from the model. For each curve shown, we simulated thousands of epidemics, sam-

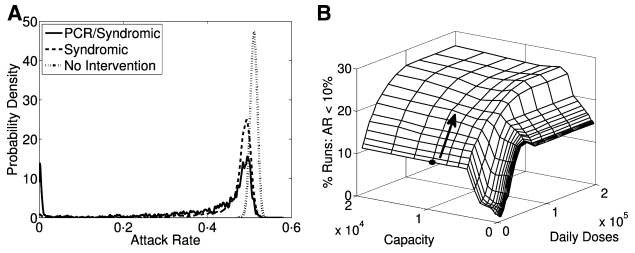

Figure 1. (A) Probability density (smoothed) for the final attack rate for different interventions assuming current capacity constraints in the Australian context. Any given intervention will either (i) almost completely control the epidemic or (ii) have a negligible impact. (B) Surface plot of the percentage of simulations in which the outbreak was controlled for a decision based on PCR while lab capacity is sufficient followed by a switch to a Syndromic (ILI) based decision. Estimated Australian PCR lab capacity appears sufficient, while significant benefits for public health outcome may be achieved if logistical delivery constraints for antiviral distribution can be ameliorated. The dot indicates the author's estimate of Australian capacity in 2009, and the arrow represents the direction in which most benefit would be found. ILI, influenza-like illness.

pling across plausible ranges of parameters describing virus, population, and intervention characteristics using a Latin Hypercube Sampling (LHS) approach. Without intervention, the proportion of the population infected either symptomatically or subclinically by the end of the epidemic is around 50%. If a syndromic strategy (ILI presentation) is used to determine provision of NAIs as treatment and prophylaxis, excessive distribution of drug to individuals who are not infected with influenza occurs early in the epidemic. Early stockpile expiry accounts for a marginal impact of the antiviral intervention on the final outbreak size, in the order of a few percent. The second strategy modelled (*PCR/Syndromic*) is one where PCR confirmation of diagnosis is required early in the epidemic to make treatment decisions until such time as laboratory capacity is exceeded. From this point, individuals are treated on the basis of symptoms alone – during an epidemic phase in which a substantial proportion of ILI presentations will be attributable to influenza. Under this strategy, the intervention is able to control the outbreak in approximately 10% of the simulated epidemics given the 'base case' constraints on diagnosis and delivery assumed in the model.

The results highlight that a successful antiviral intervention requires a highly sensitive diagnostic strategy in the initial stages of the epidemic and comprehensive distribution of post-exposure prophylaxis. A PCR/Syndromic strategy for decision to treat and provide contacts with prophylaxis is thus optimal. The surface in Figure 1(B) shows the percentage of simulation runs for the PCR/Syndromic strategy that have a final population attack rate of <10% (a substantial reduction from the no intervention case of approximately 50%) as a function of PCR capacity and NAI daily distribution capacity. As indicated by the arrow, the estimated Australian PCR laboratory capacity appears to be sufficient, while significant benefits for the public health outcome may be achieved if logistical delivery constraints for NAI distribution can be ameliorated.

However, the probability that such an intervention – even with substantial increases in PCR and NAI distribution capacity – would successfully mitigate an epidemic is low (12–25%), and consequently it is difficult to universally recommend an antiviral intervention.[15]

In this study, we introduce a statistical model that predicts whether or not an NAI distribution strategy based on a PCR/Syndromic antiviral distribution policy will be successful in mitigating an epidemic. We thereby provide proof-of-principle for the design of a decision support tool that may be used by public health policy makers during an epidemic when faced with formulation of context specific NAI distribution policy.

Materials and methods

Synthetic data of hypothetical outbreaks and interventions were generated using the LHS simulations developed in Ref. [15]. We selected a random sample of 100 outbreaks from a total of 2000 simulated epidemics (5% of model simulations). Using these data, we identified independent model parameters that were most highly rank-correlated with the final attack rate. These parameters were included in a logistic regression model to assess their ability to predict whether an influenza epidemic would be successfully mitigated by an antiviral intervention (AR < 10%). Model predictions were then validated against the full simulated dataset.

Full details of the simulation model, its structure, parameterisation and parameter distributions are available in Ref. [15]. Use of the LHS simulation approach, and the method of model analysis and evaluation was similar to that previously described.[16] MATLAB 2010A (Mathworks, Natick, MA, USA) was used for the analysis and statistical model fitting.

Results

Table 1 shows results from our logistic regression model. Key parameters sufficient to predict whether or not an outbreak may be controlled by the deployment of AV agents are:

1. R_0, the basic reproductive number of the outbreak (assigned values between 1·35 and 1·45 for this example). As the value of R_0 increases, the epidemic progresses more rapidly and is more difficult to control, explaining the negative correlation coefficient.
2. e_t, the relative infectiousness of treated individuals (assigned values between 0·8 and 1·0). Higher values

Table 1. Coefficients and standard errors for the logistic regression model used to predict the success of an antiviral intervention

Parameter	Coefficient	Standard error	*P* value
R_0	−23·13	4·25	<0·0001
e_t (relative infectiousness of treated individuals	−33·26	2·73	<0·0001
η (proportion of infections that are severe)	14·76	2·97	<0·0001
Constant	7·31	0·80	<0·0001

for this parameter indicate only modest drug effects on transmission, explaining the negative correlation coefficient.

3. η, the proportion of infections that are severe (assigned values between 0·001 and 0·1), and which in turn determines the presenting proportion (derived values between 0·11 and 0·56). As the presenting proportion increases, the ability to identify and treat cases and deliver prophylaxis to contacts also rises, increasing the impact of the antiviral intervention.

The ROC curve (1-specificity versus sensitivity, not shown) for the logistic regression model specified in Table 1 has an area under the curve of 0·937, demonstrating that the model predicts the success of an antiviral intervention extremely well. For example, with a sensitivity of 95% we still have a specificity of approximately 80%.

Discussion

Evaluation of the 2009 pandemic response has emphasised the need for early informed decision-making to implement proportionate disease control measures. Our model identifies a low probability of successful epidemic mitigation using targeted antivirals alone (Figure 1 and Ref. 15), in distinction to results from models that fail to account for the diagnosis and delivery constraints inherent in any public health response.

The decision support tool (Table 1) highlights key epidemic characteristics that are predictive of a high likelihood of effective mitigation. The reproduction number was one of the earliest parameters estimated from early outbreak data during the 2009 H1N1 outbreak.[17,18] Our findings reinforce the importance of characterising epidemic severity as early and as accurately as possible, in order to inform a proportionate pandemic response. Critically, a typically mild pandemic (low η), such as that experienced in 2009, is predictably difficult to contain using a targeted antiviral strategy due to the low proportion of infectious cases that present to health authorities.

The relative infectiousness of treated individuals, e_t, is strongly negatively correlated with successful mitigation, perhaps a surprising result given the model's underlying assumption (based on available epidemiological and human clinical trials data) that e_t lies in the range [0·8, 1]. That is, NAIs provided as treatment have a *maximum* impact of just a 20% reduction in infectiousness. However, our previous results[4] show a strong synergistic effect of treatment when overlayed on a contact prophylaxis strategy, explaining the observation here that e_t is critical in determining likely success of an intervention. Despite the limited impact of treatment at the *individual-level*, the model outcomes are highly sensitive to the value of the relative infectiousness of treated cases. It follows that determination of e_t is important for predicting the *population-level* outcome of a control effort. A 'small' reduction (of the order approximately 10%) may be extremely valuable in terms of success of a public health control strategy, and so should not be discounted.

Using a mathematical model which takes into account some of the key logistic constraints that are inherent to healthcare responses, we have derived a logistic regression model for estimating the probability that an antiviral intervention based on liberal distribution of NAIs as treatment and prophylaxis could successfully mitigate an influenza epidemic. The model demonstrates an excellent degree of accuracy when applied to synthetic data. The choice of parameters for the regression model was restricted to those that were both highly correlated with the success of the intervention and hopefully feasible to measure during the early stages of an emerging epidemic. The model could therefore be a useful near real-time decision support tool for public health policy in the face of an influenza epidemic, although further validation on a range of synthetic data (and real-world data where available) is required.

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Quantitative assessment of early intervention strategies: a case study of 2009 H1N1 pandemic in Taiwan

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Introduction

In 2009, Taiwan established the Central Epidemic Command Center and convened its first meeting on April 28; the same day the World Health Organization (WHO) raised the influenza A (H1N1) pandemic alert level to phase 4. In addition to the routine fever screening that had been in place since the severe acute respiratory syndrome (SARS) scare in 2003, on-board quarantine inspections were carried out on all flights originating in the United States and Mexico since April 29. All passengers were encouraged to report any flu-like symptoms, and work closely with government officials on all health related matters. Starting at midnight on May 19, a comprehensive notification on-board quarantine measure replaced the on-board quarantine inspection. On May 20, a 52-year-old foreign resident of Taiwan, who returned from the United States via Hong Kong, was identified as the first imported case. The patient was escorted to a designated hospital, isolated, and treated with oseltamivir. He recovered fully. All passengers seated within three rows of the ill passenger were contacted, given 10 day chemoprophylaxis and self-quarantined. Nine more imported cases were identified by airport screening in the following week. The first domestic case was diagnosed on May 24. By June 19, there were 61 confirmed cases, but only two were indigenous. Details of the response measures and clinical characteristics of the first 61 confirmed cases have been published in the Taiwan Epidemiology Bulletin[1] by the Taiwan Centers for Disease Control (CDC).

As the 2009 H1N1 pandemic (H1N1pdm) came to an end, many countries had long since downgraded the H1N1

influenza to seasonal flu status to avoid overstretching the demands on healthcare services. A great deal of information has emerged as the result of the pandemic response exercises conducted by affected countries. However, uncertainties remain regarding the effectiveness of intervention measures, as well as the feasibility and the timing of their implementation. Mathematical and computational models[2–4] have been used to project the outcomes of influenza outbreaks under various scenarios and epidemiological hypotheses. Motivated by the events of 2009 and public health measures adopted by the Taiwan CDC, we use a stochastic, individual-based simulation model[5] to study the spatio-temporal transmission characteristics of the H1N1 virus, so as to quantitatively assess the effects of early intervention strategies.

Materials and methods

Our stochastic disease simulation model[5] builds upon a highly connected network of individuals interacting with each other via social contact groups. To represent the daily interactions of approximately 23 million people living in Taiwan, we constructed a computer-generated mock population based on national demographic and employment statistics (to derive daily commute patterns) from the 2000 Taiwan Census (http://www.stat.gov.tw/). Each individual is created with a set of attributes, including age, sex, residence, family structure, and social standing (employment status, etc.). Based on their attributes and the time of day, each individual is assigned to miscellaneous contact groups, where the potential of interactions between any two individuals resulting in flu virus transmission occurs. Such epidemiological properties are defined by empirically parameterized attributes such as basic reproduction number R_0, transmission probability, contact probability and associated probability distributions outlining the disease's natural history. Additionally, intervention measures are implemented as scheduled events that could alter control parameters during the course of a simulation run.

The targeted basic reproduction number (R_0) in all our simulations is 1·6, following the suggested range by WHO of 1·2–1·7.[6] As the latent/incubation and infectious periods for H1N1 have not yet been reliably ascertained, we adopt the natural history of the 1957 and 1968 pandemic influenza viruses.[2,7] Here, the latent period ranged from 1 to 3 days, with a median value of 1·9 days. The infectious periods begin 1 day prior to symptom onset and can continue for 3–6 days, with a median value of 4·1 days. Two-thirds of the infected individuals will develop clinical symptoms, and the asymptomatic cases will have half the infectious strength. The efficacy of antiviral drugs (oseltamivir) and vaccines are based on these studies.[8,9]

For the source region of the infected cases, we use the North American continent (Canada, Mexico and United States) with an estimated total population of 450 527 697 and an average 16 hours of flight time to Taiwan. The average daily passenger number is 2489 based on the 2009 Annual Statistical Report on Tourism, Tourism Bureau, Taiwan (http://admin.taiwan.net.tw/english/statistics/year.asp?relno=61).

Each simulation lasts 365 days and starts with a baseline simulation of $R_0 \approx 1{\cdot}6$ H1N1pdm outbreak at the source region. The outbreak was adjusted to approximate clinical attack rate (CAR) in the United States, April 2009–March 13, 2010.[10] We estimate the daily number of imported cases according to average daily passenger numbers and their probability of holding a disease status. We then apply airport exit/entry screening per corresponding success rates, by subtracting the number of identified symptomatic cases. We also consider latently infected passengers with in-flight disease progression, by fitting a gamma distribution to the cumulative distribution of time to onset data with 16 hours average flight-time, as presented by Pitman *et al.*[11] The daily imported cases are seeded according to the traveling patterns of foreign tourists and residents returning home. From the disease's natural history, we derive that roughly 50% of the infected travelers present no symptoms; the percentage increases if most symptomatic individuals elect not to travel in their condition, or are stopped by airport screening.

We use the official epidemic data provided by the Taiwan CDC to calibrate the simulation model and perform regression analysis on scenario parameters. This data is a close estimation of the weekly new clinical cases of H1N1pdm patients. It consists of weekly OPD (outpatient department) ICD-9 code 487 (influenza) tallies collected by the Bureau of National Health Insurance, Taiwan – adjusted to exclude seasonal flu patients and to account for uninsured patients. We formulate our scenario settings according to 2009 events in Taiwan, and establish settings to approximate the actual events. With domestic events and intervention schedules fixed in time, the start date determines the simulation outcomes and the data range for selected indicators, such as the mean CAR, the epidemic peak, and several significant dates for the incoming index case events. We plot the 2009 Taiwan weekly H1N1 OPD487 cases alongside the weekly new clinical cases from our simulation results in Figure 1.

Our simulations not only capture the epidemic trend, but also pick out the most likely date, May 20, for identifying the first symptomatic case at airport screening based on practical assumptions. We further analyze the effectiveness of various mitigation measures with February 6, 2009 as the empirical start date for H1N1pdm in North America.

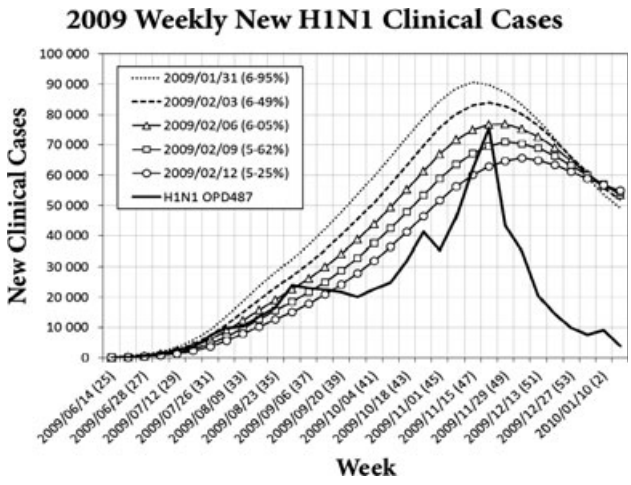

Figure 1. Epidemic curves of 2009 Taiwan weekly H1N1 OPD487 and new clinical cases from the simulation with pragmatic start dates, 5% estimated efficacy for school closure policy. The start date and attack rate are used in the label for each simulation scenario.

Results

The simulation result confirms that by the time we identified the first symptomatic case at the border screening, infected cases had already made their way to the public. By our calculation, roughly four such cases had passed in each of our scenario settings, with the first case happening as early as 3 weeks before detection. Figure 1 also highlights the importance of the timing for the implementation of mitigation measures; for example, a 6-day-delay of the identical intervention plan results in nearly an additional 1% of the population being infected. Therefore, the rule of thumb for healthcare officials is to implement intervention measures as early as possible.

In our study, we have ignored the possibility of in-flight transmission and any false positive results by airport screening procedures. To assess the effectiveness of each mitigation strategy of interest and their combinations, we take the calibrated simulation model and perform 100 simulation realizations for groups of scenarios containing only those intended mitigation measures, and analyze the averaged results. For example, in the airport exit screening policy only scenario, the first imported symptomatic case can be delayed up to 2 months, and the epidemic peak can be delayed up to 13 days. As the data suggests, the exit screening policy alone has very little impact on CAR.

Combining various screening success rates for both exit and entry screening allows us to quantitatively assess their beneficial ramifications on the epidemic. For example, there is very little additional benefit between 100% and 80% success rates for entry screening policies when exit screening policies are adequate, as the enhanced border screening only delayed the epidemic peak by 1 day, and reduced CAR by <0·01%. Base on this result, the government should not attempt to exhaust all its resources in securing the border during a pandemic event, because the return of such a policy will be disappointing. Instead, a response plan with a shifting focus on health resource allocation and the capacity of adjusting intervention strategies in line with the developing epidemic will be most effective.

Based on the same principle, we perform experiments with assorted scenarios, including relaxing entry screening policies after identifying the first imported symptomatic case, mass vaccination based on the actual vaccination schedule of H1N1pdm in Taiwan, and altering the start dates of the vaccination schedule. Our results show that with a reasonable reduction in the airport entry screening success rate, we conserve valuable healthcare resources, but loose a few days for the strategic planning and preparation of subsequent response measures. In other simulation scenarios, a national vaccination campaign has very little impact on the outcome, due to the late start of the vaccination schedule. We then explore the effect of a national vaccination campaign with various starting dates. The simulation results are illustrated in Figure 2, where the benefit of an early start date for mass vaccination is clearly demonstrated. Considering a scenario with an 80% airport exit screening success rate, 80% airport entry screening success rate and 80% symptomatic case tracing success rate, the combined intervention strategy results in: a 2% reduction in CAR if the vaccination campaign starts in mid-November; 11% reduction if the campaign starts in mid-October;

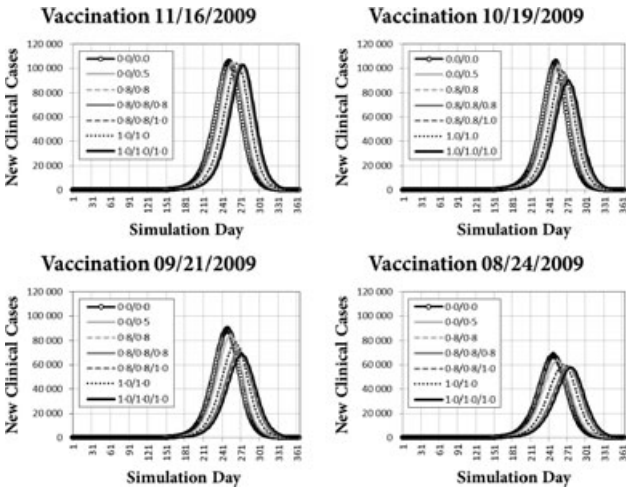

Figure 2. Simulation results with various vaccination start dates. The label for each epidemic curve shows the success rate settings for airport exit screening, airport entry screening and symptomatic case tracing policies.

26% reduction if the campaign starts in mid-September; and 37% reduction if the campaign starts in mid-August.

In retrospect, the Taiwanese government's response to H1N1pdm proved to be effective. First and foremost, it initiated enhanced border monitoring and on-board quarantine inspection as soon as the threat of a flu pandemic became clear. At the same time, the domestic preparations towards H1N1pdm were escalated, such as antiviral drug stockpiling and distribution, and vaccine acquisition. As the H1N1 cases increased worldwide, various revised plans were adopted and implemented; such as the shift from labor-extensive on-board quarantine inspection to the Notifiable Infectious Disease Reporting System and Real-time Outbreak and Disease Surveillance System in order to effectively track down symptomatic and exposed passengers, apply prophylaxis treatment and mandatory in-home quarantine. As a result, all H1N1pdm related statistics are well below the international average.

Discussion

In modern society, countries rely heavily on the global economy for their own prosperity. Shutting down the border for any length of time is not only costly, but could have disastrous economic effects that linger long after the event is over. Moreover, with nearly 50% of the infected passengers presenting no symptoms whatsoever, they are not detectable by any port authority's screening procedures, and the importation of the novel flu virus is therefore inevitable. Many studies conclude that entry screening is unlikely to be effective in preventing or delaying the importation of influenza, and has negligible impact on the course of subsequent epidemic. However, these studies are based on the assumption that effective exit screening is in place. Our study shows that as the exit screening success rate decreases, the sensitivity of the entry screening policy becomes more pronounced. With the same methodology, we can also study the effects of varying the length of flight time, or the disease's incubation time.

Lastly, the benefit of entry screening is even more crucial for a small island country such as Taiwan, since all incoming traffic must go through the port authority where entry screening can be enforced.

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Proof of principle for an immunological model to explain mortality variations over the three waves of the 1918–1919 pandemic

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Keywords Immunological model, mathematical modelling, pandemic mortality, parameter estimation.

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Introduction

In England and Wales, three waves of the pandemic struck in summer, autumn, and winter seasons of 1918–1919. Although the proportion of people reporting symptoms was often greater in the first wave,[1–3] a puzzling feature was the much higher mortality in the second wave, in which 0.27% of the population died, compared with 0.03% in the out-of-season first wave and 0.10% in the third wave.[4]

An obvious hypothesis to explain the changes in mortality from wave to wave would be that the 1918 virus mutated to higher virulence after the (lower mortality) first wave. Although pandemic virus reconstituted from the high mortality waves has proven to have high virulence in animals,[5] it has not been possible to recover virus from the first wave in 1918 for comparative purposes. Indeed it is questionable whether virulence mutation(s) occurring between wave 1 and wave 2 could have spread to so many different populations in the time-frames observed. Furthermore, in all three pandemic waves, there was the same age-distribution of mortality, with more deaths occurring amongst younger adults than older adults.[1–3] This 'pandemic signature', arguably due to immune protection of older adults who were exposed to a similar virus in the years before 1890,[6,7] suggests that the 1918–1919 viruses were at least immunologically similar in all three waves.

A second hypothesis would be that the higher case fatality in the later waves was due to higher rates of complicating bacterial pneumonia,[8] to increased transmission of influenza virus in the cooler months of the year, or to other seasonal effects.[9]

We have considered a third (immunological) hypothesis to explain the greatly increased mortality in waves 2 and 3. The underlying idea is that the mortality rate in the first wave was lower than in later waves because most persons were protected by prior immunity in the first wave, and that the mortality was higher in later waves because of waning of that short-lived immunity. This hypothesis builds on our earlier modelling papers suggesting that even before the first wave in 1918, military,[10] school, and urban[11] populations in England and Wales apparently had (short-lived) immune protection, presumably induced by recent prior exposure to seasonal influenza.[10–12] We suggest that this short-lived strain-transcending protection was in addition to the longer-lasting immunity, presumably induced by exposures to a similar virus circulating prior to 1890, that arguably reduced pandemic mortality for older adults in 1918–9.[6,7]

Materials and methods

Cumulative mortality rates attributed to pandemic influenza were available for each of the three waves in 1918–1919 for 330 populations in England and Wales.[13] We have built immunological models to potentially explain the variation in mortality rates across waves and populations. To show proof of principle, we have fitted these models to mortality data from a randomly selected sub-set of twenty populations. Our key assumption was that the risk of a fatal infection would be limited to persons with inadequate immunity who were being exposed to the pandemic virus for the first time. Persons who were exposed and who survived an earlier wave were assumed to be protected against death in a later wave.

Model A and assumptions (see Figure 1)

Before the first wave, we assumed that people could be fully susceptible (S_0), or partially protected (Q_0), or fully protected (P) by prior immunity which was not necessarily specific for the new virus. We assumed that exposure to the new pandemic virus would be fatal (M) in a proportion θ of fully susceptible persons who were actually exposed (E) in the relevant wave. For those surviving that first exposure, it was assumed that they would be permanently protected against death in later waves by an immune

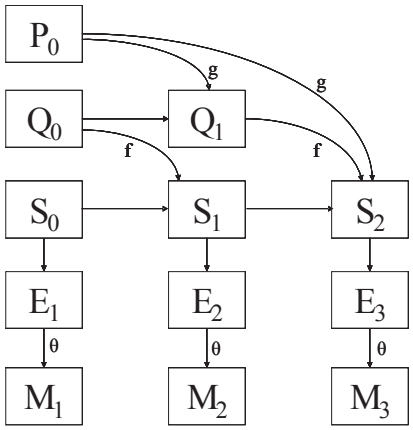

Figure 1. States for model A.

response specific for the pandemic virus. For persons in a state of intermediate immune protection, *Q*, it was assumed that viral exposure and multiplication would induce an immune response specific for the pandemic virus that would protect them against death in that wave and in subsequent waves. In contrast, for persons with strong prior immune protection, *P*, the virus would not be able to multiply to induce pandemic-specific immune protection. Between waves, it is assumed that due to the waning of non-specific prior immunity, persons in the *P* state can move to the *Q* state, and persons in the *Q* state can move to an S state before the next wave. The proportion (*E*) of susceptible persons exposed to productive infection in each population was estimated by applying the following version of the final size equation[11] to the proportion susceptible (*S* & *Q*) in each wave, for each population:

$$E = (S + Q)\left(1 - e^{-E.R_0}\right)$$

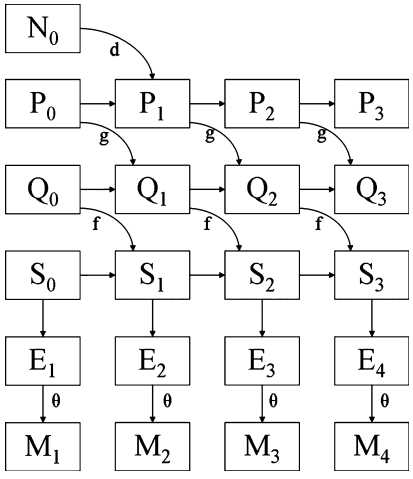

Figure 2. States for model B.

Note: In both Figures 1 and 2, we have omitted the flows out of the Q and E states that removed persons from the risk of death.

Parameters: S_0 = proportion fully susceptible to infection and death before wave 1; Q_0 = proportion susceptible to immunising infection, but not to death from exposure in wave 1; P_0 = proportion temporarily protected against both immunising infection and death from exposure in wave 1; N_0 = proportion even more protected against both immunising infection and death from exposure in wave 1 (model B only); R_0 = basic reproduction number (the average number of secondary cases for each primary case) in a fully susceptible population; f = proportion moving from Q to S between waves; g = proportion moving from P to Q between waves; d = proportion moving from N to P between waves (model B); θ = proportion of E that actually move to M and die.

Inadequacy of model A

Model A could provide a very good fit for the summer, autumn, and winter waves of the 1918–1919 pandemic (results not shown). However, because of the replenishment of the pool of susceptible persons over time, model A also predicted a fourth wave of influenza in the spring season of 1919. As no such wave was seen, and as we could not find parameters values for model A that did not predict a fourth wave, we must regard model A as inadequate.

Model B was similar to model A, but with an additional stage of prior immunity (*N*), which could wane to *P*. Model B allowed us to not only fit the three observed waves, but also to fit the imputed data (zero cases) corresponding to the absent fourth wave.

Fitting algorithm

Following earlier work,[10,11] we used a Bayesian approach with Markov Chain Monte Carlo (MCMC) procedures to estimate model parameters, and we used hyper-parameters to allow for parameter variation between populations.[11] The initial conditions were specified by the parameters: P_0, Q_0, S_0 and N_0. From these and the other parameters, it was possible to simulate the behaviour of model A over three waves, and of model B over four waves, and to estimate the expected numbers dying in each wave in each population. We calculated the log likelihood of the observed numbers of deaths given the parameter estimates, and we used MCMC simulation to generate the posterior distributions of parameters.

Results

Although we obtained an excellent fit between observed and expected numbers of deaths in each of the three waves for the 20 populations for model A, we could not find parameter values for model A that would fit the three

observed waves without giving rise to a fourth wave in the spring of 1919.

Accordingly, in the modified model B, we allowed for an additional stage of prior immunity (Figure 2), and we fitted the model to the same data, plus imputed data corresponding to 'the absent fourth wave'. We obtained a very good fit to the three observed waves and the absent fourth wave in each population. The 95% credibility intervals for parameter estimates, derived from the posterior distributions of the hyper-parameters were: $\theta = 0.05$–0.08, $S_0 = 0.015$–0.032, $Q_0 = 0.40$–0.47; $N_0 = 0.2$ (fixed); $P_0 = 1 - S_0 - Q_0 - N_0$; $f = 0.33$–0.44; $g = 0.87$–0.94; $d = 0.94$–0.97 and $R_0 = 2.6$–3.6.

This analysis had allowed all parameters to vary from population to population under the constraints of the hyper-parameters. However, several of the biologically determined parameters might be expected to be more constant from population to population, whereas those dependent on mixing history and other social characteristics which vary more widely from population to population. To test this possibility, we fixed the mean values for the more biological parameters ($f = 0.385$; $d = 0.955$; $g = 0.905$) and estimated the 95% credibility intervals for the others as: $\theta = 0.05$–0.10; $S_0 = 0.012$–0.032, $Q_0 = 0.36$–0.44; and as before $N_0 = 0.2$ (fixed); $P_0 = 1 - S_0 - Q_0 - N_0$; $R_0 = 3.1$–4.5.

In a subsequent paper we will be able to provide more details of the method, the robustness of the assumptions, and the results from fitting to many more populations.

Discussion

This short report suggests that the observed patterns of mortality in England and Wales over the three waves of the 1918–1919 influenza pandemic[4,13] can be explained by an immunological model. In particular, the lower mortality in wave one can be explained by the assumption of protective immunity antedating the first wave, arguably induced by prior exposure to seasonal influenza.[10,11] The much greater mortality in wave two can be explained by the waning, between wave one and wave two, of that short-lived and less-specific immune protection. The somewhat lesser mortality in wave three and the 'absent fourth wave' can be explained in terms of the progressive acquisition of immunity specific to the pandemic virus.

The credibility estimates for parameters are of potential interest. For example, R_0 estimates of 3.1–4.5 across different populations are consistent with our earlier findings.[10,11] If all persons had been susceptible, such R_0 values imply that the virus would have infected most people in all populations. However, even in the first wave, the proportion susceptible, $S_0 + Q_0$, was <50% in all populations, so that a considerable number of persons escaped productive infection in that wave; as their immunity waned, they became susceptible to infection in the later waves. It is likely that the variation in R_0 between populations is due to different rates of population mixing. Estimates for θ indicate that between 5% and 10% of infections in the most susceptible persons were fatal; the higher values of θ could reflect higher rates of secondary bacterial infection in the most socially disadvantaged and overcrowded populations.[4]

Although we have shown the plausibility of an immunological explanation for wave to wave changes in pandemic mortality, we cannot assume that our particular model is even approximately correct. Nor can we exclude the possibility that the higher mortality in the later pandemic waves in 1918–1919 was because of genetic change in the virus in later waves, or because of changing rates of secondary bacterial infection[8] or seasonal effects.[9] Nevertheless, there is growing evidence that the population spread of pandemic influenza, whether in 1918–1919[10,11] or in 2009,[12,14] can be constrained by significant prior immunity, even for viruses that are ostensibly novel. Previous reports, reviewed in Ref. [10,11], support the idea of strain-transcending immune protection, which can wane over periods of a few months. This form of protection, probably induced by recent exposure to seasonal influenza, may not be mediated by HI or neutralizing antibody.[11] In contrast, strain-specific immunity, most often mediated by HI or neutralizing antibodies can be so long-lasting that after several decades it will still provide significant protection against any closely-related virus that re-appears in the population.[14]

It has not escaped our notice that although attack-rates in the H1N1 2009 pandemic were low in many countries, with generally mild symptoms, the virus did cause life-threatening illness in a small proportion of younger affected persons.[15] It seems likely that those who were most severely affected in 2009 were doubly unlucky: they had missed out on seasonal influenza infection or vaccination in the preceding season(s), and they were born too late to have been protected by the closely-related viruses that are thought to have circulated before 1950.[14]

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Evaluation of model prediction during the early phase of the 2009 influenza pandemic in Italy

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Abstract

During the early phases of the 2009 influenza pandemic in Italy, real-time modeling analysis were conducted in order to estimate the impact of the pandemic. In order to evaluate the results obtained by the model we compared simulated epidemics to the estimated number of influenza-like illness (ILI) collected by the Italian sentinel surveillance system (INFLUNET), showing a good agreement with the timing of the observed epidemic. By assuming in the model mitigation measures implemented in Italy, the peak was expected on week 44 (95% CI: 44, 45). Results were consistent with the INFLUNET data showing that the peak in Italy was reached in week 46. These predictions have proved to be a valuable support for public health policy makers for planning interventions for mitigating the spread of the pandemic.

Introduction

Mathematical models have recently become a useful tool to analyse disease dynamics of pandemic influenza virus candidates.[1–11] As of April 2009, after the pandemic threat emerged worldwide,[12] it was crucial for policy makers to have early predictions on the possible spread of the 2009 pandemic influenza virus in order to support, with quantitative insight into epidemic, policy decisions. Thus, after the first pandemic alert was announced by the World Health Organization (WHO) in late April 2009, a National Crisis Management Committee headed by the Minister of Health was established in Italy in order to provide weekly advice to the Italian Ministry of Health. Real-time analyses using an individual based model were undertaken. The transmission model was previously used for evaluating the effectiveness of the control measures adopted in the National Pandemic Preparedness Plan[3] and for assessing the age-prioritized distribution of antiviral doses during an influenza pandemic.[2] To parameterize the transmission model, we used data derived from the National Surveillance System until 17 June 2009 and estimates of key epidemiological parameters as available at that time.

In order to provide a preliminary assessment of the model predictions performed during the early stages of the epidemic, we compare model predictions with surveillance

data of influenza-like illness (ILI) available since August 2009.

Materials and methods

Data description

After the first pandemic alert was announced by the WHO in late April 2009, a national active surveillance system for the 2009 pandemic influenza was set up from 28 April to July 2009.[13] However, over the period from April to October 2009, surveillance systems, laboratory testing, and diagnostic strategies have varied considerably in Italy. Since end of July 2009, following WHO recommendations,[14] the focus of surveillance activities has changed in reporting requirements, as active case-finding became unsustainable and unnecessary. For this reason, the Ministry of Health (Ministry of Health, available in Italian at the website: http://www.normativasanitaria.it) requested regional Health Authorities to report the weekly aggregated ILI cases according to a new case definition (sudden onset of acute respiratory symptoms and fever >38°C plus at least one of the following systemic symptoms: headache, malaise, chills, sweats, fatigue; plus at least one of the following respiratory symptoms: cough, sore throat, nasal obstruction).

By October 2009, following the increasing number of cases, the Sentinel Influenza Surveillance System (INFLUNET available at: http://www.flu.iss.it) became the official surveillance system for ILI cases in Italy (Ministry of Health, available in Italian at the website: http://www.normativasanitaria.it). Since 1999, INFLUNET is routinely based on a nation-wide, voluntary sentinel network of sentinel community based physicians in the 21 regions and autonomous provinces of the country. Incidence rates are, therefore, not based on consultations, but on the served population of each reporting physician each week. INFLUNET usually consists of an average of 830 (range 648–902) general practitioners (including physicians and pediatricians) per year, covering about 1·5–2% of the general population, (representative for age, geographic distribution, and urbanization level) reporting ILI cases (according with a specific case definition). Italian INFLUNET surveillance system is part of the European Influenza Surveillance Scheme (EISS).

The model

A stochastic, spatially explicit, individual-based simulation model[3] was used. Individuals are explicitly represented and can transmit the infection to household members, to school/work colleagues, and in the general population (where the force of infection is assumed to depend explicitly on the geographic distance). The national transmission model was coupled with a global homogeneous mixing Susceptible-Exposed-Infectious-Removed (SEIR) model accounting for the worldwide epidemic, which is used for determining the number of cases imported over time.

Model parameterization

Regarding the epidemiological assumptions (e.g., length and shape of the infectivity period, which lead to an effective generation time of 3·2 days), this study is consistent with Refs [3,4,11,15], but for the proportion of symptomatic individuals, which is assumed to be 66·7%.[16] The basic reproductive number of the national transmission model was set to 1·4, according to the early estimates as obtained during the initial phase of the epidemic in Mexico in a community setting.[17,18] We initialized our simulations through the global homogeneous mixing model in such a way that 158 imported cases were generated until 7 June 2009. This gives a reliable way for fixing the time in the simulations and thus determining the timing of school closure and vaccination in the simulations. The model accounts for school closure for both summer and Christmas holidays: we assumed that in these periods contacts among students decrease, while contacts in the general community increase, as in Ref. [19]. We also considered scenarios accounting for partial immunity in the population.[20]

Intervention options – non-pharmaceutical interventions

In order to investigate the effects of recommendations of the Ministry of Health (confirmed cases coming from affected areas were isolated for 7–10 days, either in hospital or at home) established in the early phase of the pandemic (April–July 2009), we assumed that a fraction of the imported symptomatic cases were isolated on the first day after the symptoms onset. This recommendation was in place until 27 July 2009. We also assumed, according to the Italian school calendar, that schools were closed from 10 June 2009 to 10 September 2009 for the summer holidays, and from 22 December 2009 to 6 January 2010 for Christmas holidays. The effects of prolonged school closure were also investigated.

Intervention options – pharmaceutical interventions

When considering vaccination, we assumed 6 weeks for the logistical distribution of doses of pandemic vaccine. Since at the time of simulation specific recommendations regarding the administration of a single dose of pandemic vaccine from EMA were not available yet, we considered the administration of 2 vaccine doses 1 month apart). The pandemic vaccine was considered effective after the administration of the second dose with a vaccine efficacy of 70%. We assumed the vaccine to be administered by priority,

vaccinating first the target population accounting for essential services workers (including health care workers and blood donors), pregnant women at the second or third trimester, and at risk patients (with chronic underlying conditions) younger than 65 years old. The vaccination coverage was assumed 90%. Regarding antiviral treatment and prophylaxis, recommendations of the Ministry of Health in the initial phase of the epidemic were to administer antivirals to all confirmed cases and to their close contacts. We assumed that the surveillance system would be able to detect 90% of symptomatic cases. After 8 July 2009, recommendations changed and antiviral treatment was considered only for cases with severe complications and in case of local clusters. Since it was difficult to establish the proportion of treated cases, we considered different scenarios: antiviral treatment from 0% to 30% of the symptomatic cases. Consistently with Ref. [3], both treatment and prophylaxis were assumed to start 1 day after the clinical onset of symptoms in the index case. Treatment was assumed to reduce infectiousness by 70%, whereas antiviral prophylaxis was assumed to reduce susceptibility to infection by 30%, infectiousness by 70%, and the occurrence of symptomatic disease by 60%.

Results

As of 26 July 2009, approximately 1238 confirmed cases have been reported to the Italian surveillance system for pandemic influenza. During July, the sudden increase of ILI confirmed cases suggests for sustained autochthonous transmission in Italy. By analyzing the number of ILI cases reported to the surveillance during the weeks from 39 to 44, we found that the exponential growth rate was 0·832/week and thus we estimated the national reproductive number to be $R_0 = 1{\cdot}38$. This estimate of the basic reproductive number supports the choice of the value adopted in the model simulations ($R_0 = 1{\cdot}4$).

In the absence of intervention measures, the predicted cumulative attack rate was 30·6% (95% CI: 30·6, 30·7), and the peak was expected on week 42 (95% CI: 41, 42) with a peak day incidence of 0·35% (95% CI: 0·329%, 0·37%). By assuming case isolation, antiviral treatment, and prophylaxis to 90% of symptomatic cases until 8 July 2009, the peak was expected on week 43 (95% CI: 43, 44). When considering 33·3% of natural immunity in the population aged more than 59 years, the peak was expected 1 week later than in the previous scenario, i.e., on week 44 (95% CI: 44, 45). To validate the model, we compared model predictions (which are based only on the available information on the early phases of the epidemic) with ILI data (Figure 1). Based on model predictions, we estimated the underreporting factor of INFLUNET ranging from 3·3 to 3·7, considering different scenarios. By aligning the simulations with the ILI data adjusted by the underreporting factor, we can observe that almost all the points in the increasing phase of the epidemic lie within the 95% CI of the model results (both considering or not natural immunity). The decay phase of the simulated epidemics shows a small delay with respect to the ILI data.

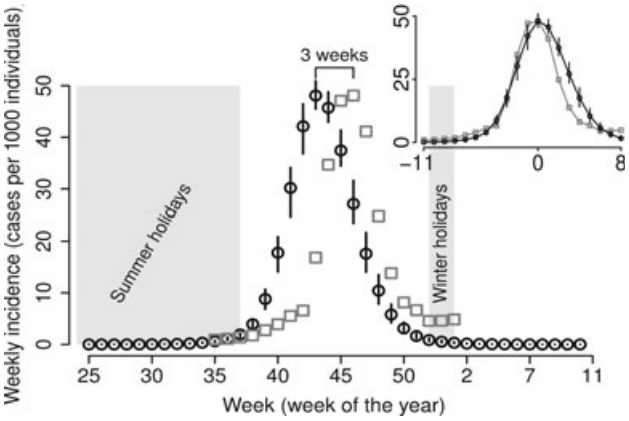

Figure 1. Model fit (black circles) and INLFLUNET surveillance data adjusted by the underreporting rate (gray squares). Vertical lines (black for model results) represent the 95% CI. The gray background represents summer and Christmas holidays, during which schools are regularly closed. The inset shows the simulated epidemic and the influenza-like illness data obtained by aligning the peak of the simulations to the peak in the dataset.

When introducing single and combined mitigation measures, such as case isolation, antiviral treatment, prophylaxis, and vaccination in the model, results showed that even a low proportion of symptomatic cases treated with antiviral drugs could have led to a relevant reduction in the epidemic size (Table 1). We simulated the planned Italian vaccination strategy (begun on 15 October 2009), obtaining a limited but not negligible reduction in the attack rate with respect to the scenarios accounting only for antiviral treatment. Moreover, the effect of vaccination would be higher if coupled with antiviral treatment; vaccination would have no effect on delaying the peak incidence.

Discussion

Model predictions produced in Italy during the early phase of the 2009 pandemic influenza are in excellent agreement with Italian surveillance data on the beginning of the epidemic (when case isolation, antiviral treatment of index cases, and antiviral prophylaxis to close contacts were implemented by the Italian regional public health authorities) and are basically consistent with the INFLU-

Table 1. Effectiveness of mitigation measures by assuming natural immunity

Intervention	Final attack rate (%)	Peak day (days*)	Peak day incidence (%)	Antiviral courses
None	30·6 (30·6, 30·7)	123·5 (121·5, 126·4)	0·35 (0·329, 0·37)	–
AVP**, case isolation**	30·6 (30·6, 30·7)	130·7 (128, 133·4)	0·35 (0·33, 0·37)	<1000
AVP**, case isolation**, antiviral treatment to 15% of the clinical cases	27·6 (27·5, 27·6)	134·9 (131·6, 140·3)	0·288 (0·272, 0·306)	2 300 000
AVP**, case isolation**, antiviral treatment to 30% of the clinical cases	24·4 (24·3, 24·4)	148·5 (145·5, 152·8)	0·226 (0·215, 0·239)	4 100 000
AVP**, case isolation**, antiviral treatment to 15% of the clinical cases, vaccination†	27·7 (27·4, 27·5)	141 (138, 145·8)	0·248 (0·235, 0·261)	2 100 000
AVP**, case isolation**, antiviral treatment to 30% of the clinical cases, vaccination†	21·7 (21·6, 21·8)	155·1 (152·6, 158·9)	0·197 (0·187, 0·206)	3 600 000

AVP, antiviral prophylaxis to households contacts of index cases.
*Measured in days from 14 June 2009.
**Until 8 July 2009. Case detection fixed at 90%.
†Vaccination begins on 15 October 2009; the target population: essential workers and chronic patients (about 8 500 000 individuals).
††Natural immunity on 33·3% of the population aged >59.

NET data during the course of the epidemic. The model has been useful for predicting the timing of the epidemic, while it has overestimated the impact of the 2009 influenza pandemic for adult and elderly individuals. However, the disalignment is probably due to the model parameterization. Based on literature values,[4,5] we assumed a similar fraction of cases in the different social contexts considered in the model (namely 1/3 in households, 1/3 in schools/workplaces, and 1/3 in the general community), since analysis on the relative transmissibility of the virus was not carried out for any country yet.

We were also able to estimate an underreporting factor for the INFLUNET data in the range 3·3–3·7. If we focus our attention on the reporting factor computed by considering the total number of cases (instead of symptomatic cases), the resulting value lies in the range 18–20·2%, which is in excellent agreement with the range estimated in Ref. [21] on previous A/H1N1 influenza seasons, namely 16·2%–21·6%.

Moreover, based on our results showing that vaccinating 40% of the Italian population was more than adequate to mitigate the pandemic, the Ministry of Health decided to stockpile a limited number of vaccines. We have also shown that starting the vaccination program in October (or later) could have had only a limited effect on reducing the impact of the epidemic, although it may have been useful to prevent a possible second wave and to protect essential workers and at-risk patients. Finally, our results have shown that antiviral treatment would have been the most efficient strategy to reduce the impact of the influenza pandemic, even with a limited antiviral stockpile.

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Logistical feasibility and potential benefits of a population-wide passive immunotherapy program during an influenza pandemic

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Introduction

Influenza pandemic plans for treating severe cases currently focus on the use of antivirals, anti-inflammatory agents, and non-pharmacologic means such as optimal ventilator and fluid management. In contrast, passive immunotherapy has received limited attention.[1] A recent meta-analysis study suggested that during the 1918 influenza pandemic, transfusion of blood products from recovered individuals reduced the mortality rate of severe cases by more than 50%.[2] The proof of principle for this therapeutic approach was demonstrated in the modern clinical setting in Shenzhen in 2006.[3] In 2007, Simmons *et al.* used a murine model of infection to demonstrate the prophylactic and therapeutic efficacy of monoclonal antibodies with neutralizing activity produced by immortalized B lymphocytes of convalescent patients recovering from influenza A/H5N1 virus infection,[4] thereby providing further pre-clinical evidence that passive immunotherapy with convalescent blood products could be a practical treatment option for pandemic influenza.

A population-wide passive immunotherapy program

In this paper, we assume that convalescent plasma (CP) is efficacious in treating severe cases of pandemic influenza.

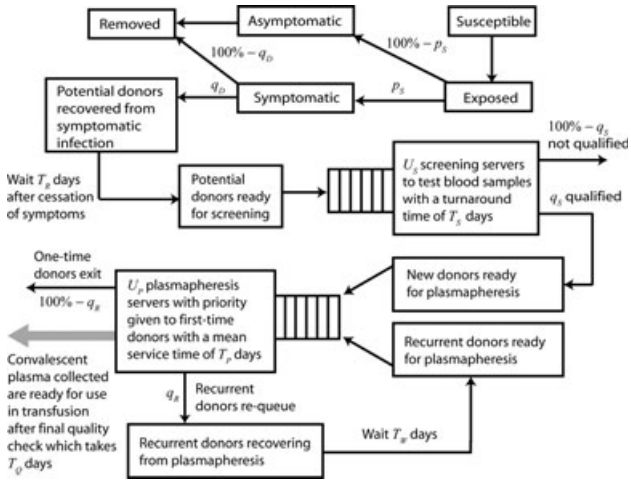

Figure 1. Schematic of the proposed passive immunotherapy program.

Under this premise, we test the hypothesis that a population-wide passive immunotherapy program that collects plasma from a small percentage of convalescent individuals can harvest sufficient CP to treat a substantial percentage of severe cases during the first wave of the pandemic. The proposed program involves recruiting adults (individuals age 20–55 years) to donate blood if they have experienced influenza-like symptoms more than 2 weeks ago (to account for the time needed for neutralizing antibodies to build up). The blood samples would be screened for infectious diseases (including HIV, HBV, HCV, HTLV, and syphilis, etc., as in routine blood donation screening) and neutralizing antibodies against the pandemic virus. Donors whose blood samples are free of known infectious agents and contain a sufficiently high titer of neutralizing antibodies would then be invited to donate plasma by plasmapheresis or routine whole blood donation. Qualified donors with higher titers may be given higher priority for plasma donation.

In this paper, we use the demographic and logistical parameters of Hong Kong as a case study. See Figure 1 for a schematic of the proposed passive immunotherapy program. We examine the following questions regarding the logistical feasibility and potential benefits of the proposed passive immunotherapy program: (i) What percentage of convalescent individuals (donor percentage) is needed in order for the program to significantly reduce pandemic mortality? (ii) How many severe cases can be offered passive immunotherapy? (iii) What are the rate-limiting factors in the supply of passive immunotherapy? (iv) What are the epidemiologic and logistical factors that determine the demand–supply balance of passive immunotherapy? A more detailed presentation of our results is now available in Ref. [5].

Materials and methods

Transmission and natural history model for pandemic influenza

We use an age-structured disease transmission model to simulate the spread of pandemic influenza. The natural history model is similar to that used by Basta *et al.*[6,7] The most important parameter in characterizing the growth of an epidemic is the basic reproductive number R_0, which is defined as the average number of secondary cases generated by a typically infectious individual in a completely susceptible population. We consider values of R_0 between 1·2 and 2, which is consistent with recent estimates.[6,8–10]

Logistical model for the passive immunotherapy program

We assume that q_D (%) of 20 to 55 year-old individuals who have recovered from symptomatic infections of pandemic influenza donate their blood for screening $T_R = 14$ days after cessation of symptoms. Follow-ups of convalescent individuals infected with H1N1pdm in an ongoing clinical trial of passive immunotherapy suggested that neutralizing antibodies level reaches maximal level around 14–21 days after recovery and stays at that level for months after.[11] We assume that q_S (%) of these donors are qualified for plasma donation of which q_R (%) are recurrent donors who return to donate plasma every $T_W = 14$ days. Screening involves both detection of infectious agents and neutralizing antibodies against the pandemic virus. The latter is the rate-limiting step because neutralization tests of pandemic viruses can only be done in a BSL3 setting. We assume that five BSL3-trained technicians are available to test the blood specimens, each running 150 viral neutralization tests in 3 days. Therefore, the capacity and turnaround time of blood screening are $U_S = 750$ and $T_S = 3$ days, respectively. Hong Kong currently has nine plasmapheresis machines which allow a maximal throughput of 162 plasma donations per day (assuming 12-hour daily operation with each donation taking 40 minutes). Therefore, the capacity and turnaround time of plasmapheresis are $U_P = 9$ and $T_P = 1/18$ days, respectively.

Collected CP are ready for use in transfusion after final quality check, which takes $T_Q = 2$ days. We assume that r_T plasma donations are required to treat one severe case on average. The expert panel of the abovementioned study of passive immunotherapy for H1N1pdm in Hong Kong suggested that $r_T < 10$.

Demand for passive immunotherapy

We assume that p_H (%) of symptomatic cases will be severe cases for whom passive immunotherapy is suitable. Although p_H will be smaller than the case-hospitalization rate (passive immunotherapy may not be suitable for some

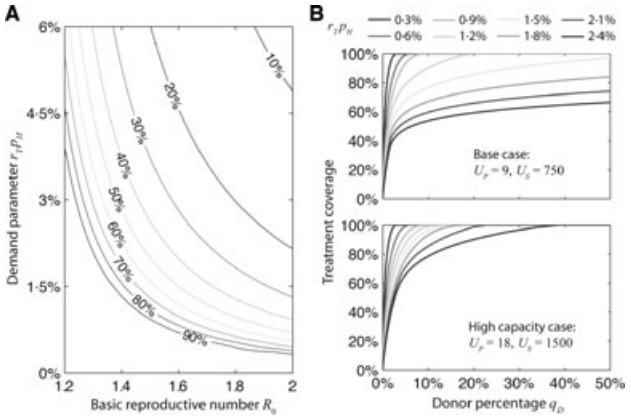

Figure 2. Treatment coverage in the base case. (A) Treatment coverage ρ when the donor percentage is $q_D = 15\%$. (B) Sensitivity of ρ against q_D with $R_0 = 1{\cdot}4$ in the base case (upper panel) and when the plasmapheresis and screening capacity are doubled (lower panel). In general, increasing q_D beyond 15% has little impact on the outcome. To interpret the grayscale, note that treatment coverage decreases monotonically as $r_T p_H$ increases.

hospitalized cases), we assume that the two have similar ranges and consider p_H ranging from 0·1% to 1%. Because each severe case requires r_T plasma donations on average, demand for CP is simply $r_T p_H$ times the number of symptomatic cases. Therefore, $r_T p_H$ can be regarded as a single parameter, which we refer to as the lumped demand parameter.

Outcome measure

We define the outcome as the percentage of severe cases that can be offered passive immunotherapy by the proposed program during the first wave of the local epidemic. We refer to this outcome as *treatment coverage* and denote it by ρ.

Results

Base case

We consider the base case scenarios assuming $q_R = 20\%$ and $q_S = 80\%$. In general, the treatment coverage ρ increases sharply as the basic reproductive number R_0 and the lumped demand parameter $r_T p_H$ decrease (Figure 2A). In particular, when R_0 is large and $r_T p_H$ is small, ρ is very sensitive to $r_T p_H$, but insensitive to R_0. Similarly, when R_0 and $r_T p_H$ are small, ρ is very sensitive to both. With a donor percentage of $q_D = 15\%$, the proposed program can supply passive immunotherapy to more than 82% of severe cases ($\rho > 82\%$) if $R_0 < 1{\cdot}4$ and $r_T p_H < 1{\cdot}5\%$, but <35% if $R_0 > 1{\cdot}8$ and $r_T p_H > 1{\cdot}5\%$. In general, the treatment coverage ρ increases sharply as the donor percentage q_D rises from 0%, but with rapidly decreasing marginal increase (Figure 2B). When $R_0 < 1{\cdot}4$ and $r_T p_H < 1{\cdot}5\%$, $\rho > 67\%$ even if q_D is as low as 5%, which is comparable to the current average blood donation rate of 38·1 donations per 1000 population in developed countries.[12] When q_D is >15%, ρ becomes largely insensitive to further increase in q_D in most scenarios. The treatment coverage ρ for $q_D = 15\%$ is more than 81% that for $q_D = 50\%$ across all values of R_0 and $r_T p_H$ considered in the base case. Therefore, increasing the donor percentage q_D beyond 15% has a relatively small impact on CP supply. This is because increasing q_D can boost supply only when plasmapheresis is not yet the supply bottleneck. For the same reason, once the donor percentage q_D has reached 15%, the treatment coverage ρ is insensitive to further increase in q_D even when the plasmapheresis and screening capacity are doubled (Figure 2B, lower panel).

Sensitivity analysis

We conduct an extensive multivariate sensitivity analysis to test the robustness of our base case observations against uncertainties in parameter values. We generate 15 000 epidemic scenarios by randomly selecting parameter values from their plausible ranges using Latin-hypercube sampling. Although there are numerous model parameters, the treatment coverage ρ is mainly determined by three lumped parameters: (i) $r_T p_H$, which indicates the magnitude of demand; (ii) $q_S q_D$, which indicates the magnitude of supply; (iii) the initial growth rate of the epidemic r (results not shown). While the dependence of ρ on $r_T p_H$ and $q_S q_D$ is readily comprehensible, it is not obvious a priori that ρ depends on the natural history and transmission dynamics of the disease via only the initial epidemic growth rate. When the plasmapheresis and screening capacity are very large, the supply–demand dynamics is further simplified: the treatment coverage ρ depends on lumped demand parameter $r_T p_H$ and the lumped supply parameter $q_S q_D$ only via their ratio. Finally, ρ becomes insensitive to $q_S q_D$ when the latter increases beyond 15–20%, which is consistent with our base case observations.

Discussion

Our results suggest that with plasmapheresis capacity similar to that in Hong Kong, the proposed passive immunotherapy program can supply CP transfusion to treat 67–82% of severe cases in a moderate pandemic (basic reproductive number $R_0 < 1{\cdot}4$, lumped demand parameter $r_T p_H < 1{\cdot}5\%$) when the donor percentage is 5–15%. Increasing the donor percentage beyond 15% has little additional benefit because CP supply is constrained by the capacity of plasmapheresis during most stages of the epidemic. Increasing plasmapheresis capacity could significantly boost CP supply, especially when there is a substantial pool of recurrent donors to alleviate the dependence of CP supply on donor percentage. In an ongoing clinical trial of passive immunotherapy for H1N1pdm virus infection in Hong Kong, 20% of convales-

cent individuals agreed to donate their plasma for the study. Therefore, the donor percentage required by the proposed passive immunotherapy program (5–15%) is likely to be feasible. In view of the logistical feasibility of such program, we recommend that further clinical studies are conducted to evaluate the safety and efficacy of passive immunotherapy as a treatment for severe cases of pandemic influenza virus infection.

Our study is based on the premise that CP will be efficacious in reducing morbidity and mortality associated with pandemic influenza. In theory, the polyclonal nature of neutralizing antibodies in CP would lower the probability of an escape mutant emerging in treated patients. Further, besides providing neutralizing antibodies against the pandemic virus, CP also might carry antibodies to other bacterial pathogens, which might decrease the severity of coexisting bacterial infections.[4] As such, CP not only might reduce the case fatality rate but might also increase the recovery rate and shorten duration of hospitalization of severe cases. The proposed passive immunotherapy program can thus significantly reduce the burden on the healthcare system, especially the intensive care unit, which will likely be stressed, if not overloaded, at the peak of an influenza pandemic wave, hence benefiting the general public and not only those receiving passive immunotherapy. Although the hypothesized efficacy of CP has yet to be proven in clinical trials, our modeling results show that a public health system similar to that in Hong Kong has the capacity to support a population-wide passive immunotherapy program that can supply CP treatment to a substantial percentage of the severe cases in a moderately severe pandemic. We estimate that compared to other developed countries, Hong Kong has a relatively low plasmapheresis capacity. Our conclusions regarding donor percentage needed and rate-limiting factors remain valid for plasmapheresis capacity ranging from 50% to 400% of what we have assumed in the base case (results not shown).

Our conclusions are robust against uncertainties in the natural history and transmission dynamics of pandemic influenza. Our sensitivity analysis shows that the outcome depends on these epidemiological characteristics only via the initial growth rate of the epidemic. As such, our results are applicable not only to pandemic influenza, but also to other emerging infectious diseases for which the time-scales of disease transmission and antibody response are similar to that for influenza virus. The three determinants of treatment coverage (the initial epidemic growth rate, the lumped demand parameter $r_T p_H$, and the lumped supply parameter $q_D q_S$) are all readily measurable in real-time during an epidemic. Therefore, our methods and results can be used as a general reference for estimating the treatment coverage of the proposed passive immunotherapy program for a given plasmapheresis capacity.

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Disclosure

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Antiviral activity of a selective COX-2 inhibitor NS-398 on avian influenza H5N1 infection

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Keywords Antiviral, H5N1, NS-398, selective COX-2 inhibitor.

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Abstract

Background Highly pathogenic H5N1 virus continues to pose a serious threat to human health and appears to have the capacity to cause severe disease in previously healthy young children and adults. At present, antiviral therapy by oseltamivir remains the mainstay for managing H5N1 patients. While early treatment improves survival, approximately 50% of patients treated within 4 days of illness still succumb to the disease. In addition to the role of viral replication, there is good evidence that the host pro-inflammatory responses contributes to H5N1 pathogenesis. This suggests that both antiviral and immune-modulatory drugs may have a role in therapy. We previously demonstrated that cyclooxygenase 2 (COX-2) plays a regulatory role in H5N1 hyperinduced pro-inflammatory responses, and its inhibitor has potent effects at modulating this host response. Now we demonstrate that, in addition to its immune-modulatory effect, a selective COX-2 inhibitor, NS-398 has a direct antiviral effect against H5N1 infection.

Materials and methods Human primary monocyte-derived macrophages or alveolar epithelial cells (A549) were pre-treated with NS-398 or drug-vehicle for 1 hour before H5N1 virus infection. H5N1 viruses at multipicity of infection (MOI) of 2 was used to infect the cells. Following virus adsorption for 30 mins, the virus inoculum was removed, and the cells were washed and incubated in corresponding medium with NS-398 or drug-vehicle as controls for 3, 6, 24, 48, and 72 hours post-infection. Cells were harvested for RNA isolation at 6 hours post-infection to study viral matrix (M) gene expression. Supernatants were collected for 50% tissue culture infection dose ($TCID_{50}$) assay to determine the virus titers at 3, 24, 48, and 72 hours after H5N1 infection.

Results NS-398 was found to suppress virus gene transcription and infectious virus yield in H5N1-infected human cells.

Conclusion We demonstrate that a selective COX-2 inhibitor, NS-398, shows an inhibitory effect on H5N1 viral replication in addition to its immune-modulatory effect that could counter the detrimental effects of excessive pro-inflammatory cytokine production. The findings suggest that selective COX-2 inhibitors may be a therapeutic target for treating H5N1 disease in combination with appropriate antiviral therapy.

Introduction

The emergence and spread of the highly pathogenic avain influenza viruses (H5N1) in poultry and wild birds with repeated zoonotic transmission to humans has raised pandemic concern. At the time of writing, 507 human cases have been reported with 302 fatalities, an overall case fatality rate of around 60% (cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to World Health Organization updated to 18 October 2010).

Our previous data demonstrated that COX-2 was markedly up-regulated in H5N1-infected primary human macrophages, and that it played a regulatory role in the H5N1-hyperinduced host pro-inflammatory responses.[1] Such cytokine dysregulation is proposed to be a major contributor to the pathogenesis of H5N1 disease in humans.[2] With the use of selective COX-2 inhibitors, we found that the H5N1-hyperinduced cytokine response was significantly suppressed by the drug in a dose-dependent manner.[1]

Selective COX-2 inhibitor is a form of a non-steroidal anti-inflammatory drug that selectively targets COX-2, and it is an inducible enzyme responsible for inflammatory process and immune response. Here, we report a novel finding of a direct antiviral effect of a selective COX-2 inhibitor, NS-398, against H5N1 infection in human primary macrophages and alveolar epithelial cells. Taken together with our previous findings that suggest an immuno-modulatory effect that can modulate virus driven cytokine dysregula-

tion, these findings highlight a role for COX-2 and its downstream signaling as potential novel targets for adjunctive therapy of severe viral pneumonia, such as that caused by H5N1. Such therapy may be combined with conventional antiviral drugs.

Materials and methods

Viruses

The H5N1 virus used was A/Vietnam/3212/04 (3212/04) (H5N1), a virus from a patient with H5N1 disease in Vietnam during 2004. The viruses were grown and titrated in Madin–Darby canine kidney cells cells as described elsewhere.[3] Virus infectivity was expressed as $TCID_{50}$. All experiments were performed in a Biosafety Level 3 facility.

In vitro cell culture

Monocyte-derived macrophages: Peripheral-blood leucocytes were separated from buffy coats of healthy blood donors (provided by the Hong Kong Red Cross Blood Transfusion Service) by centrifugation on a Ficoll-Paque density gradient (Pharmacia Biotech) and purified by adherence as reported previously.[3] The research protocol was approved by the ethics committee of the University of Hong Kong. Macrophages were seeded onto tissue culture plates in RPMI 1640 medium supplemented with 5% heat-inactivated autologous plasma. The cells were allowed to differentiate for 14 days *in vitro* before use in the infectious experiments.

Alveolar epithelial cells: A549 cells were obtained from ATCC and maintained in culture using Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 0.6 mg/l penicillin, and 60 mg/l streptomycin.

Influenza virus infection and treatment of cells with NS-398

Differentiated macrophages or A549 cells were pre-treated with a selective COX-2 inhibitor, NS-398 (Cayman), at concentrations as indicated or drug-vehicle for 1 hour before infection. Cells were infected with H5N1 viruses at MOI of 2. Following virus adsorption for 30 min, the virus inoculum was removed, the cells were washed and incubated in corresponding medium with NS-398 or drug-vehicle as controls throughout the experiments. Cells were harvested for RNA isolation at 6 hours post-infection to study viral M gene expression. Supernatants were collected for $TCID_{50}$ assay to determine the virus titers at 3, 24, 48, and 72 hours after H5N1 infection.

Real-time quantitative RT-PCR assays

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized from mRNA with poly(dT) primers and Superscript III reverse transcriptase (Invitrogen). Transcript expression was monitored by real-time PCR using Power SYBR® Green PCR master mix kit (Applied Biosystems) with specific primers. The fluorescence signals were measured using the 7500 real-time PCR system (Applied Biosystems). The specificity of the SYBR® Green PCR signal was confirmed by melting curve analysis. The threshold cycle (CT) was defined as the fractional cycle number at which the fluorescence reached 10 times the standard deviation of the base-line (from cycle 2 to 10). The ratio change in target gene relative to the β-actin control gene was determined by the $2^{-\Delta\Delta CT}$ method as described elsewhere.[4]

Results

NS-398 reduced the viral M gene expression in H5N1-infected human macrophages in a dose-dependent manner (Figure 1). Similarly, production of infectious virus yield in H5N1 infected macrophages was found to be suppressed in the presence of NS-398 at 100 μm compared to vehicle-treated cells (Figure 2A). A comparable effect of NS-398 was observed in H5N1-infected human alveolar epithelial cells (Figure 2B).

Discussion

We have previously demonstrated that COX-2 expression was dramatically upregulated following H5N1 infection in human macrophages *in vitro* and in epithelial cells of lung tissue samples obtained from autopsy of patients who died of H5N1 disease.[1] This suggests that COX-2 may be an important host factor involved in H5N1 pathogenesis and also provide a possible explanation on why H5N1 virus replication is susceptible to a selective COX-2 inhibitor.

COX-2 was previously reported to play an important role in the pathogenesis of other influenza A viruses.[5] An *in vivo* study has highlighted the importance of COX-2 in H3N2-

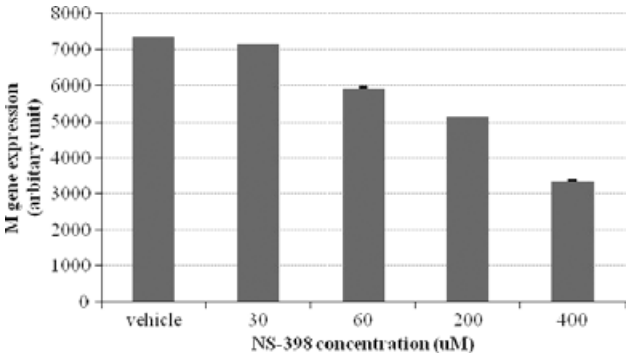

Figure 1. Effect of NS-398 on viral M gene expression in H5N1-infected human macrophages.

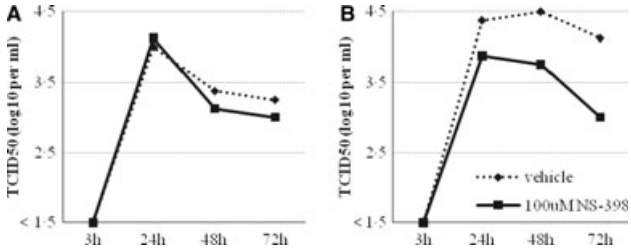

Figure 2. NS-398 reduces the progeny virus production in H5N1-infected human macrophages (A) and alveolar epithelial cells (B).

infected mice. Findings showed that infection induced less severe illness and reduced mortality in COX-2 knock-out mice than in wild-type mice. On the other hand, COX-1 knock-out mice had enhanced inflammation and earlier appearance of proinflammatory cytokines in the BAL fluid, whereas the inflammatory and cytokine responses were dampened in COX-2 knock-out mice. These data suggests that COX-1 and COX-2 may lead to opposite totally contrasting effects on influenza H3N2 infected mice. COX-1 deficiency is detrimental, whereas COX-2 deficiency is beneficial to the host during influenza viral infection. Therefore in the present study, instead of blocking COX enzymes in general as reported by others,[5] we have chosen NS-398 that selectively block COX-2 but preserve COX-1 activity and showed that this drug significantly reduced H5N1 virus replication in a dose-dependent manner. Taken together with our previous report suggesting its immuno-modulatory effects,[1] we believe that selective COX-2 inhibitors and COX-2 signaling pathways deserve investigation as a promising approach for targeting therapy in H5N1 diseases.

However, a few reports have suggested the importance of COX-2 in the late stage of inflammation for the resulution of inflammation,[6–8] and this raises concern whether inhibition of COX-2 may be harmful in treating diseases related to dysregulation of host inflammatory response such as acute lung injury,[9] which is a leading cause of death in H5N1 patients. We previously looked at the autopsy samples of lung tissues from H5N1 patients and found that COX-2 expression was markedly up-regulated compared with that from persons who died of non-respiratory causes.[1] Moreover, data also demonstrated that pro-inflammatory cytokines, such as TNF-α, was markedly elevated in the H5N1 infected lung autopsies.[10] Taken together, with the histo-pathological findings, which showed predominant features of exudative inflammatory phase in autopsy lung samples from H5N1 patients,[11,12] we may therefore speculate that people who had fatal H5N1 infection died during acute inflammation phase, and before the resolution could occur, especially for the cases with a short disease duration (<10–12 days).[13]

In conclusion, the roles of COX-2 in both pro-inflammation and pro-resolution phases deserves detailed investigation. The timing of selective COX-2 inhibitor therapy in H5N1 infected patients may be extremely critical. Therefore a time-dependent study using selective COX-2 inhibitors on H5N1-infected animal models will be particularly important in order to address the effectiveness of this drug in treating H5N1 disease.

Acknowledgements

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Avian antibodies to combat potential H5N1 pandemic and seasonal influenza

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Abstract

Highly pathogenic avian influenza A virus (HPAIV) strain A/H5N1 with unprecedented spread through much of Asia and parts of Europe in poultry remains a serious threat to human health. Passive immunization (transfer of protective immunoglobulins) offers an alternative and/or additional strategy to prevent and cure influenza. Here, we report that virus-specific immunoglobulin Y (IgY) isolated from eggs of immunized hens provide protection in mice against lethal H5N1 virus infection by neutralization of the viruses in the lungs upon intranasal administration. Importantly, chicken eggs obtained from randomly selected supermarkets and farms in Vietnam, where mass poultry vaccination against A/H5N1 is mandatory, contain high levels of IgY specific for A/H5N1 virus. When administered before or after the infection, IgY prevented and significantly reduced replication and spread of HPAIV H5N1 and related H5N2 strains. Thus, the consumable eggs readily available in markets of countries that impose poultry vaccination against A/H5N1 could offer an enormous source of valuable biological material that provides protection against A/H5N1 virus with pandemic potential. The approach could be used to control seasonal influenza.

Introduction

Since 2004, HPAIV of the H5N1 subtype has resulted in more than 430 cases of laboratory-confirmed human infection in 15 countries with a death rate of more than 50% (http://www.who.int/csr/disease/avian_influenza/). H5N1 influenza virus remains a global threat because of its continued transmission among domestic poultry and wild birds. Passive immunization (the transfer of antigen-specific antibodies (Abs) to a previously non-immune recipient host) offers an alternative and/or additional countermeasure against influenza.[1] Development of human monoclonal antibodies (mAbs) against H5N1 influenza haemagglutinin (HA) using Epstein-Barr virus (EBV) immortalization of B cells isolated from patients infected with H5N1,[2] phage display,[3] humanized mAbs,[4] and human recombinant Abs[5] has been attempted. Chickens produce a unique immunoglobulin molecule called IgY that is functionally equivalent to mammalian IgG.[6] IgY is found in the sera of chickens and is passed from hens to the embryo via the egg yolk.[7] Egg IgY has been used to prevent bacterial and viral infections (see review [8]) of the gastrointestinal tract and recently for protection against *Pseudomonas aeruginosa* infection of the respiratory tract of patients with cystic fibrosis (CF).[9]

The epidemic of HPAIV H5N1 virus has resulted in serious economic losses to the poultry industry, mostly in Southeast Asia. Therefore, many countries including China, Indonesia, Thailand, and Vietnam have introduced mass vaccination of poultry with H5N1 virus vaccines that controls the H5N1 epidemic to some extent.[10] Chickens immunized with recombinant H5 and/or inactivated H5N1 reassortant vaccines produced a high level of virus-specific serum antibodies (Abs) and were protected from H5N1 virus challenge.[11] Theoretically, these Abs could be found in egg yolk and separated for use in humans to prevent and cure H5N1 HPAIV infection and disease, respectively. Here, we examined the possibility that IgY isolated from consumable eggs available in supermarkets in Vietnam, where mandatory H5N1 vaccination has been implemented, provide prophylaxis and therapy of HPAIV H5N1 infection in mice.

Materials and methods

Six- to 8-week-old female BALB/cAnNCrl (H-2d) mice (Charles River and Jackson Laboratory) and Hy-Line

Leghorn hens (Kyunggi Poultry Farm) were used according to approved protocols for care and use of animals. The H5N1 human influenza isolate A/Vietnam/1203/2004 (VN/1203) was obtained from the World Health Organization (WHO) influenza collaborating laboratory at the U.S. Centers for Disease Control and Prevention (CDC), Atlanta, GA. The A/Aquatic bird/Korea/W81/2005 (H5N2) was isolated from a wild bird in Korea in 2006 and adapted by multiple passages (15 times) in BALB/c mice. Inactivated reassortant avian H5N1 influenza virus (A/Goose/GD/96-derived, strain Re-1) (Harbin, China) was used for mass vaccination of poultry in Vietnam, and A/ck/Scotland/59 (H5N1) was used for determination of haemagglutination inhibition (HAI) titres of sera and IgY. Eggs laid by hen raised in the poultry unit of Konkuk University, Seoul, Korea, and purchased from randomly selected supermarkets in Hanoi, Vietnam and Seoul, Korea and farms in Vietnam were used. Hens were immunized intramuscularly with heat-inactivated A/PR8/34 (H1N1) mixed with Freund's adjuvant (FA) (Sigma, MO, USA). IgY Abs were extracted from egg yolks as previously described.[12] The 50% egg infectious dose (EID_{50}) was determined by serial titration of virus stock in eggs, and EID_{50}/ml values were calculated according to the method of Reed and Muench.[13] Human virus stocks were grown in MDCK cells as described previously[14] with viral titers determined by standard plaque assay. The 50% tissue culture infectious dose ($TCID_{50}$) of virus was determined by titration in MDCK cells.[15] The standard ELISA was performed for detection of anti-IgY in the sera of IgY-immunized mice. Fifty percent lethal dose (LD_{50}) titers were determined by inoculating groups of eight mice i.n. with serial 10-fold dilutions of virus as previously described.[16] For infection, ketamine-anesthetized mice were inoculated intranasally with a lethal dose with 250 pfu ($5 \times LD_{50}$) of A/PR/8/34 (H1N1) virus as previously described,[17] $10 \times LD_{50}$ of VN/1203 (H5N1) or $5 \times LD_{50}$ A/Aquatic bird/Korea/W81/2005 (H5N2) resuspended in 50 μl PBS per animal. Ketamine-anesthetized mice were treated intranasally with 50 μl of IgY before or after infection. Mice were observed for weight loss and mortality. Subsets of animals were scarified for virus titre.

Results

Hemagglutination inhibition (HAI) and virus neutralizing (VN) activities of IgY isolated from consumable eggs available in public markets

We found comparable HAI titers in the sera and egg yolks obtained from a farm in Vietnam that was participating in a national mass vaccination program. Furthermore we found 90% of eggs purchased in randomly selected supermarkets in Hanoi, Vietnam containing H5-specific IgY. The HAI and VN titers of pooled egg yolk IgY are comparable with those of sera obtained from hens selected randomly from the farm that underwent supervised H5N1 vaccination. In contrast, IgY separated from eggs purchased in Korean markets where poultry are not vaccinated against avian influenza H5N1 has no detectable H5-specific HAI or VN activity.

Protection against infection with H5 influenza viruses

We first treated naïve mice intranasally with H5N1-specific IgY before infection with HPAIV H5N1 strain, A/Vietnam/1203/2004, isolated from a fatal case. Such treated mice displayed mild weight loss and recovered completely by the end of the first week after inoculation (Figure 1A). When animals were treated once with H5N1-specific IgY after H5N1 inoculation they exhibited minimal weight loss during the first week after inoculation, and virus titers in the lungs were substantial reduced at day 3 after infection; however, 50% of treated mice succumbed to infection during the second week after inoculation (Figure 1B). It is possible that not all the HPAIV A/H5N1 viruses were neutralized upon the single treatment with IgY, and escaping viruses can spread systemically to organs outside of the lungs. These viruses may reappear in lung tissue later when specific IgY is absent. Indeed, VN/1203 virus injected intravenously or into the brain can spread to the lungs.[18] To circumvent the virus escape, we administered multiple treatments with H5N1-specific IgY after the infection. As a result, all infected mice recovered completely by the second week post-infection (Figure 1C), and virus titers in the lungs were substantially reduced to the level that seen in protected mice that received single prior-infection treatment (Figure 1D). Similarly, the protective efficacy of H5N1-specific IgY was observed in mice infected with lethal dose of mouse-adapted avian influenza virus strain A/Aquatic bird/Korea/W81/2005 (H5N2). This virus shares 94.4% nucleotide sequence homology with HA (H5) but has different NA (N2) from the one used for mass immunization in Vietnam (reassortant avian H5N1 influenza virus A/Goose/GD/96-derived, strain Re-1). The results indicate that H5N1-specific IgY isolated from eggs purchased in markets have preventive and therapeutic effects against infection with HPAIV H5N1 and the related strain H5N2. The findings suggest that while a single treatment with IgY prior to lethal infection was sufficient to protect the animals from the infection, multiple treatment is required for complete therapeutic effect after infection with HPAIV such as VN/1203 strain.

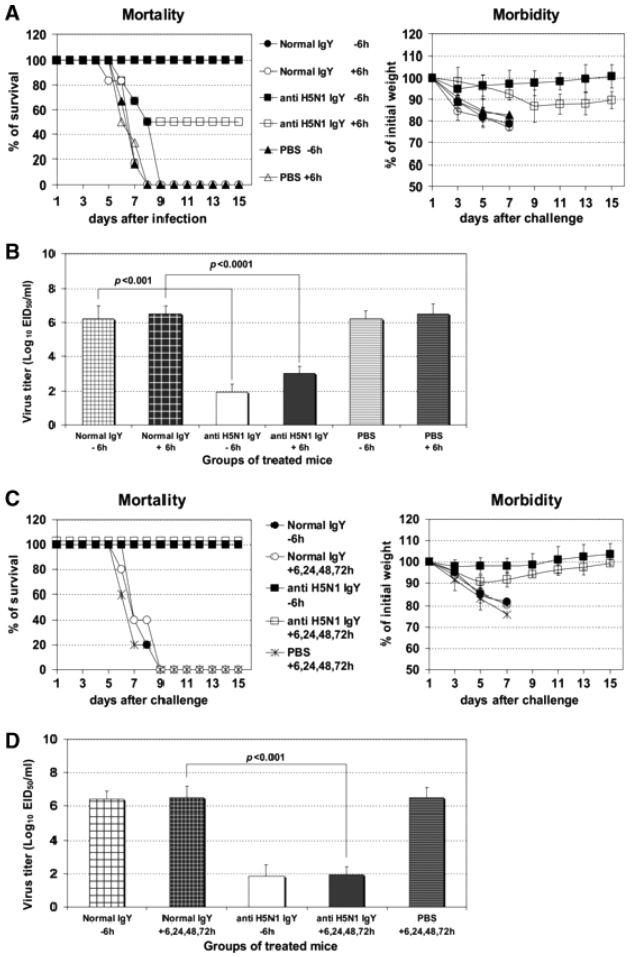

Figure 1. Protection against challenge with HPAIV H5N1. Morbidity and mortality of BALB/c mice treated with H5N1-specific IgY (anti H5N1 IgY) at 6 hours pre- (−6 hours) or post- (+6 hours) infection with VN/1203 (H5N1) virus (Single pre- or post-infection treatment, (A) – 6 mice per group); or at 6, 24, 48, and 72 hours after infection (Multiple post-infection treatments, (C) – 5 mice per group). Ten LD_{50} of VN/1203 H5N1 virus and 50 μl of H5N1-specific IgY were used for intranasal infection and treatment, respectively. The mice protected from disease did not die even after 3 weeks monitoring. Virus titers (EID_{50}) in the lungs were determined on day 3 after infection (B, D). The values are the mean of 4 mice in each group. The group of mice receiving single pre-infection treatment was included as control.

Protection against infection with human influenza A/H1N1 virus

We further examined the protective efficacy of IgY isolated from eggs laid by hens immunized in the laboratory with heat-inactivated human influenza A/H1N1 virus, A/PR/8/34. We found substantial levels of HAI and VN Abs in the sera and yolks derived from immunized hens. When naïve mice were administered intranasally with such anti-PR/8 IgY at 6–8 hours before or after infection with lethal dose of PR/8 virus, they were protected from the infection or lethal disease, respectively. The virus titers in the lungs of A/PR8 specific IgY-treated mice at day 3 after infection were also significantly lower than those seen in untreated mice or mice receiving normal IgY. Intranasal administration is the most effective route as compared to oral or peritoneal or intravenous administration for protection against lethal challenge, and the presence of virus-specific IgY in bronchoalveolar lavage (BAL) is required for the protection. The results provide a proof-of-concept that intranasal administration of virus-specific IgY prevents influenza virus infection and cures the disease. The concept could be applied to control influenza outbreaks including seasonal and pandemic influenza. The protection was correlated with HAI and VN activities of the IgY and reduced virus titers in the lungs after treatments, suggesting that the protection is mediated by VN.

IgY treatment induced anti-IgY Abs that do not prevent protection mediated by IgY

We asked if administration of IgY in the respiratory tract induces anti-IgY Ab response in mice. If this is the case, the next question is whether pre-existing anti-IgY Abs block IgY-mediated protection. Indeed, significant levels of anti-IgY were observed in animals that received single or multiple administration of IgY. When IgY-immune mice were treated with virus-specific IgY before or after lethal challenge, the results were identical to those obtained from treated naive mice, indicating that pre-existing anti-IgY Abs do not interfere with the protection mediated by virus-specific IgY. Consistently, incubation with anti-IgY serum did not interfere with HAI and VN activity of the virus-specific IgY, indicating that anti-IgY Abs do not block virus binding by virus-specific IgY (Figure 2). The finding suggests that the IgY treatment could be applied to persons who have developed anti-IgY during the individuals' life, and such treatment strategy could be repeated if multiple treatment is required and/or necessary later on to protect infections with other pathogens.

Discussion

The approach using specific IgY for prevention and therapy of HPAIV H5N1 infection offers a practical alternative to immunotherapy using convalescent plasma [19] and an additional therapeutic option to antiviral drugs since widespread drug resistance has been recently reported among influenza virus strains. IgY is relatively stable. We found no change in protective activity after at least 12 months storage at 4°C, and lyophilization does not affect the activity, making production of IgY practical. The use of IgY immunotherapy has many advantages, since IgY does not activate the human complement system or human Fc-receptors, which all are well-known cell activators and mediators of inflammation.[20] We chose the water dilution method for

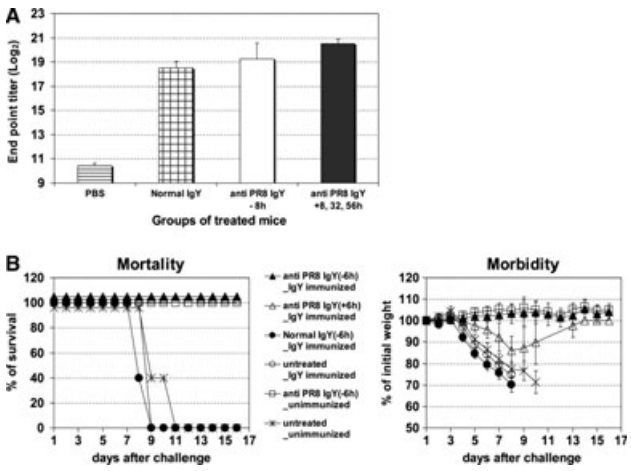

Figure 2. Induction of anti-IgY Abs and IgY treatment in mice with pre-existing anti-IgY. Anti-IgY in the sera of mice immunized with normal IgY (IgY immunized), treated once intranasally with PR8-specific IgY 8 hours before (anti PR8 IgY −8 hours) or three times after infection (anti PR8 IgY +8, 32, 56 hours) (A). Endpoint titers (log_2) were determined by ELISA. Morbidity and mortality of IgY-immunized mice treated with PR8-specific IgY (anti PR8 IgY) before (−6 hours) or after (+6 hours) infection with mouse-adapted PR8 (B). The values are the mean of 5–10 mice in each group.

preparation of IgY. The method is simple, efficient and does not require any toxic compounds or any additives. Such IgY preparations by this method have been used in other human study.[9,21] Eggs are normal dietary components, so there is minimal risk of toxic side effects, except for those with egg allergy. Thus, our study demonstrated that influenza virus-specific IgY can be used in passive immunization that provides great help for immunocompromised patients and elderly who have weaken immune response to influenza vaccines. Importantly, the consumable eggs readily available in the markets of countries that impose mandatory H5N1 vaccination offer an enormous source of valuable, affordable, and safe biological material for prevention and protection against potential H5N1 pandemic influenza.

Disclosure

Parts of the information and data presented in this manuscript were previously published in http://www.plosone.org/article/info:doi%2F10.1371%2Fjournal.pone.0010152.

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The polyphenol rich plant extract CYSTUS052 is highly effective against H5N1 and pandemic H1N1v influenza A virus

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Introduction

The 2009/2010 H1N1influenza A virus pandemic clearly demonstrates that influenza is still a major risk for the public health. Although the pandemic swine origin influenza A virus (SOIV) caused only mild symptoms, the control of the outbreak still remains difficult. Even as vaccine is available against this virus, the possibility of reassortment between the pandemic and a seasonal or avian A/H5N1 influenza virus strain is indeed a frightening, but a likely event. This reassortant strain might be able to transmit easily between humans causing fatal infections, and the current SOIV vaccine might no longer be sufficient to protect against the reassorted virus. In such a case, we can only rely on effective antiviral drugs. Today, neuraminidase-inhibitors, such as oseltamivir, represent the most common clinically approved medication against influenza A viruses. Unfortunately, the frequency of reports describing the appearance of drug-resistant seasonal H1N1 and also H5N1 influenza A viruses dramatically increased in the recent past.[1–4] Drug resistance to the known antivirals highlights the urgent need for alternative antiviral compounds with novel defense mechanisms. Recently, we have reported that a polyphenol rich plant extract, CYSTUS052, which showed antiviral activity against influenza A viruses in cell culture and in mice.[5,6] Moreover, the antiviral activity of CYSTUS052 against seasonal influenza virus and common colds was also demonstrated in humans.[7] However, the efficiency of CYSTUS052 against SOIV and A/H5N1 isolates was unknown so far. Therefore, we investigated CYSTUS052 effectiveness against the pandemic strain and seven natural influenza A/H5N1 isolates detected in several avian species during 2006/2007 avian influenza outbreak. Additionally, the potency of the most common neuraminidase inhibitor oseltamivir was also investigated against these isolates. Here, we show that CYSTUS052 treatment was effective in *in vitro* studies against SOIV and A/H5N1 influenza virus.

Material and methods

Viruses

Avian H5N1 isolates were originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany. The SOIV A/Hamburg/4/2009 was obtained from the Robert-Koch-Institut, Berlin, Germany. All H5N1 viruses were further propagated in embryonated chicken eggs or MDCK II (H1N1v) cells at the Friedrich-Loeffler-Institut, Tübingen, Germany.

Antiviral compounds

CYSTUS052 extract was supplied and originally developed by Dr. Pandalis NatUrprodukte GmbH & Co. KG (Charge-Nr.:40121T01B/04; Glandorf, Germany). CYSTUS052 granulate was dissolved in sterile PBS (10 mg/ml) at 60°C for 1 hour. Oseltamivir carboxylate was obtained from Toronto Research Chemicals Inc. (TCR, North York, Canada) and dissolved in sterile PBS.

Viral cytopathological effect inhibition screening

For the cytopathological effect (CPE) inhibition screening, in accordance with Sidwell,[8] MDCK II cells were infected with different viruses at MOI of 0·005. Virus-infected cells were then treated with antiviral compounds CYSTUS052 from 0·1 to 1000 μg/ml or oseltamivir from 0·01 nm to 1 mm. After incubation for 48 hours at 37°C and 5% CO_2, cells were fixed, and viable cells were stained with crystal violet. After extraction of crystal violet from viable cells with 100% methanol, the extinction was measured with an ELISA reader.

Infectivity assay

Immediately before infection, MDCK II cells (8×10^4 cells/well) were washed with PBS and subsequently incubated with virus diluted in PBS/BA (0·2% BA) 1 mm $MgCl_2$, 0·9 mm $CaCl_2$, penicillin and streptomycin to a multiplicity of infection (MOI) of 0·001 for 30 minutes at 37°C. CYSTUS052 was added in a concentration of 50 μg/ml directly to the virus-stock and on the cell monolayer simultaneously with the infection. After 30 minutes incubation period, the inoculums were aspirated and cells were incubated with either MEM or MEM containing 1 μm oseltamivir. At indicated time points, supernatants were collected. Infectious particles (plaque titers) in the supernatants were assessed by a plaque assay under Avicel as described previously.[9]

Results

In order to investigate the antiviral potential of CYSTUS052, EC_{50} values based on the inhibition of the CPE on MDCK II cells were determined for CYSTUS052 and in addition for oseltamivir. The EC_{50} values for CYSTUS052 ranged from 1·53 to 18·88 μg/ml. CYSTUS052 demonstrated the highest sensitivity against the SOIV, SN1 and MB1 isolates with EC_{50} values below 5 μg/ml. Compared to these virus strains, CYSTUS052 showed a slightly increased EC_{50} value for GSB1 (18·88 μg/ml). In contrast the EC_{50} values for BB1 and BB2 were notably elevated (65·68 and 76·22 μg/ml). Thus, the weakest antiviral effect of CYSTUS052 was observed against these two isolates.

The EC_{50} values evaluated for oseltamivir ranged from 0·07 to 512·76 μm (Table 1), indicating that BB2 (512·76) and GSB1 (356·92 μm) can be considered resistant against oseltamivir. To confirm these results we investigated the ability of CYSTUS052 to block virus replication as published before.[1] As a control, virus infected cells were treated with oseltamivir as described earlier.[6] In the absence of the drugs all influenza strains showed similar growth properties (Figure 1, black squares).

First progeny viruses were detectable between 8 and 20 hours post infection (Figure 1, black squares). Treatment with CYSTUS052 resulted in reduction of virus titers of all influenza virus strains (Fig. 1A–H, open triangles). Surprisingly, oseltamivir failed to inhibit the replication of two H5N1 influenza virus strains (GSB1 and BB2), supporting the data of EC_{50} values (Figure 1D+H, grey rhombes).

Discussion

We assessed the antiviral activity of CYSTUS052 against the newly emerged SOIV and seven avian H5N1 influenza viruses. CYSTUS052 showed efficient antiviral activity against the pandemic H1N1v strain and was effective to a wide range of H5N1 viruses. Furthermore, CYSTUS052 demonstrated a broader and more efficient antiviral potential than oseltamivir. CYSTUS052 treatment leads to a stronger reduction of progeny virus titers, and more importantly, CYSTUS052 was effective against all tested viruses, while oseltamivir was unresponsive against two of seven A/H5N1 viruses. Even though the pandemic strain in general is still sensitive to oseltamivir treatment, there are increasing numbers of reports of emerging resistant variants. The treatment with CYSTUS052 does not result in the emergence of viral drug resistance since the mode of action is an unspecific physical binding of the virus particle that is also beneficial to reduce opportunistic bacterial

Table 1. In vitro effect of oseltamivir and CYSTUS052 on different influenza A viruses

Isolate	Abbreviation	EC_{50}*	
		CYSTUS052 (μg/ml)	Oseltamivir (μm)
A/Hamburg/4/2009	SOIV	3·58 ± 0·42	$0{\cdot}07 \pm 4{\cdot}14^{-03}$
A/mute swan/Germany/R1349/07	SN1	1·53 ± 0·61	0·49 ± 0·001
A/mallard/Bavaria/1/2006	MB1	2·51 ± 0·82	0·51 ± 0·004
A/common buzzard/Bavaria/11/2006	BB1	65·68 ± 1·59	11·09 ± 0·01
A/common buzzard/Bavaria/2/2006	BB2	76·22 ± 1·62	512·76 ± 10·21
A/great crested grebe/Bavaria/22/2006	CGB1	15·78 ± 2·68	8·05 ± 0·003
A/goldeneye duck/Bavaria/19/2006	GEB1	16·87 ± 2·72	9·5 ± 0·004
A/goosander/Bavaria/20/2006	GSB1	18·88 ± 1·61	356·92 ± 2·59

*The percent of cell viability after treatment with the antiviral compound was calculated after correction for the background values (virus-infected cell control) as follows: Percent inhibition = 100/[(OD 590) cell-control sample: (OD 590) treated sample]. The EC_{50} value (i.e. the concentration of compound required to reduce the viral cytopathological effect on MDCKII cells to 50%) was determined with the GraphPad Prism 5 Software by plotting the percent cell viability as a function of compound concentration.

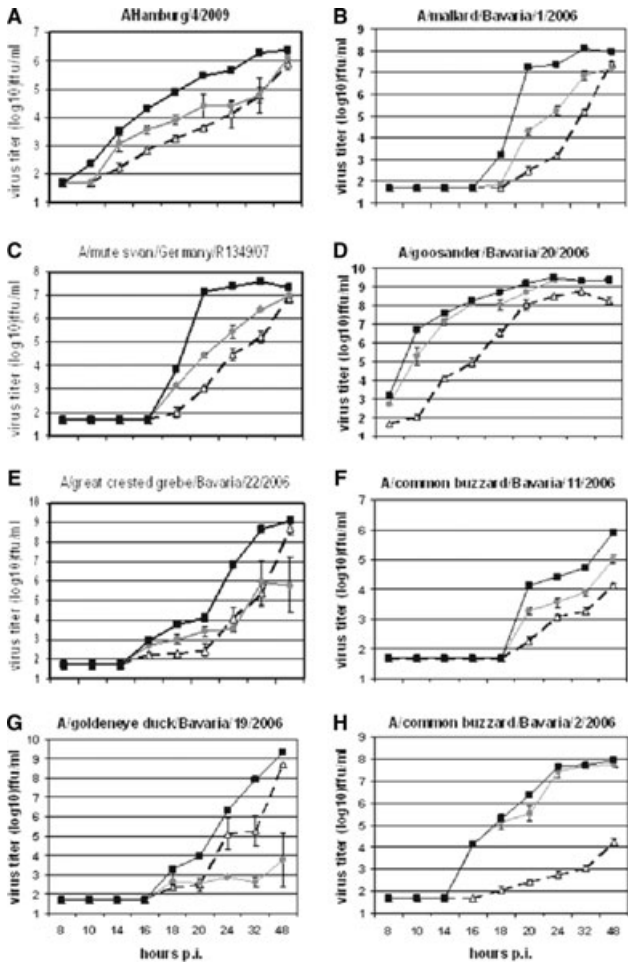

Figure 1. Infection of MDCK II cells with SOIV (A) and various H5N1 isolates (B–H) either untreated (black squares), treated with CYSTUS052 (50 μg/ml) (open triangles) or oseltamivir (1 μm) (grey rhombs). For (A)–(F) and (H) CYSTUS052 treatment of the virus inoculum was performed with 50 μg/ml 30 minutes prior to infection, whereas treatment of the virus inoculum in (G) was performed with the standard protocol, using 100 μg/ml following supplementation of the culture medium with CYSTUS052 (100 μg/ml).

infections.[5,7,10] CYSTUS052 is an extract from a special variety of the plant *Cistus incanus*, and it is very rich in polymeric polyphenols.[11] It is well known that polyphenols exhibit protein-binding capacity.[12] However, CYSTUS052 exhibited no neuraminidase inhibiting activity. Therefore, ingredients of CYSTUS052 may act in a rather unspecific physical manner by interfering with the viral hemagglutinin at the surface of the virus particle as demonstrated before.[5] While this prevents binding of the virion to cellular receptors, it does not block accessibility and action of the viral neuraminidase. Since, infections with influenza A viruses are still a major health burden and the options for control and treatment of the disease are limited, plant extracts such as CYSTUS052 should be considered as a new candidate drug for a save prophylactic and therapeutic use against influenza viruses.

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Attenuation of respiratory immune responses by antiviral neuraminidase inhibitor treatment and boost of mucosal immunoglobulin A response by co-administration of immuno-modulator clarithromycin in paediatric influenza

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Abstract

Oseltamivir (OSV) is widely used to suppress viral replication in the treatment of influenza. Here, we report that OSV administration significantly suppressed respiratory mucosal secretory IgA (sIgA) responses, but not systemic IgG responses, in weanling mice as a model of pediatric influenza. Treatment with clarithromycin (CAM), a macrolide with immunomodulatory activities, in pediatric influenza boosted the production of sIgA against influenza A virus (IAV) in nasal washes (NWs) and alleviated the respiratory disease manifestations, particularly in patients treated with OSV. Our findings suggest that CAM boosts mucosal anti-viral sIgA responses and prevents the risk of re-infection in the OSV-treated patients.

Introduction

The antiviral neuraminidase inhibitor OSV and zanamivir are widely used treatment options for influenza infection and are being stockpiled in many countries. Although mucosal immunity is the frontline of defense against pathogens, the effects of neuraminidase inhibitor treatment on airway mucosal immunity have not been reported. The suppression of viral RNA replication and viral antigenic production by these drugs may result in a limited immune response against influenza virus.[1] Macrolides, such as CAM and azithromycin, have anti-inflammatory and immunomodulatory properties that are separate from their antibacterial effects.[2–4] This study examined the impact of OSV treatment on immune responses in the airway mucosa and plasma in mice infected with IAV and pediatric influenza patients. We also assessed the immuno-modulatory effects of CAM in influenza patients who were treated with or without OSV.

Materials and methods

Female 3·5-week-old weanling BALB/C mice were nasally inoculated with 25 pfu of IAV/PR8/34 H1N1 at day 0. Immediately after infection, mice were given 50 μg of OSV orally or vehicle at 12-hours intervals for 11 days. The levels of virus-specific sIgA in NWs and bronchoalveolar fluids (BALF) and IgG in plasma were measured by ELISA as reported previously.[5]

A retrospective clinical study was conducted. For the study, 40 children with acute influenza were recruited and grouped according to the treatment received: 5 days treatment with OSV ($n = 14$), CAM ($n = 8$), OSV + CAM ($n = 12$), and untreated ($n = 6$). Since parents in Japan are well aware of the adverse effects of OSV especially the neuropsychiatric complications,[6] the decision on whether to administer OSV or not and to prescribe CAM was made by the parents and the attending paediatricians, based on their anti-viral and immuno-modulatory activity.[3,6] Comparisons were made of the levels of sIgA against IAV/H3N2 and IAV/H1N1, total sIgA, in NWs and disease symptoms before and after treatment. Anti-HA sIgA and total sIgA in NWs of patients were determined from the standard regression curves with human IgA of known concentration in a human IgA quantitation kit (Bethy Laboratories). Because an affinity purified

human anti-HA-specific sIgA standard of each influenza A subtype is not available, the relative value of anti-HA-specific sIgA amount was expressed as unit (U). One unit was defined as the amount of one μg of human IgA detected in the assay system as reported previously.[6] The concentrations of sIgA in individual NWs were normalized by the levels of total sIgA (μg/ml).

Results

Attenuation of inducible respiratory immune responses, but not systemic immune responses by OSV treatment in mice infected with IAV

Oseltamivir suppresses viral RNA replication and viral antigenic protein production. To investigate the influence of daily treatment with OSV on HA-specific mucosal and systemic immune responses, we analyzed HA-specific sIgA levels in NWs and BALF as well as IgG levels in plasma at days 8 and 12 post-infection in mice treated orally with OSV or methylcellulose (MC) as vehicle. The OSV treated mice showed lower antibody responses in NWs and BALF than control mice treated with MC solution (Table 1). Significantly reduced HA-specific sIgA responses were particularly noted in the OSV group at day 12, the period of maximal mucosal sIgA induction. The airway secretions and plasma from mice at day 0 did not contain detectable levels of HA-specific antibodies. These findings were supported by other data whereby mice treated with OSV displayed significantly lower numbers of HA-specific IgA antibody-forming cells (AFCs) in the nasal lamina propria, mediastinal lymph nodes, and lungs compared with MC-treated mice. These results clearly indicate that oral administration of OSV downregulates HA-specific sIgA responses in mucosa. On the other hand, there were no significant differences in the elevated levels of HA-specific plasma IgA and IgG antibodies or the increased numbers of HA-specific IgA and IgG AFCs in the spleen between OSV- and MC-treated mice. Taken together, these results implicated the oral administration of OSV in a suppressed induction of HA-specific sIgA responses in respiratory lymphoid tissues, although systemic HA-specific antibody responses were not significantly affected by OSV.

Effects of CAM on production of antiviral sIgA in NWs of paediatric influenza patients treated with or without OSV

Since CAM up-regulates IL-12, a mucosal adjuvant cytokine in the airways, and promotes the induction of sIgA and IgG in the airway fluids of mice infected with IAV,[4,7] we assessed the impact of treatment with OSV and/or CAM on the levels of anti-influenza sIgA in NWs and clinical status of influenza patients. The concentration ratio of

Table 1. Comparison of mucosal and systemic HA-specific antibody responses on weanling mice infected with IAV(H1N1)

	Reciprocal $\log_2$ titer in external secretions and plasma				Numbers of AFCs/10^6 on mucosal and systemic lymphoid tissues					
			Plasma						Spleen	
Post-infection day	sIgA in NWs[a]	sIgA in BALF[a]	IgA	IgG	sIgA in NLP[b]	sIgA in MLNs[b]	sIgA in lungs[b]	IgA	IgG
Day 0	ND	ND	ND	ND	ND	ND	ND	4·1 ± 2·3	5·2 ± 4·4
Treatment with OSV									
Day 8	0·2 ± 0·4	0·6 ± 1·2	4·6 ± 0·9	7·9 ± 1·4	3·1 ± 5·4	9·7 ± 2·0*	3·8 ± 2·7	92·1 ± 57·5	163·8 ± 46·1
Day 12	0·5 ± 1·2*	0·8 ± 0·6**	5·6 ± 1·7	7·9 ± 0·8	8·2 ± 1·2**	17·2 ± 2·7*	27·9 ± 3·6**	99·8 ± 40·2	151·6 ± 91·9
Treatment with MC									
Day 8	1·4 ± 0·3	1·9 ± 0·5	6·0 ± 1·2	8·2 ± 0·8	8·8 ± 1·0	46·1 ± 24·4	15·3 ± 3·3	101·8 ± 25·4	184·7 ± 65·1
Day 12	2·3 ± 0·9	4·2 ± 1·4	6·1 ± 1·4	8·7 ± 1·3	38·6 ± 18·8	73·8 ± 34·0	118·9 ± 28·5	104·1 ± 26·7	222·1 ± 40·4

[a]NWs, BALF and plasma were collected at days 0 (n = 5), 8 (n = 5), and 12 (n = 5). HA-specific antibody levels in plasma and external secretions were determined by ELISA. [b]Mononuclear cells from the nasal lamina propria (NLP), mediastinal lymph nodes (MLNs), lungs and spleen were isolated at days 0 (n = 5), 8 (n = 5), and 12 (n = 5) and subjected to ELISPOT assay. Values are mean ± SD. *P < 0·05, **P < 0·01 versus vehicle (MC) at the corresponding day after infection. ND, not detected; OSV, oseltamivir; IAV, influenza A virus; BALF, bronchoalveolar fluids; MC, methylcellulose; AFC, antibody-forming cells.

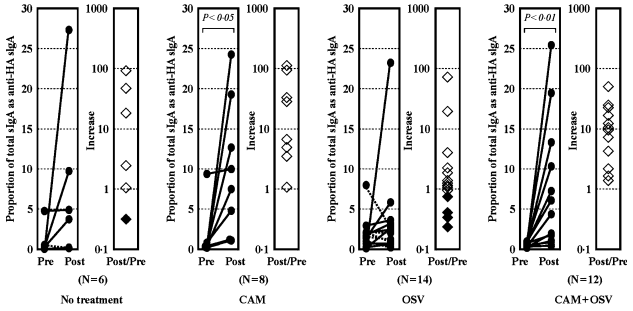

Figure 1. Proportion of total sIgA as anti-HA sIgA in NWs collected before and after 5 days of treatment in children studied, by the four treatment groups (CAM, OSV, OSV + CAM and no treatment). The proportion of total sIgA that was anti-HA sIgA was expressed as: anti-HA-specific sIgA (U/mg)/total sIgA (μg/mg) × 100. The increase in the proportion during the treatment (Post/Pre) is also indicated for each patient. A significant increase in the mean proportion was observed after treatment with CAM ($P < 0{\cdot}05$) and OSV + CAM ($P < 0{\cdot}01$). (◇): increased or unchanged; (♦): decreased.

anti-HA-specific sIgA to total sIgA in NWs was expressed as titer: anti-HA-specific sIgA (U/mg)/total sIgA (μg/mg) × 100. Figure 1 shows changes in the anti-HA(H3N2) sIgA ratio (titer) and fold of increase in sIgA titer in each patient during the 5-days' treatment for the four different treatment groups. It is noteworthy that, upon admission to the hospital, the sIgA titers were <3 in 93% of patients. During the 5 days of treatment, rapid increases in the titers were observed in almost all patients in CAM, OSV + CAM, and no treatment groups. In contrast, in the OSV group, the anti-HA-specific sIgA titers remained unchanged or decreased in the majority of patients. The finding of significant low induction of anti-viral sIgA in the OSV group was supported by the results of animal experiments. However, the addition of CAM to OSV augmented sIgA production and restored mucosal sIgA levels; 75% of patients treated with OSV + CAM showed >5-fold increase in the titers during treatment. These observations suggest that CAM stimulated the local mucosal immunoresponse in the nasopharyngeal region of patients treated with OSV.

Decrease in cough prevalence at end of treatment with CAM + OSV

The prevalence of disease manifestations was also analyzed.[6] Among the symptoms listed, a significant decrease in the prevalence of cough was recorded between the no treatment group and the OSV + CAM group and between the OSV group and the OSV + CAM group (**$P < 0{\cdot}01$), despite the limited number of patients in each group. The duration of the febrile period was significantly shorter in the OSV and OSV + CAM groups than the no treatment group. However, no significant difference was observed between the OSV group and OSV + CAM group.

Discussion

It has been reported that OSV does not affect the cellular immune responses, such as cytotoxic T lymphocytes and natural killer cells.[8] However, the effects of OSV on mucosal immunity have not been studied so far. The present study showed that OSV treatment of mice infected with IAV induced insufficient protective mucosal sIgA responses in the respiratory tract, although treated mice showed the similar levels of systemic IgG and IgA antibody responses in plasma to those in mice treated with vehicle (Table 1).[1] The observed effect of OSV on mucosal immunity was probably due to a suppression of viral replication and viral antigen production in the mucosal layer. These observations in mice are further supported by our clinical reports of sIgA in NWs and BALF of OSV treated influenza patients.[6]

The 14 membered- and 15 membered-ring macrolides have been found to possess a wide range of anti-inflammatory and immuno-modulatory properties,[2,3] and to be effective in the treatment of respiratory syncytia and IAV infection.[9,10] The efficacy of low doses administered on the long term against pathogens that are insensitive to macrolides indicates a mode of action that is separate from their antibacterial activity.[2,3,9,11] In the present study, we evaluated the immunomodulatory effects of CAM on mucosal immune responses in pediatric influenza.

A decrease in the proportion of total sIgA that was anti-HA-specific sIgA during treatment was observed in 21.4% of patients in the OSV group (those represented by the dotted lines and closed diamonds in Figure 1), whereas an increase in the proportion was observed in most patients of the other groups (except for one patient of the untreated group). Despite the low or unchanged induction of anti-HA-specific sIgA in the majority of OSV-treated patients, the additional use of CAM with OSV boosted the mucosal immune response and restored local mucosal sIgA levels. We are currently engaged in detailed immunological studies of the effects of CAM and OSV on the levels of mediators controlling IgA class switching in NWs of influenza patients and airway secretion of mice infected with IAV. Further studies should clarify the boost mechanisms of CAM and the suppression mechanisms of OSV in IgA class switching.

Our findings suggest the risk of re-infection in patients showing a low mucosal response following OSV treatment and CAM effectively boosts the sIgA production for protection of re-infection.

Acknowledgements

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Protective role of protease-activated-receptor 2 against influenza viruses occurs through an ERK independent pathway: implication for new influenza therapy

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Abstract

To date there is an urgent need to develop new antivirals against influenza. Most of the molecules reported target influenza proteins that acquire rapid mutations of resistance. The development of new molecules that have a broad antiviral activity and are not subjected to influenza mutation is of particular interest. Our laboratory and others recently showed that proteases can participate to the innate immune response in the airways through the activation of a family of receptors called PAR. In particular, through the release of interferon, PAR2 agonists curbed viral replication significantly in infected cells. In this study, since ERK activation is crucial for virus replication, we investigated whether PAR2 could inhibit virus replication through inhibition of the ERK pathway. Results showed that while influenza A infection alone or PAR2 stimulation alone induced ERK activation, PAR2 stimulation does not inhibit ERK activation in influenza infected cells. Thus, PAR2 agonists may be a potential new drug against influenza viruses that could be used in combination with other anti flu therapy such as the inhibition of the ERK pathway.

Introduction

Respiratory tract-resident proteases are key players during influenza virus type A infection.[1,2] In addition to their direct activating effect on surface viral proteins, lung mucosal proteases can regulate cellular processes by their ability to signal through protease-activated receptors (PARs).[3] After cleavage of the receptor by proteases, the new amino-terminal sequence of PAR binds and activates the receptor internally. These receptors are highly expressed at epithelial surfaces, in particular in the lung, where human influenza virus replicate *in vivo*. PARs are thus directly exposed to proteases present in the airways. Among the four different PARs, PAR2 acts as an antiviral through an interferon-dependent pathway.[4,5] Thus, agonists of PAR2 are potential

new drugs against a broad range of influenza viruses, which is in accordance with the broad antiviral action of interferon. However, the signalling pathway induced by PAR2 agonists in influenza A infected cells has still to be investigated. In this manuscript, we showed that influenza infection or activation of PAR2 induced ERK activation, a crucial step for efficient virus replication.[6,7] However, PAR2 agonists do not impaired ERK activation in influenza A virus infected cells. Since the pathway of PAR2 protection is likely to be ERK-independent, the use of anti ERK molecules in combination with PAR2 agonists maybe of potential interest in future anti-influenza therapy.

Material and methods

Virus strains, cells, antibodies, and reagents

Influenza viruses A/WSN/33 (H1N1) (a kind gift from Nadia Naffakh) was used in the present study. MDCK (Madin-Darby Canine Kidney) and the human alveolar type II A549 cell were obtained from ATCC and grown as previously described.[8] For Western blot analysis, the following antibodies were used: monoclonal antibody for phospho-ERK1/2 (T202/Y204) and for ERK1/2 antibodies from Cell Signaling Technology (Beverly, MA), horseradish peroxydase (HRP)-coupled rabbit polyclonal antibodies against mouse or rabbit IgG from PARIS (Compiègne, France).

Infection, stimulation, and western blot analysis

A549 cells were infected with IAV at an MOI of 1 in EMEM medium, as previously described.[9,10] At various time points post infection, cells were collected and proteins were analysed as previously described.[6,11] PAR2 stimulation was performed at 37°C in EMEM medium as previously described.[4] After infection and/or stimulation, cells were lysed in ice-cold lysis buffer. Lysates were centrifuged at 12 000 ***g*** for 20 min, and total proteins of the supernatants were analyzed by western blot analysis as previously described.[12,13]

Results

ERK activation after influenza infection or PAR2 stimulation

Since activation of the ERK pathway is essential for efficient influenza replication,[7] we first investigated the kinetics of ERK activation after influenza infection in human A549 alveolar epithelial cells. For this purpose, A549 cells were infected with influenza viruses at a MOI of 1 at different time point post-infection, and activation of ERK1/2 pathway was assessed by Western blot analysis using an anti-ERK antibody. Results showed that ERK was phosphorylated after influenza infection in a time course dependent manner when compared to uninfected cells. In contrast, ERK phosphorylation was not observed with heat-inactivated viruses, suggesting that productive infection is needed for ERK activation (Figure 1A). Antibodies against ERK1/2 were used as controls. Since ERK is activated after influenza infection, we then tested whether activation of PAR2 in uninfected cells also leads to activation of this pathway. For this purpose, A549 cells were stimulated with the selective human (H) or mouse (M) PAR2 agonist or a control peptide for the indicated time (Figure 1B). When exposed to the PAR2 agonists and compared to control-treated cells, ERK phosphorylation increased over the time course of stimulation. Thus, influenza infection or stimulation of PAR2 without infection in A549 cells induced activation of the ERK pathway at different time point post-infection.

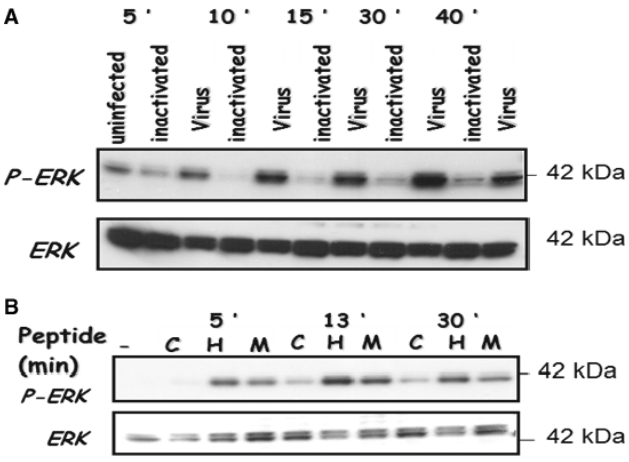

Figure 1. (A) Time course of ERK activation after influenza (virus) or heat-inactivated viruses (inactivated) infection. Numbers on the right are molecular weight in kDa. (B) Time course of ERK activation after Par2 stimulation or not (–) with a control peptide (C), human SLIGKV peptide (H) or mouse SLIGRL peptide (M).

ERK activation is not inhibited by PAR2 activation in influenza stimulated cells

Since influenza infection and PAR2 stimulation induced ERK activation, we then investigated whether PAR2 could inhibit ERK activation in influenza infected A549 cells. Results in Figure 2 showed that in influenza infected cells, PAR2 activation for ten minutes does not inhibit ERK activation after influenza infection. Thus, ERK activation is not inhibited by PAR2 activation in influenza stimulated cells.

Discussion

In this manuscript, we studied the activation of the ERK pathway after PAR2 stimulation and or influenza infection.

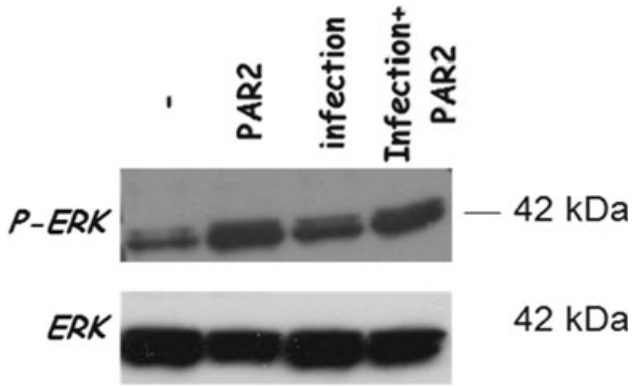

Figure 2. ERK activation after influenza infection and or PAR2 stimulation (PAR2).

Particularly interesting is the fact that either influenza infection or PAR2 stimulation alone induce ERK phosphorylation in A549 epithelial cells, while ERK activation is not inhibited in A549 infected cells compared to uninfected ones after PAR2 stimulation.

Proteases are key factor in the pathogenicity of influenza viruses. In addition to the cleavage of HA, necessary for IAV replication, extracellular proteases also play a role in the modulation of the immune system against influenza viruses through the activation of PARs. Particularly PAR2, activated by extracellular trypsin-like proteases, could inhibit virus replication through the release of interferon,[4,5] thus, strengthening the immune system via agonist peptides and providing new therapeutic potential against a broad range of influenza strains. In addition, targeting the host instead of the virus could provide a way to escape from virus resistance.[14] Thus, a better understanding of how virus escapes from immune surveillance may provide new therapeutic strategies to block IAV. In addition, combinations of drugs that block virus replication via different pathways are of interest. The non classical molecules HLA-G maybe an interesting new target as we recently showed that it is upregulated after influenza infection,[13] and it is a well known immunotolerant molecule.[15] Indeed, it inhibits the innate immune response [16] as well as the adaptive immune response.[17,18] Also, as previously suggested, the ERK signal transduction cascade is also of potential interest since it is crucial for virus replication and particularly influenza replication.[6,7] As shown here, it is unlikely that PAR2 protection occurs through an ERK-dependent pathway. Thus strengthening the immune response with PAR2 agonists and blocking nuclear retention of the viral ribonucleoprotein complexes with inhibitors of the MEK/ERK pathway may be alternative combinatory approaches for influenza therapy. In addition, since those potential drugs target the host instead of the virus, this could help in the design of new antivirals molecules more resilient to IAV mutations and thus to virus resistance.

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Treatment of severe influenza with immunomodulatory agents

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Introduction

The initial waves of the first influenza pandemic of the 21st Century have passed. In June 2009, vaccine companies estimated they could produce in 6 months almost 2.5 billion doses of pandemic vaccine.[1] Instead, they actually produced only 534 million doses, of which 64% were non adjuvanted preparations. Had these doses been produced with adjuvants (i.e., 3.75 μg instead of 15 μg HA per dose), an additional 1 billion doses could have been made available. Yet there was public opposition to adjuvants in many countries, especially by regulatory officials in the United States. Misperceptions about the safety of both adjuvanted and nonadjuvanted vaccines were widespread. Added to this, shortfalls in vaccine production, delays in vaccine delivery, and the ''mildness'' of the pandemic itself meant that only a few countries achieved reasonable levels of vaccine coverage. Millions of doses went unused and had to be destroyed. Supplies of antiviral agents were even more limited. Thus, despite the best efforts of influenza scientists, health officials, and companies, more than 90% of the world's people did not have timely access to affordable supplies of vaccines and antiviral agents. Instead, they had to rely on 19th century public health ''technologies.'' Given current understanding of biology in the early 21st century, they should have had – and probably could have had – something better.

This report reviews evidence for an alternative approach to serious and pandemic influenza that could be used in all countries with basic health care systems. Instead of confronting the influenza virus with vaccines and antiviral agents, it suggests that we might be able to modify the host response to influenza virus infection by using anti-inflammatory and immunomodulatory agents. This idea was introduced several years ago [2] and has been reviewed in several publications.[3–8]

The central importance of the host response

In the 1918 pandemic, young adults had high mortality rates. Ever since, influenza virologists have sought to answer the question ''why did young adults die?'' by defining the molecular characteristics of the 1918 virus that were responsible for its virulence.[8] In doing so, they have overlooked a crucial piece of clinical evidence from the 1918 pandemic: compared with young adults, children were infected more frequently with the same virus, yet they seldom died. Consequently, the more important question is ''why did children live?'' This can only be explained by recognizing that children must have had a different host response to the 1918 influenza virus than adults. Physicians have long recognized that for several other medical conditions, both infectious (e.g., pneumococcal bacteremia) and non-infectious (e.g., multiple trauma), children have a more benign clinical course than adults.[6,8] A corollary of this observation is that secondary bacterial pneumonia, although commonly found in young adults in 1918, could not have been the primary cause of death. Children must have had the same or higher rates of nasopharyngeal colonization with the same bacteria that were associated with pneumonia deaths in adults, yet children seldom died of secondary bacterial pneumonia.[6] If young adults died with secondary bacterial pneumonia, underlying host factors must have made them more susceptible.

Few people who die of influenza do so during the first few days of illness when pro-inflammatory cytokine levels are high. Instead, like patients with sepsis, they usually die in the second week, when anti-inflammatory cytokines and immunosuppression dominate.[6,8,9] Influenza deaths occur more frequently in older persons with cardiopulmonary conditions, diabetes, and renal disease, but as seen in the 2009 H1N1 pandemic, they also occur in younger adults with obesity, asthma, and in women who are pregnant. Regardless of age, people with all of these conditions share one characteristic in common: they have chronic low-grade inflammation. In effect, their ''innate immune rheostats'' have been set at different, and perhaps more precarious, levels that make them more vulnerable to influenza-related complications.[10]

Laboratory studies of influenza virus infection confirm the importance of the host response. In several studies in

mice in which the host response has been modified (e.g., cytokine knockout), survival has been improved without increasing virus replication in the lung.[5] In fact, severe disease can be induced without any influenza virus replication. For example, fatal acute lung injury has been induced in mice by inactivated (not live) H5N1 virus.[11] In this model, antiviral agents would be useless; only the host response could be responsible for disease.

These observations raise the following question: could the host response be modified so patients with severe seasonal and pandemic influenza might have a better chance of surviving?

Influenza, acute coronary syndromes, statins and related agents

Influenza is associated with acute coronary syndromes, and influenza vaccination and statins reduce their occurrence. These associations led to the suggestion in 2004 that statins might be used to treat pandemic influenza.[2] Other agents that might also be effective include PPARα and PPARγ agonists (fibrates and glitazones, respectively) and AMPK agonists (e.g., metformin).[5,8] These agents have been studied in laboratory models of inflammation, sepsis, acute lung injury, ischemia/reperfusion injury, energy metabolism, mitochondrial function, and programmed cell death. The results of these studies cannot be reviewed in detail here, but the major findings for cell signaling are summarized in the Table 1. Unfortunately, the results of experimental studies are not always clear cut. For example, in one study of influenza virus infected mice, IL-10 was necessary for containing infection,[12] but in another study IL-10 appeared to be harmful.[13] Nonetheless, overall understanding of cell signaling pathways in influenza virus infections and the actions of statins, glitazones, fibrates, and AMPK agonists strongly suggest that these agents could benefit patients with severe influenza.

Effectiveness of immunomodulatory treatment in laboratory and clinical influenza

Laboratory studies in mice infected with PR8 (H1N1) H2N2 and pandemic H1N1 viruses show that resveratrol, fibrates, glitazones, and AMPK agonists reduce mortality by 30–50%, often when treatment is started 2–4 days following infection.[14–17] (Resveratrol is a polyphenol found in red wine. It shares with these other agents many of the same cell signaling effects.) In H5N1-infected mice, treatment with celecoxib and mesalazine, together with zanamivir, showed better protection than zanamivir alone.[18] Remarkably, these immunomodulatory agents have not increased virus replication. Even more remarkable, in another model of a highly inflammatory and frequently fatal condition – hepatic ischemia/reperfusion injury – glitazone treatment ''rolled back'' the host response of ''young adult'' mice (8–10 weeks old) to that of ''children'' (3–4 weeks old).[19] This unique study suggests that immunomodulatory treatment might roll back the damaging and sometimes fatal host response of young adults with influenza to the more benign and rarely fatal response of children.

Several, but not all, observational studies have shown that outpatient statins decrease hospital admissions and mortality due to community-acquired pneumonia.[8] For influenza itself, preliminary evidence presented in October 2009 suggests that immunomodulatory treatment of influ-

Table 1. Cell signaling targets that might be affected by immunomodulatory treatment of severe seasonal and pandemic influenza*

Down regulate pro-inflammatory cytokines (e.g., NF-kappaB, TNFα, IL-1, IL-6)
Up regulate anti-inflammatory cytokines (IL-10, TGFβ)
Up regulate pro-resolution factors (lipoxin A4, resolvin E1)
Up regulate HO-1 and decrease TLR signaling by PAMPs and DAMPs
Up regulate eNOS, downregulate iNOS, restore iNOS/eNOS balance and stabilize cardiovascular function
Decrease formation of reactive oxygen species and decrease oxidative stress
Improve mitochondrial function and restore mitochondrial biogenesis
Decrease tissue factor and its associated pro-thrombotic state
Stabilize the actin cytoskeleton in endothelial cells and intracellular adherins junctions, and thereby increase pulmonary barrier integrity and decrease vascular leak
Differentially modify caspase activation and apoptosis in epithelial and endothelial cells, macrophages, neutrophils and lymphocytes in the lung and other organs
Increase the Bcl-2/Bax ratio in influenza virus-infected cells and prevent the apoptosis necessary for virus replication.

*See references[2,5,7,8] for details. NF-kappaB, nuclear factor kappaB; TNFα, tumor necrosis factor alpha; TGFβ, transforming growth factor beta; HO-1, heme oxygenase -1; TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; DAMP, damage associated molecular pattern; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase.

enza patients with severe illness could be beneficial. In a study of almost 4000 patients hospitalized with laboratory–confirmed seasonal influenza, inpatient statin treatment reduced hospital mortality by 66%.[20] In these patients, the cell signaling effects of statin treatment, summarized in the Table 1, probably acted to reduce pulmonary infiltrates, maintain oxygenation, stabilize myocardial contractility and the peripheral circulation, reverse immunosuppression, restore mitochondrial biogenesis, and prevent multi-organ failure. Achieving these clinical effects led to a decrease in mortality. Because of the molecular cross-talk between statins, fibrates, glitazones, and AMPK agonists,[5,8] similar clinical benefits might be expected from other members of this ''family'' of immunomodulatory agents.

The global implications of immunomodulatory treatment

Simvastatin, pioglitazone, and metformin are produced as inexpensive generics in developing countries. They are used throughout the world in the daily treatment of millions of patients with cardiovascular diseases and diabetes. Global supplies are huge. Because most people with influenza recover without specific treatment (this was true in 1918), not all patients would require immunomodulatory agents. Instead, only those at risk of ARDS, multi-organ failure, and death would need to be treated. Importantly, the cost of treatment for an individual patient would be less than $1.00 (D.S. Fedson, Unpublished observations). Moreover, unlike vaccines they could be used on the first pandemic day.

Conclusions

Thus far, influenza scientists and the institutions that support their work (e.g., NIH and CDC, national health agencies in many countries, the Bill and Melinda Gates Foundation, the Welcome Trust, and the World Health Organization) have shown little interest in immunomodulatory treatment. Nonetheless, when more than 90% of the world's people have no access to influenza vaccines and antiviral agents, their physicians must have access to an effective ''option,'' especially one that might be life-saving.

Research on immunomodulatory agents for influenza must involve investigators in many fields outside influenza science – those with expertise in the molecular and cell biology of inflammation, immunity, sepsis, cardiopulmonary diseases, endocrinology and metabolism, ischemia/reperfusion injury, mitochondrial function, and cell death. Laboratory studies needed to identify promising treatment agents would probably cost $5–15 million (D.S. Fedson, Unpublished observation). The results of these studies would inform clinical trials that critical care physicians are already eager to undertake.[21,22] This work will be especially important for people in developing countries where critical care capacity is extremely limited and not likely to improve.[23] Like critical care physicians, influenza scientists too must recognize that they cannot afford not to undertake research to determine whether generic immunomodulatory agents might be useful in managing severe seasonal and pandemic influenza.

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The NF-kappaB-inhibitor SC75741 efficiently blocks H5N1 influenza virus propagation *in vitro* and *in vivo* without the tendency to induce resistant virus variants

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Introduction

Influenza is still one of the major plagues worldwide. The appearance of highly pathogenic avian influenza (HPAI) H5N1 viruses in humans and the emergence of resistant H5N1 variants against neuraminidase inhibitors highlight the need for new and amply available antiviral drugs. We and others have demonstrated that influenza virus misuses the cellular IKK/NF-kappaB signalling pathway for efficient replication, suggesting that this module may be a suitable target for antiviral intervention.[1] Here, we show that the novel NF-kappaB inhibitor SC75741 efficiently blocks replication of influenza A viruses, including avian and human A/H5N1 isolates *in vitro* in concentrations that do not affect cell viability or metabolism. In a mouse infection model with HPAI A/H5N1 and A/H7N7 viruses, we were able to demonstrate reduced clinical symptoms and survival of SC75741 treated mice. Moreover, influenza virus was reduced in the lung of drug-treated animals. Besides this direct antiviral effect, the drug also suppresses H5N1-induced overproduction of cytokines and chemokines in the lung, suggesting that it might prevent hypercytokinemia we hypothesise to be associated with pathogenesis after infections with highly pathogenic influenza viruses, such as the A/H5N1 strains. Thus, a SC75741-based drug may serve as a broadly active nontoxic anti-influenza agent.

Material and methods

Antiviral compound

SC75741 was supplied by 4SC AG (Planeeg-Martinsried, Germany). SC75741 was dissolved in 10% DMSO/30% Cremophor (Merck).

Virus

Avian influenza A/mallard/Bavaria/1/2006 (H5N1) virus, grown in embryonated chicken eggs, was used throughout this study. The avian influenza A virus isolate was originally obtained from the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany and further propagated at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Tübingen, Germany. In addition the avian strain FPV (fowl plague virus) H7N7 was grown on MDCK cells. For cell culture experiments a multiplicity of infection (MOI) of 0·001 was used. For infection, the animals were anaesthetized by intraperitoneal injection of 150 μl of a ketamine (Sanofi)/rompun (Bayer) solution (equal amounts of a 2%-rompun-solution and a 10%-ketamin-solution were mixed at the rate of 1:10 with PBS) and infected i.n. with 5×10^3 pfu MB1 (H5N1) in a volume of 50 μl PBS via the intranasal route. According to the German animal protection law, the mice were sacrificed as soon as they lost 25–30% of their weight. Five animals per group were monitored for 14 days after infection. The experiments were performed three times.

Influenza virus titration (AVICEL® plaque assay)

To assess the number of infectious particles (plaque titers) in organs a plaque assay using Avicel® was performed in 96-well plates as described by Mastrosovich and colleagues.[2] Virus-infected cells were immunostained by incubating for 1 hour with a monoclonal antibody specific for the influenza A virus nucleoprotein (Serotec) followed by 30 minutes incubation with peroxidase-labeled anti-mouse antibody (DIANOVA) and 10 minutes incubation with True Blue™ peroxidase substrate (KPL). Stained plates were scanned on a flat bed scanner and the data were acquired using Microsoft® Paint software. The virus titer is given as the logarithm to the basis 10 of the mean value. The detection limit for this test was $<1{\cdot}7$ $\log_{10}$ pfu/ml.

RNA isolation and reverse transcription real-time PCR

Organs of infected and control mice were homogenized and incubated over night in 1 ml TriZol® Reagent (Invitrogen) at 4°C. Total RNA isolation was performed as specified by the manufacturer (Invitrogen). RNA was solubilised in 50 μl RNase free water and diluted to a working concentration of 50 ng RNA/μl. Reverse transcription real-time PCR was performed using QuantiFast™ SYBR® Green RT-PCR Kit and QuantiTect Primer Assays (Qiagen). All samples were normalized to GAPDH and fold expression analyzed relative to uninfected controls.[3] Ct values were obtained with the SmartCycler® (Cepheid).

Results

To answer the question whether the NF-kappaB inhibitor SC75741 shows antiviral properties against influenza virus, H5N1 infected MDCK cells were treated with different concentrations of the inhibitor (Figure 1). Already treatment with 1 nm of SC75741 led to a reduction of viral CPE of more than 70%. Almost 100% protection of cells was achieved when cells were treated with 50 μm SC75741. The results indicated that SC75741 has antiviral properties at concentrations ranging from 1 to 5 nm.

We next tested whether SC75741 would also be effective in the mouse model of influenza virus infection. When H7N7 mice were treated i.v. once daily for 5 days with 5 mg/kg SC75741, survival rate of the animals increased significantly ($P < 0{\cdot}05$). The same results were found when H7N7 influenza virus infected mice were treated i.p. with 15 mg/kg SC75741 (data not shown). Moreover, SC75741 treatment was not only effective when the inhibitor was given prior to H5N1 influenza virus infection, but also in a therapeutic setup when SC75741 was applied to the animals 4 days after infection (data not shown).

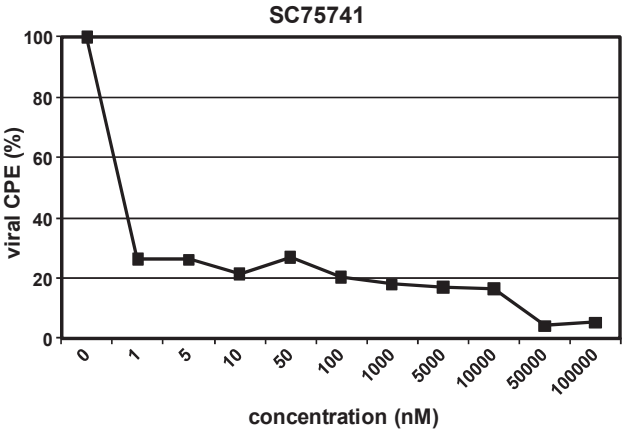

Figure 1. Virus reduction assay after SC75741 treatment of MDCK II cells. Virus infected cells (MOI 0·001) were treated with different SC75741 concentrations ranging from 0 to 100 000 nm). Virus titer was determined by plaque assay.

Since influenza virus infected mice showed increased survival after lethal infection, we next questioned whether the amount of influenza virus was reduced in the lung. Therefore, we performed quantitative real-time (qRT) PCR to detect viral mRNA. Mice were treated with either SC75741 or the solvent, and 48 hour later the lungs were prepared to perform qRT-PCR. As shown in Figure 2A the amount of viral mRNA was reduced by 90% in SC75741 treated mice compared to solvent treated controls, indicating that SC75741 leads to a reduced expression of H5N1 specific mRNA in the lung of infected mice. Since infection of mice with H5N1 leads to hypercytekinemia,[4] we also investigated the expression of cytokines in SC75741 treated mice. As shown in Figure 2B the amount of IL-6 specific mRNA was drastically reduced in SC75741 treated mice compared to solvent treated controls. Moreover, also the expression of IP-10 was altered in SC75741 treated H5N1 influenza virus infected mice. Here, roughly 90% reduction of specific

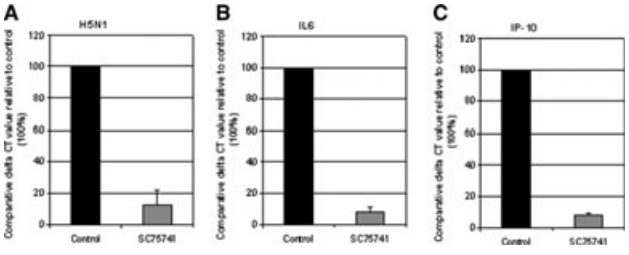

Figure 2. mRNA levels of cytokines and chemokines 2 days p.i. after infection of mice with 5×10^3 pfu H5N1 after a reverse-transcriptase real-time PCR according to manufacturer's protocol (QIAGEN). Solvent treated control (black bars) and SC75741 treated mice (grey bars) (A) H5N1, (B) IL-6, (C) IP-10. The bars represent the mRNA levels of three infected mice according to Boeuf.[3] This experiment was performed twice with similar results.

mRNA was detectable (Figure 2C). Thus, SC75741 leads to a reduced transcription of IL-6 and IP-10 in H5N1 infected mice.

Discussion

There is an urgent need for new concepts to develop antiviral drugs against influenza virus. Targeting cellular factors is a promising but challenging approach, and the concerns about side effects are obvious. However, it should be considered that drugs targeting viral factors, such as amantadine or oseltamivir, also exhibit a wide range of side effects in patients. Thus, drug safety has to be rigorously tested in clinical trials regardless whether a drug targets a cellular or a viral factor. Moreover, resistance against human H1N1 influenza viruses and highly pathogenic avian H5N1 virus strains to oseltamivir and amantadine have been reported.[5] In that respect, the strategy to target cellular factors [6,7] might be one way to ensure that new drugs against influenza virus will be useful and effective for a long time without causing the development of resistant virus variants.

We were able to demonstrate that the NFkappaB inhibitor SC75741 is able to reduce influenza virus activity in cell culture. Moreover, the compound was also effective against highly pathogenic avian influenza viruses of the H5N1 and H7N7 subtypes in the mouse model. Next to the reduction of virus SC75741 was also able to reduce H5N1-induced overproduction of cytokines and chemokines in the lung in the lung of mice after infection with H5N1. Most importantly, the drug did not show any tendency to induce resistant virus variants (data not shown). Thus, a SC75741-based drug may serve as a broadly active non-toxic anti-influenza agent.

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Community psychological and behavioral responses through the first wave of 2009 pandemic (H1N1) in Hong Kong

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Background

A new strain of H1N1 influenza virus (pH1N1) emerged in North America in early 2009, and the World Health Organization (WHO) raised the level of influenza pandemic alert from phase 5 to phase 6 in June 2009. Prior the development of new vaccines, non-pharmaceutical interventions were one of the most important tools for mitigating the impact of influenza outbreaks.[1–5] In Hong Kong, the first confirmed case was a tourist from Mexico reported on May 1, 2009. The local government made its first attempt to contain the spread of H1N1 in the local community by closing the Metropark Hotel where that tourist was staying, and quarantining 350 guests and staff for 7 days. Following identification of the first local case around 6 weeks later on June 11, 2009, the government closed all kindergartens and primary schools from June 12 until early July. Fever clinics were also opened, the alarm levels in hospitals were raised to the highest, and a public education campaign was implemented.

Previous studies of the community responses to severe acute respiratory syndrome (SARS) and human-to-human H5N1 avian flu identified the importance of understanding the background perceptions of risk and psychological impact on the community.[6–10] In this study we investigated the psychological and behavioral responses of the general local community throughout the first wave of pH1N1, and we also examined the factors associated with greater use of preventive measures.[11]

Methods

A total of 13 surveys were conducted between April and November 2009, covering the entire first wave of the pH1N1 pandemic. Computer generated random-household telephone numbers from all land-based local telephone numbers covering over 98% of Hong Kong households were used to recruit a total of 12 965 local adults. One Cantonese-speaking adult (age ≥18) was invited for interview in each selected household on the basis of a Kish grid. The survey instrument was based on previous experience in SARS and avian influenza projects. Information, including knowledge on modes of transmission, psychological responses to pandemic influenza, preventive behaviors, attitudes towards the new vaccines and socio-demographics, was collected. Informed consent was obtained prior to the interview. Ethics approval was obtained from the Institutional Review Board of the University of Hong Kong.

Descriptive statistics were weighted by sex and age based on the reference population data provided by the Hong Kong Government Census and Statistics Department.[12] Multivariable logistic regression analyses were used to examine the association between the use of preventive measures and knowledge, perceptions and behaviors, socio-demographic characteristics, and psychological responses to pandemic influenza. Multiple imputation was used to cope with a small proportion of missing data and make the best use of all available data.[13] Statistical analyses were conducted in R version 2.9.1 (R Development Core Team, Vienna, Austria).

Results

Twelve thousand and nine hundred and sixty-five local adults were recruited throughout the study period, with a total of 127 715 telephone calls being made; the response rate among eligible participants was 69.9%.[11] Hong Kong entered the containment phase after the World Health Organization (WHO) announced a global alert, and policies including border screening, tracing, and quarantine of

suspected cases were implemented. Hong Kong transitioned to the mitigation phase on June 10, 2009 when the first local case was reported. The chronology of these and other events plus the epidemic curve of laboratory-confirmed pH1N1 cases are shown in Figure 1(A).

The anxiety scores and risk perception of the respondents are shown in Figure 1(B,C). Anxiety, measured by the State Trait Anxiety Inventory, remained steady throughout the study period. In response to the announcement made by WHO and the unknown nature of the new virus, a higher proportion of the respondents expressed worry (more, much more, or extremely more worried than normal) if developed ILI and perceived pH1N1 severity (same, more, or much more serious than SARS) initially in early May 2009. Fewer respondents reported worry if they developed ILI as the pandemic proceeded, with a slight perturbation around the first deaths in July 2009 and a steady decline to 40.0%, while perceived severity of pH1N1 declined more dramatically after an early high. Perceived risks of infection of respondents (absolute susceptibility) and risk relative to others (relative susceptibility) were also investigated and found to remain relatively stable throughout the first wave, with no indication of an increase during the period of peak pH1N1 activity in September (Figure 1C).

As the first wave of pH1N1 progressed, knowledge on modes of transmission did not improve. On the contrary, later in the epidemic increasing proportions of respondents reported oral-fecal and cold weather as modes of transmission of pH1N1. Around 35–40% of the respondents did not recognize direct and indirect contact or touching infected persons and contaminated objects as transmission routes for pH1N1 throughout the first wave (Figure 1D).

Higher proportions of respondents avoided crowded places and rescheduled travel plans in the second half of June 2009 when local kindergartens and primary schools were closed and the first pH1N1-associated deaths were announced. Social distancing measures such as avoiding crowded places and rescheduling travel plans remained stable with slightly decreasing trends thereafter. The use of hygiene measures and other social distancing strategies was relatively stable with slightly decreasing trends during the study period (Figure 2).

Female sex and older age were generally associated with greater reported use of hand hygiene measures, home disinfection, avoidance of crowded places, and rescheduling of travel plans.[11] Female sex was also positively correlated with use of face masks and cough etiquette. We found a negative correlation between anxiety and use of all hand hygiene measures and cough etiquette, but a positive correlation between anxiety and use of home disinfection and

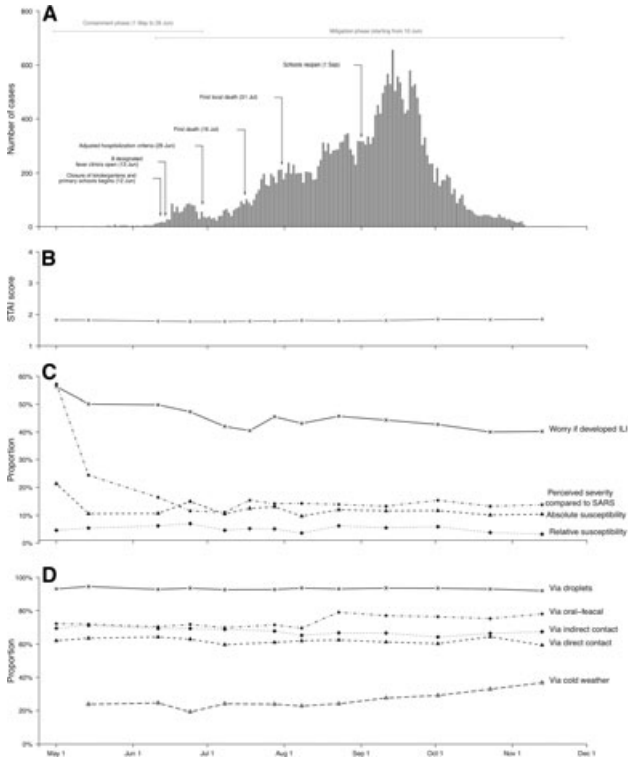

Figure 1. (A) Event chronology and the number of confirmed laboratory H1N1 cases. (B) Mean state trait anxiety inventory (STAI) score (1 is not anxious, and 4 is very anxious). (C) Proportion of the respondents reporting higher worry if developed flu-like symptoms (more, much more, or extremely worried), higher perceived seriousness of H1N1 compared to SARS (much more or more severe), higher probability to contract H1N1 over the next 1 month (certain, much more, or more likely), higher probability to contract H1N1 over the next 1 month compared to others outside family (certain, much more, or more likely). (D) Proportion of the respondents identifying 5 possible modes of transmission as the actual modes of transmission of H1N1.

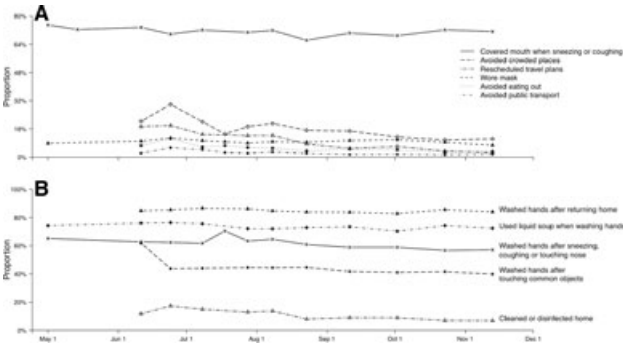

Figure 2. (A) Proportion of the respondents reporting covering mouth when sneezing or coughing (always or usually), wearing face masks (always, often, or sometimes) in the previous 3 days, and social distancing in the previous 7 days before the interview. (B) Proportion of the respondents reporting use of hand hygiene (always or usually) in the preceding 3 days and cleaning or disinfecting the home in the previous 7 days.

social distancing measures.[11] Other significant factors contributing to greater use of preventive measures were worry and knowledge.[11] Greater worry was associated with higher probability of home disinfection, social distancing measures, and use of face masks. Knowledge that H1N1 could be spread by indirect contact was associated all the investigated preventive measures, and knowledge that H1N1 could be spread by droplets was associated with cough etiquette, but not face masks. There were no consistent trends between all the investigated preventive measures and absolute and relative susceptibility.[11]

Conclusions

Community transmission emerged in Hong Kong in mid-June 2009, and prior to emergence of community transmission, perceived risk and perceived severity were high. As pH1N1 spread in Hong Kong, risk perception declined, even at the same time as incidence was increasing. Anxiety was low throughout, at around 1.8 on the 4-point scale, compared to a maximum of 2.5 during SARS on the same scale.[9] Anxiety has been showed to be positively correlated to personal hygiene measures and social distancing in previous studies;[9,14] however, we found a negative correlation between anxiety and use of all hand hygiene measures, cough etiquette, and face masks, and a positive correlation between anxiety and home disinfection.[11] The differences in findings may be due to the fact that our anxiety measure was not specific to H1N1, and the score could be affected by other factors including economics. Unlike hygiene measures, higher anxiety level, greater worry, and higher risk of perception were all associated with more social distancing.[6,7,9,14] Social distancing is the most direct strategy in avoiding infection from other people, and it is commonly observed in an outbreak that the general public avoids crowded places, travelling to other countries, and social gatherings,[9,14] but the economic impact could be substantial.[15]

As community incidence of H1N1 peaked, we did not observe any increase in use of preventive measures (Figure 2). We found that face mask use peaked at the early stage of the pandemic, while hand hygiene remained fairly constant, and the knowledge on the modes of transmission of pH1N1 did not improve over time. The lack of substantial change in preventive measures or knowledge about the modes of pH1N1 transmission in the general population suggests that community mitigation measures played little role in mitigating the impact of pH1N1 in Hong Kong. On the other hand, knowledge that pH1N1 could be spread by indirect contact was associated with all of the preventive measures studied. Consistent with reports during the SARS period,[9,16] this study also showed that females and those of older age were more likely than others to use hygiene measures, avoid crowded places, and reschedule travel plans.

This study has some limitations. First, this was a cross-sectional study that was carried out at different time points, rather than a longitudinal study following the same individuals over time, and so the inferences on changes in behavior may need to be interpreted more cautiously. Second, we recruited samples from all land-based local telephone numbers that cover 98% of Hong Kong households, but the response rate was not high enough to guarantee a representative sample, and this could be a source of selection bias. Third, the responses were self-reported, and this may lead to social desirability bias in estimating knowledge, attitudes, and preventive behaviors. Fourth, since the Hong Kong population has previously gone through unique experiences from SARS in 2003 and avian flu in 1997, our results may not be comparable to other countries or settings.

In conclusion, this study revealed that the pH1N1 pandemic failed to generate an increase use of preventive measures in the local community. There was no association between anxiety level and the events of the pandemic. With a relatively low mortality and morbidity rates compared to SARS, pH1N1 was not a matter of concern in the Hong Kong community. The lack of substantial change in the use of preventive measures and improvement in knowledge on the modes of transmission of pH1N1 suggested that public health campaigns during the pandemic may not have had substantial effects on the general public.

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Managing the 2009/10 influenza A/H1N1v pandemic in London

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Keywords emergency preparedness, issues identified, lessons learned, London, response.

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Introduction

London is a major tourist destination, the seat of government and finance in the UK, and in 2012 will host much of the Olympic and Paralympic Games. Along with the rest of the global community, in 2009 and early 2010 London faced the challenges of responding to the first pandemic of the 21st century. At the time, NHS in London was composed of 72 organisations, including the London Ambulance Service, acute hospitals, mental health and primary care trusts, and the strategic health authority. While London's NHS is well practiced at responding to large, big bang incidents, the influenza A/H1N1v pandemic was a rising tide event that lasted many months.

Significant preparatory work had been undertaken prior to April 2009, which meant that the NHS in London was ready to respond. NHS London (the strategic health authority for London) led the response in partnership with local managers in all NHS organisations. The first UK cases of influenza A/H1N1v were reported in Scotland on 27 April, with the first in London on 30 April. Cases continued to increase, and the first wave peaked in London in July. Cases reduced over the school summer holidays, but increased again when children returned to school at the start of September, and a second, smaller wave occurred. It is essential that the NHS learns from the 2009/10 influenza A/H1N1v pandemic to ensure it is prepared for future challenges.

Methods

NHS London provided a standardised debriefing pack to all NHS organisations in the region to identify, capture, and learn lessons. Each debrief event involved health and inter-agency partners to ensure all viewpoints were considered and brought together in a single local report. All local reports were compiled in an over-arching document, which brings together common themes to inform ongoing preparedness in the region.[1]

Results

The debrief process identified a number of common themes, such as the need for clear and appropriate communication, the importance of working with partners, and the benefits of strong and early leadership. However, differences between and within organisations were also highlighted; for example, some wanted more freedom for local decision making, whereas others would have preferred more stringently applied central direction. The following paragraphs considers individual areas assessed in the debrief process.

Command and control was in the main effective, with clear direction delivered from the national centre through NHS London to local NHS organisations. Effective leadership is essential; the identification of senior local individuals to lead the response with teams of people to support them was critical. Appropriate use of technology to communicate messages and coordinate command and control processes greatly aided the response. This included the development of the NHS London Noon Brief, a daily digest and associated web portal, and regular teleconferencing.

Key points are:

- Operational management at all levels must be considered in pandemic planning.
- Appointing an executive lead in each organisation was invaluable in the response.
- Pandemic flu planning for London must continue to be regionally led.

Communication is an essential component of the response to any incident. It must be clear, timely, and accurate. In the main, communication was excellent and met these criteria. One of the most challenging aspects was when messages from partner organisations differed, which occasionally led to confusion, unnecessary work, or frustration. The use of technology greatly aided communication across the region and supported the response; this included secure web sites, Bluetooth, and text messaging etc.

Key points are:

- Regular internal communications and staff briefings are critical in the response to emergencies.
- Regular teleconferencing should be incorporated into future plans.
- Organisations should consider proactive and innovative methods for communicating during emergencies.

Robust *partnership working* was an essential component of pandemic preparedness work; however in the event, the A/H1N1v pandemic had little impact on sectors in London other than health. Resilient communication networks between organisations, a common understanding, and the ability to make decisions were essential to the response at local level. IPCs proved an excellent mechanism to maintain local working relationships and resolve problems. Clarity on the seniority of those attending these meetings and whether multi-site organisations such as mental health trusts should attend every IPC should be considered on a local and regional basis.

Key points are:

- Pandemic planning must remain part of inter-agency working.
- Social care resilience and planning must be embedded and integrated in health planning.

'*Vulnerable groups*' is a universal term that covers a large and fluid group of individuals with different needs. Ensuring access to healthcare during the pandemic for those who became vulnerable due to the situation, or those identified as such prior to the event, was the role of the PCT in partnership with the local authorities. Work continues to ensure that communication with vulnerable people is appropriate and timely in all incidents, and that organisations work together to achieve this.

Key points are:

- Planning to support the breadth of vulnerable people must continue.
- Pandemic preparedness for the prison sector should be further developed.
- Red/Amber/Green ratings for assessing vulnerabilities of mental health service users in an emergency should be further developed across the region.

Correct and appropriate usage of *PPE* is an essential component of reducing influenza spread, particularly in healthcare settings. London's NHS had been working towards developing local stockpiles of PPE when the pandemic commenced; however, there was little in place. The unanticipated national stockpile, while providing PPE to all organisations, was accompanied with some challenges in that it was often unfamiliar stock.

Key points are:

- Work around local stockpiling of non-standard consumables should continue.
- Regular training and fit testing of respirators should be embedded in all organisations.

Antiviral treatment was a core component of the response to influenza A/H1N1v, and was provided free of charge from a national stockpile. NPFS reduced pressure on frontline NHS services once it was activated; however, there were concerns that patients could 'cheat' the system and obtain the drugs prior their clinical need. Information about storage requirements of countermeasures must be clearly explained when they are delivered to frontline services, and the potential for recall into national stockpiles should be planned for.

Key points are:

- Regular exercising of local mass countermeasures centres and Antiviral Collection Points (ACPs) should continue.
- The use of community pharmacies as ACPs should be further considered in the capital.

Pandemic influenza *vaccine* uptake by healthcare workers was better than usual seasonal influenza uptake in the majority of NHS organisations, but could have been even better. This was largely due to the second pandemic wave not being as significant as expected, lack of clarity around when the vaccine would be delivered, and limited amounts being available initially.

Key points are:
- GP-led and mass vaccination models for pandemic vaccination should be considered in local plans.
- Local lessons from the pandemic vaccination campaign should be applied to seasonal flu vaccination.

The ability to *maintain or increase capacity* in response to a surge in demand, no matter what the cause, must be planned for. Any of a number of situations could result in reduced staff or more patients, such as industrial action, transport disruption, disease outbreak, major incident, or poor weather. The work undertaken during planning for and responding to the pandemic will stand organisations in good stead for future disruptions. The importance of robust business continuity planning locally cannot be overlooked, as this is a key component of maintaining and increasing capacity.

Key points are:
- Local GP 'buddy schemes' should be encouraged for response to extreme pressure events.
- Organisations should regularly run staff skills audits so as to be aware of their overall capability for managing emergencies.
- Less emphasis should be placed on the use of retired staff when planning service continuity.

Reporting is a necessary but onerous task, and is often one of the most time-demanding parts of any incident response. It is also the aspect least likely to be tested through exercising. NHS London worked with organisations to endeavour to reduce reporting pressures, but much of this was dictated by central government. It is essential that future reporting requirements are proportional, informative, and realistic. While recognising it is not possible to predict the detail of information that may be requested, some broad assumptions can be made.

Key points are:
- Organisations should consider how they would collect and collate data from disparate parts of their organisation, rather than focussing on the detail of what that might be.
- National and regional planning should consider the need for information and how this is balanced with the demand this places on organisations.
- The introduction of the concept of a daily dashboard to identify areas of pressure should be incorporated into pandemic flu planning.

The winter and pandemic influenza *resilience assurance* process undertaken in autumn 2009 was a useful process to inform planning for the first winter when the pandemic virus would be circulating in the UK. This consisted of a regional inter-agency exercise and a comprehensive review of the winter and pandemic plans of all NHS organisations in London.

Key points are:
- Regular assurance of pandemic flu preparedness should be maintained.
- Future resilience assurance processes should be undertaken in a timely and measured manner.
- Local organisations should continue to undertake regular pandemic flu exercises.

The *recovery* period is as important as the response, but often receives minimal attention and has the potential to suffer as staff return to their normal jobs. One of the aspects that was not anticipated during the pandemic was the amount of stock (PPE, antivirals, and vaccine consumables) that would be recalled into national stockpiles. This proved particularly challenging for PCTs who had to coordinate the process across their local areas.

Key points are:
- The recovery period of an emergency must be given the same status and importance as the response.
- Future pandemic flu planning must include the recovery of national stockpiles of equipment and medicines.

Discussion

It is essential the lessons from the 2009/10 influenza A/H1N1v pandemic are learnt and embedded into business-as-usual and emergency response processes in preparation for the next pandemic and other incidents. Even though the A/H1N1v pandemic was generally milder than previous pandemics, it still presented challenges to the NHS in London. The biggest challenge that remains is to ensure that the public and NHS staff are aware that a more virulent virus could cause significantly more illness, death, and disruption, and that we must maintain our preparedness should this happen.

The influenza A/H1N1v pandemic has been a major stimulus to business continuity planning and emergency preparedness across health in London, and many of the experiences during the pandemic proved invaluable in the unusually severe weather in early 2010. It is important that this impetus and focus is maintained.

Changes to the NHS landscape in London will be considered in ongoing pandemic and emergency preparedness to ensure we remain as well prepared as possible for future events, particularly as London approaches the 2012 Olympic and Paralympic Games.

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WHO public health research agenda for influenza: road mapping for information gaps and research needs in a pandemic scenario

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Abstract

Experience to respond to influenza pandemic events accumulated over years around the world corroborates that health authorities often lack sufficient evidence-based information to address issues, particularly in a pandemic situation. The WHO Global Influenza Programme (GIP) in cooperation with international influenza experts developed a *Public Health Research Agenda for Influenza* to provide a framework of research topics that reflects public health research priorities for influenza, including immediate needs during pandemic periods.

Research recommendations for the influenza pandemic scenario were developed and compiled by invited experts during the breakout group discussions held at the first global consultation meeting on *Public Health Research Agenda for Influenza* in November 2009. The suggested research roadmap prioritizes topics and questions relating to rapid action and response during pandemic periods per five key Agenda's research areas: Stream 1. Reducing the risk of emergence of pandemic influenza; Stream 2. Limiting the spread of pandemic, zoonotic and seasonal epidemic influenza; Stream 3. Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza; Stream 4. Optimizing the treatment of patients; Stream 5. Promoting the development and application of modern public health tools. Realization of these recommendations is expected to provide an evidence-based public health practices to reduce the impact of pandemic influenza.

Keywords influenza, pandemic period, public health, research agenda, WHO.

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Background

One of the major lessons learnt from all global pandemic events is that better preparedness of national health systems to deal with influenza viruses could make a significant difference. The way national health systems operate during inter-pandemic and the pandemic alert periods and the methods they use to address potential threats posed by zoonotic viruses with pandemic potential, as well as seasonal influenza epidemics, can clearly indicate whether the countries have enough capacities to respond adequately to unexpected influenza outbreaks. These public health decisions to ensure the maximum of efficiency require a robust scientific knowledge base.

The *WHO Public Health Research Agenda for Influenza* developed by the Global Influenza Programme (GIP) in cooperation with international influenza experts identified specific research topics and their importance in meeting

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public health needs for pandemic periods according to its five key research streams:

- Stream 1. Reducing the risk of emergence of pandemic influenza.
- Stream 2. Limiting the spread of pandemic, zoonotic and seasonal epidemic influenza.
- Stream 3. Minimizing the impact of pandemic, zoonotic and seasonal epidemic influenza.
- Stream 4. Optimizing the treatment of patients.
- Stream 5. Promoting the development and application of modern public health tools.

Materials and methods

Stream-specific breakout discussion groups during the global consultation meeting included representatives of researchers and public health professionals. Funding organizations were invited to observe the process with no direct participation in the deliberations.

The methods used to design the research roadmap for an influenza pandemic scenario are closely related to the process of development of the final document of *WHO Public Health Research Agenda for Influenza*. During a pandemic scenario, the group prioritized topics and questions relating to rapid action and response.

Results

Five to 10 key public health needs associated with a pandemic scenario have been identified for each of the research agenda streams:

Stream 1: Reducing the risk of emergence of pandemic influenza

Five priority public health topics were identified for a pandemic scenario as follows:

- Examination of host range and transmission dynamics of animal influenza viruses to guide surveillance, control strategies, and risk communication.
- Enhanced surveillance in animals and humans to monitor virus evolution:
  - Early detection of novel reassortants or changes in genotype and/or phenotype related to virulence.
  - Development of epidemiological and laboratory diagnostic tools and capacity building to optimize case finding.
  - Develop a framework for surveillance in animals that address ethical, legal, and social barriers to intra-pandemic surveillance and reporting.
- Deconstruct the origins of the pandemic virus to identify factors that permitted efficient human transmission.
- Develop strategies to limit economic, social, and cultural disincentives of animal-based interventions to reduce intra- and inter-species transmission.
- Operational research to optimize risk communication in the early phases of the pandemic linked to animal husbandry and food safety.

Stream 2: limiting the spread of pandemic, zoonotic and seasonal epidemic influenza

Ten priority research topics were identified for both pandemic and inter-pandemic scenario as follows:

Transmissibility of influenza across the progression of infection and spectrum of disease:

- Relative contributions of the different modes of transmission for influenza.
- Biological, behavioral, and social host factors that influence the risk of transmission and infection.
- Patterns, drivers, and mechanisms affecting the seasonality of transmission.
- Viral and population factors that influence transmission and spread of different influenza types, subtypes, and strains.
- Strategies to reduce the transmission of influenza in community, household, and health care settings, especially in less-resourced areas.
- Impact and cost effectiveness of social measures, such as school closures, and the role of surveillance in assessing timing of these interventions.
- Impact, effectiveness, and cost effectiveness of individual measures, such as isolation and quarantine.
- Role of vaccination in limiting the spread of influenza and strategies for its use.
- Impact of antiviral treatment and prophylaxis in reducing transmission of influenza.

Stream 3: Minimizing the impact of pandemic, zoonotic and seasonal epidemic influenza

Five priority public health topics were identified for a pandemic scenario as follows:

- Identification of groups at higher risk of infection and severe disease outcome through enhanced surveillance.
- Understanding disease severity and identification of predictors of severe outcomes.
- Investigation of vaccine effectiveness, especially in high risk groups in diverse geographic areas.
- Establishment/enhancement of pharmacovigilance, particularly for adverse events among at-risk groups.
- Optimization of strategies for rapid and targeted vaccine deployment.
- Rapid assessment to optimize acceptance of pandemic vaccine.

Stream 4: Optimizing the treatment of patients

Six priority public health topics were identified for a pandemic scenario as follows:

- Collaboration and coordinated sharing of data, protocols, regulatory, and other implementation strategies and databases from different countries on all aspects of patient management and outcome to accelerate improvements in patient care.
- Development of best practices in patient management in different settings, including checklists and algorithms for clinical care and treatment, prognostic parameters, and tests to predict potential for the development of severe disease.
- Rapid, reliable, simple, low-cost point-of-care diagnostic tools for influenza.
- Best use of current antiviral drugs and optimal formulations in different target populations, such as parenteral and other routes of administration for severe infections.
- Use of combination therapies, including use of adjunctive therapies (e.g., use of convalescent serum and immunomodulators).
- Role of ongoing viral replication, host responses, and the effect of co-infections in the pathogenesis of severe disease.

Stream 5: Promoting the development and application of modern public health tools

Modern tools for early detection and monitoring of disease

The group on surveillance tools concluded that the agreed topics of interest were equally applicable during a pandemic or inter-pandemic period:

- Studies to appraise and adapt modern technologies for early detection of influenza outbreaks in surveillance at the human-animal interface.
- Develop, integrate, and evaluate innovative approaches for influenza surveillance and monitoring with other existing disease monitoring systems.
- Study efficient mechanisms on sharing data, clinical specimens, and viruses with consideration for local, ethical, legal, and research perspectives.
- Examine the timeliness and quality of data required for early detection from local to national and global levels for the respective stakeholders.

Role of modeling in public health decision making

Five priority public health topics were identified for a pandemic scenario as follows:

- Identify environmental determinants of seasonal variation in influenza transmissibility in tropical and temperate regions.
- Estimate the transmission risk associated with types of contacts by comparing measured contact patterns with outbreak data.
- Incorporation of validated models of behavioral responses to risk and control measures in virus transmission.
- Development and implementation of novel technology for real-time sero-surveillance during a pandemic.
- Develop experimental and theoretical framework to assess host adaptation to study host receptor, antigenicity, and virulence.

Modern tools for strategic communication

Three priority public health topics were identified for a pandemic scenario as follows:

- Evaluate tools to more rapidly and accurately assess and monitor knowledge, attitudes, beliefs, and practices in different population groups to guide future communication efforts; develop tools and methods to more rapidly and accurately assess and monitor knowledge, attitudes, beliefs, and practices in different population groups, and thereby, guide future communication efforts.
- Evaluation of communication tools and approaches for communicating in different cultural settings, which engage and empower individuals and communities to practice and promote appropriate risk reduction measures.

Conclusions

Implementation of the identified research priorities is expected to underpin public health decision making at all levels with proven knowledge that will help to save large numbers of lives, reduce health costs and economic loss, and mitigate potential social disruption.

Reference

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WHO public health research agenda for influenza: road mapping for information gaps and research needs in an inter-pandemic scenario

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Abstract The aptitude of countries to adequately respond to pandemic events is quite often determined by the extent of preparedness of national health systems to deal with influenza viruses during inter-pandemic periods. The *WHO Public Health Research Agenda for Influenza* developed by the WHO Global Influenza Programme (GIP) in cooperation with international influenza experts aims to generate evidence to support policy development related to influenza. The first global consultation meeting convened by WHO in November 2009 provided a forum for public health professionals and other experts in influenza-related disciplines to design research roadmaps for inter-pandemic periods including seasonal and zoonotic infections. The outcome of research recommendations to address information needs in inter-pandemic scenario is organized around the five key research areas: Stream 1. Reducing the risk of emergence of pandemic influenza; Stream 2. Limiting the spread of pandemic, zoonotic and seasonal epidemic influenza; Stream 3. Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza; Stream 4. Optimizing the treatment of patients; Stream 5. Promoting the development and application of modern public health tools. Complemented by an analogous research roadmap for a pandemic influenza scenario, the research recommendations for an inter-pandemic period represent a framework to provide evidence to guide public health policies on influenza control.

Keywords influenza, inter-pandemic period, public health, research agenda, WHO.

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Introduction

One of the major lessons learnt from all global pandemic events is that better preparedness of national health systems to deal with influenza viruses could make a significant difference. These public health decisions to ensure the maximum of efficiency require a robust scientific knowledge base.

The *WHO Public Health Research Agenda for Influenza*[1] developed by the Global Influenza Programme (GIP) in cooperation with international influenza experts identified specific research topics and their importance in meeting public health needs for inter-pandemic periods according to its five key research streams:

- Stream 1. Reducing the risk of emergence of pandemic influenza.
- Stream 2. Limiting the spread of pandemic, zoonotic, and seasonal epidemic influenza.

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- Stream 3. Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza.
- Stream 4. Optimizing the treatment of patients.
- Stream 5. Promoting the development and application of modern public health tools.

Materials and methods

Stream-specific breakout discussion groups during the global consultation meeting included representatives of researchers and public health professionals. Funding organizations were invited to observe the process with no direct participation in the deliberations.

The methods used to design the research roadmap for an influenza inter-pandemic scenario are closely related to the process of development of the final document of *WHO Public Health Research Agenda for Influenza*. During an inter-pandemic phase, a more comprehensive approach was applied to establish research topics and prioritizing a range of questions that will build a solid foundation to guide research activities to support public health decision making.

Results

Five to ten key public health needs associated with an inter-pandemic scenario have been identified for each of the research agenda streams:

Stream 1: Reducing the risk of emergence of pandemic influenza

1. Integration of animal and human surveillance systems	• Development of sustainable joint surveillance programmes • Refinement of tests to detect animal influenza infection in humans • Development and improvement of veterinary diagnostic tools • Evaluation of surveillance systems • Identification, integration, and sharing of critical epidemiological and laboratory data
2. Factors associated with increased public health risk from animal influenza viruses	• Viral determinants of host range, transmissibility, pathogenicity, and antiviral resistance • Host factors involved in virus transmissibility and pathogenesis • Effect of cross-protection induced by seasonal influenza viruses • Ecological and epidemiological factors involved in environmental persistence and interspecies transmission
3. Strategies to reduce the risk of emergence of zoonotic and pandemic influenza viruses	• Reducing high risk interactions between humans and animals • Clarifying the effectiveness of animal vaccination programmes
4. An information, education, and communication strategy to improve situational awareness	• Development and validation of strategies to assess their sustainability in different contexts • Identification of key messages for different stakeholders to increase awareness and facilitate behavior change • Advocacy for resources from governments and international donors

Stream 2: Limiting the spread of pandemic, zoonotic, and seasonal epidemic influenza

Ten priority research topics were identified for *both pandemic and inter-pandemic* scenario as follows:

1. Transmissibility of influenza across the progression of infection and spectrum of disease
2. Relative contributions of the different modes of transmission for influenza
3. Biological, behavioral, and social host factors that influence the risk of transmission and infection
4. Patterns, drivers, and mechanisms affecting the seasonality of transmission
5. Viral and population factors that influence transmission and spread of different influenza types, subtypes, and strains
6. Strategies to reduce the transmission of influenza in community, household, and health care settings, especially in less-resourced areas
7. Impact and cost effectiveness of social measures, such as school closures, and the role of surveillance in assessing timing of these interventions
8. Impact, effectiveness, and cost effectiveness of individual measures, such as isolation and quarantine
9. Role of vaccination in limiting the spread of influenza and strategies for its use
10. Impact of antiviral treatment and prophylaxis in reducing transmission of influenza

Stream 3: Minimizing the impact of pandemic, zoonotic, and seasonal epidemic influenza

1. Identify higher risk groups and severe disease through surveillance; disease severity and identification of predictors of severe outcomes
2. Evaluate vaccination preventable disease burden and the potential impact of immunization programs through vaccine demonstration projects
3. Enhancement of the properties of existing vaccines, including duration and breadth of protection, safety, immunogenicity, and dose-sparing
4. Development of new vaccines and vaccine platforms, especially suitable for under-resourced country settings
5. Study the effectiveness of vaccine strategies to reduce disease burden in children and other high risk groups in a wide range of settings
6. Improved uptake and acceptability of vaccines for both seasonal and pandemic influenza

Stream 4: Optimizing the treatment of patients

Seven priority public health topics were identified for an *inter-pandemic seasonal influenza* scenario as follows:

Inter-pandemic seasonal influenza scenario

1. Research on the burden of severe disease with a focus on regional-specific factors, such as the burden of TB and HIV and optimization of pandemic and management
2. Development of new antiviral strategies and validation of surrogate endpoints which may aid in advancing understanding of disease progression
3. Further clinical evaluation of current antiviral drugs, particularly in populations at risk
4. Integration of seasonal influenza with pandemic preparedness; strengthen surveillance, health care systems, capacity, and preparedness planning
5. Improving diagnostics (e.g., multiplex assays for viruses and bacteria), including antiviral resistance testing at point-of-care
6. Dissemination of best practices, situation analysis, preparation for next epidemic (e.g., establish protocols for rotating stockpiles of antiviral drugs)
7. Increased attention to basic science research such as studying immunomodulatory drugs

Five priority public health topics were identified for an *inter-pandemic zoonotic influenza* scenario as follows:

Inter-pandemic zoonotic influenza

1. Antiviral susceptibility of circulating zoonotic viruses (e.g., H5, H9, H7 influenza viruses)
2. Reassortment between zoonotic and human influenza viruses and the potential for inter sub-type spread of antiviral resistance and virulence markers
3. Development and sharing of clinical treatment protocols in anticipation of the emergence of a new zoonotic virus
4. Further characterization of human infections with avian H5N1 and other animal influenza viruses, including geographic variability in morbidity and mortality, genetic susceptibility factors, and sites of infection and viral replication
5. Monitoring for the development of antiviral resistance during treatment of A(H5N1) virus infection

Stream 5: Promoting the development and application of modern public health tools

Modern tools for early detection and monitoring of disease

The group focusing on surveillance tools concluded that the agreed topics of interest were equally applicable during *both pandemic and inter-pandemic period*:

1. Identify modern technologies for early detection of influenza outbreaks as well as their application in surveillance at the human-animal interface
2. Develop and evaluate innovative approaches for influenza surveillance and monitoring with other existing disease monitoring systems
3. Studies to address challenges on data, clinical specimens, and viruses sharing with consideration for local, ethical, legal, and research perspectives
4. Examine the timeliness and quality of data required for early detection from local to regional, national, and global levels

Role of modeling in public health decision making

Five priority public health topics were identified for an *inter-pandemic seasonal influenza* scenario as follows:

1. Integration of genetic and epidemiological data to understand spatiotemporal spread to forecasts evolution for vaccine strain selection and to anticipate likely burden of disease
2. Quantifying the relative contributions of different modes of transmission of human influenza and developing mechanistic modeling of transmission processes
3. Research using data-capture technologies to characterize human contact and mobility patterns at local, regional, and global scales, and their correlation with transmission risk
4. Integration of genetic, antigenic, and epidemiological analyses to optimize surveillance for newly emerging pathogens at the animal/human interface
5. Identifying and quantifying human and environmental ecological, behavioral, and demographic determinants of the risk of cross-species transmission and pandemic emergence

Modern tools for strategic communication

Four priority public health topics were identified for an *inter-pandemic seasonal influenza* scenario as follows:

1. Review of evidence and experience related to health crisis communication from fields to organize knowledge and support evidence-based practice in strategic communication
2. Identify and develop tools to rapidly and accurately monitor knowledge, attitudes, and practices in different population groups and guide future communication efforts
3. Identify and develop communication tools and approaches for cultural settings and communities to practice and promote appropriate risk reduction measures
4. Understand the potential ethical, social, economic, and political communication in crisis and develop strategies to work within constraints while maximizing opportunities

Discussion

Complemented by an analogous research roadmap for a pandemic influenza scenario, the research topic recommendations for an inter-pandemic period represent an

important outcome of joint international efforts by WHO, academicians, and public health experts.

Implementation of the identified research priorities is expected to underpin public health decision-making at all levels with proven knowledge that will help to save large numbers of lives, reduce health costs, and economic loss and mitigate potential social disruption over a medium-to-long term period.

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The impacts of school resumption on the incidence of pandemic (H1N1) 2009 in school students

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Keywords school closure, non-pharmaceutical intervention, social distancing.

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Introduction

School closure is one non-pharmaceutical intervention that is often suggested in pandemic preparedness plans, and it was widely implemented in Pandemic (H1N1) 2009 to reduce transmission amongst school students. However, from past epidemiological studies, the effect of school closure in reducing respiratory disease transmission was inconclusive.[1] Given this public health intervention causes major disruption to the education system and potentially raises childcare issues to working parents, evaluating its effect in the recent pandemic is necessary to improve future pandemic planning.

In Hong Kong, since school closure was implemented early in the pandemic and closure was effectively continued with the commencement of summer holiday, the lack of incidence data in the absence of school closure makes it difficult to analyse its effect directly. This has prompted us to analyse the situation indirectly from the angle of school resumption after summer holiday.

Materials and methods

In Hong Kong, public health surveillance on Pandemic (H1N1) 2009 was effective from 25th April–30th September 2009: healthcare professionals were advised to report suspected cases of infection to Centre for Health Protection, Department of Health, HKSAR, for further laboratorial confirmation. Demographics of reported cases were subsequently recorded into a computerised system (the ''e-flu'' database). Following institutional approval, a dataset of all confirmed cases diagnosed from May to September 2009 was obtained, which included the age, gender, confirmation date, and notification date of each report. All cases were classified into four defined socio-economic classes by age: pre-schoolers (0–5), school students (6–19), adults (20–60), and retirees (≥61). Assuming cases had contracted infection on the earlier date between confirmation and notification, daily incidence in each age class was counted for epidemic curve construction.

Upon observing an unusual rise in the epidemic curve of school students when school season resumed in September, interrupted time series analysis (also known as intervention analysis)[2] was applied to obtain the statistical significance of this observation. The analysis was applied to the incidence in school students from 12th July to 26th September 2009, which covered the period from the start of summer holiday to the end of the 4th week of new school season. Incidence in school students before summer holiday was deliberately dropped since not all schools were closed when the school closure policy was effective: all primary schools were closed proactively, whereas secondary schools were individually closed on a reactive basis if students were identified to have contracted the infection. School activity was formulated as a step function, which takes value from 1st September 2009 onwards (St = 0: $t <$ 1st September,

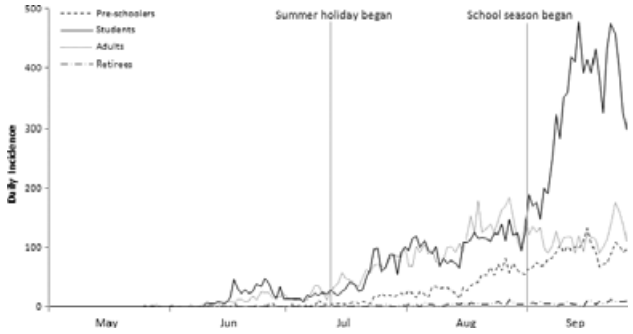

Figure 1. Epidemic curve of pre-schoolers (age: 0–5), school students (age: 6–19), adults (age: 20–60) and retirees (age: ≥61) from 1st May to 26th September 2009.

St = 1 otherwise). A range of times series models were fitted by the maximum likelihood method and AIC (Akakine Information Criterion) was used to select the one with best fit. All computations were performed in SAS version 9.2.

Results

A total of 3905 (14·8%) pre-schoolers, 13758 (52·1%) school students, 8383 (31·8%) adults, and 338 (1·3%) retirees were diagnosed with the infection in the surveillance period. The epidemic curves of preschoolers, school students, and adults showed a steady rise from 11th June onwards when local transmission of pandemic influenza was identified. An upsurge in the epidemic curve of school students can be observed in early September, coinciding with the commencement of the new school year (Figure 1).

Interrupted time series analysis on the epidemic curve of school students returned an ARIMA(0,1,0) model with equations:

$$Yt = Nt + 60 \cdot 27St$$

$$Nt = yt - 1 + 2 \cdot 73$$

where St, Yt, yt denote school activity, predicted and actual incidence in school students on day t, respectively. Standard error and significance for model constants were: 60·27 (SE = 33·5, $P = 0{\cdot}08$), 2·73 (SE = 3·84, $P = 0{\cdot}48$). In short, the model can be interpreted as: the number of infected school students rose by 2·73 per day on average during the entire study period, with a sharp increase by 60·27 coming into effect when the new school year began.

Discussion

Time series analysis showed, at the marginally significance level, that daily incidence in school students had a major increase when school season resumed. On the assumption that the increase was not caused by any change in health seeking behaviour, this result suggests that school resumption had facilitated transmission amongst school students. On the basis that school activity significantly increases incidence of pandemic influenza in school students, this study suggests closures of schools in the early phase of pandemic (H1N1) 2009 and subsequently in the summer holiday probably had a major effect in mitigating transmission amongst school students.

Youngsters were postulated to be major vector for transmission in Pandemic (H1N1) 2009. If this were true, it would be reasonable to expect the epidemic curves of the other age classes to show a similar upsurge when one is observed in school students. The absence of such observation in the epidemic curve of Hong Kong suggests school students were mostly disseminating the virus amongst themselves, but not to the other age groups.

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WHO public health research agenda for influenza: tools for strategic communication during pandemic and inter-pandemic periods

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Keywords influenza, inter-pandemic periods, pandemic, research, strategic/risk communication, WHO.

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Abstract

Clear, credible, and appropriate communication is an important tool for the dissemination of information essential to the implementation of public health measures for influenza control. Many countries repeatedly reported that they need technical assistance on evidence-based communication strategies and approaches to support public health policy development. The Global Influenza Programme (GIP) of the World Health Organization (WHO) responded to these and other influenza-related public health needs by convening a global consultation to initiate the development and implementation of a *WHO Public Health Research Agenda for Influenza.* Outcomes of the discussions highlighted strategic/risk communication as one of its pivotal cross-cutting areas that requires further development. A workshop on ''Modern Tools for Strategic Communication'' was organized by GIP in May 2010 to examine these issues by bringing together experts from relevant fields, including anthropology, marketing, communication, broadcasting, media research, as well as the behavioral and social sciences. The expert group of the workshop identified important public health needs in relation to communication during pandemics, as well as in the inter-pandemic times, and classified them into four areas: Area 1. Increasing our understanding of communication; Area 2. Designing communication interventions; Area 3. Implementation and evaluation research; Area 4. Research methods. Each area encompasses a list of suggested research topics reflecting the immediate public health priorities. Outcomes of these research activities are expected to widen the evidence base which will support developing communication strategies for influenza by countries, institutions, and individuals.

Introduction

Clear, credible, and appropriate communication is an important tool for the dissemination of information essential to the implementation of public health measures for influenza control. Such information should be aimed at diverse communities and retaining public trust. The experience of the H1N1 pandemic of 2009 highlighted the need to strengthen risk and strategic communication by bringing together expertise from relevant fields, including anthropology, marketing, communication, broadcasting, media research, as well as the behavioral and social sciences.

The World Health Organization (WHO) received requests from many Member States asking for technical assistance on evidence-based communication strategies and approaches to support public health policy development. To respond to these acute needs, in 2009, the Global Influenza Programme (GIP) organized a global consultation to initiate the development and implementation of a *WHO Public Health Research Agenda for Influenza*.[1] Outcomes of the discussions highlighted strategic/risk communication as one of its pivotal cross-cutting areas that requires further development. GIP convened a workshop on ''Modern Tools for Strategic Communication'' in May 2010 to address these needs.

Materials and methods

Global consultation on public health research agenda for influenza

In November 2009, GIP convened the first global consultation on a Public Health Research Agenda for Influenza to identify key research topics in each of the five main streams of public health research. During this meeting, the Scientific Working Group (SWG) of the sub-stream in ''Modern Tools for Risk Communication'' identified the requirements in research during influenza pandemics and inter-pandemic periods to provide clear, credible, and appropriate messages which meet the needs of diverse communities. The SWG suggested that WHO hold a follow-up workshop to assess the use of modern tools related to strategic and risk communication and to further promote research in these areas.

Workshop on modern tools for strategic communication

GIP convened a workshop on ''Modern Tools for Strategic Communication'' in May 2010. One of the main objectives

of the meeting was to generate a roadmap of public health research priorities related to strategic and risk communication. The research roadmap was developed by the group of invited experts on the basis of an analysis of available evidence and experience on public health and health crisis communication from relevant disciplines across global regions, as well as critical assessment of existing communication methods related to influenza control in different cultural, social, and ethnic settings.

The workshop consisted of a series of presentations by experts in relation to experiences and lessons learned about communication during the SARS, H5N1 epidemic, and H1N1 pandemic. There were also a series of group discussions on identifying research needs for pandemic and inter-pandemic periods in order to strengthen the Research Agenda.

Results

The expert group identified important public health needs in relation to communication during pandemics as well as in the inter-pandemic times. The main topics of discussion centered on communicating issues of influenza virus transmission, the use of influenza vaccines safety and efficacy, and use of antivirals as well as definition of the severity of the pandemic and the phase changes. In this context a number of research areas were identified, which can be broadly classified into four areas:

Area 1. Increasing our understanding of communication

Understanding of communication principles and mechanisms is associated with an array of research topics covering different subject areas. One of the key questions here relates to the link between communication and ''behaviour change'' models and their application and appropriateness for different settings. The expert group defined the term ''behaviour change'' in this context as the modification of behaviour towards better health practices that are supported by clinical and scientific evidence for personal protection against infectious diseases and other adverse health risks. Research topics related to these models require understanding and differentiating information and ''behaviour change'' needs of different audience segments, such as stakeholder mapping, target audience analysis, research into behaviour motivation, social norms, and the cultural, religious, social, legal, and political barriers and enablers of particular behaviors that are beneficial in influenza control.

This research area also includes the analysis of media consumption among different audiences, role models, including ways to analyse how rumours and misinformation are spread, and ways to provide evidence-based information correctly. Other important areas of investigation embrace methods to communicate uncertainty, learning how to build trust while communicating about a pandemic, and understanding what needs to be done before, during, and after a pandemic in order to create the best environment for influenza pandemic communication. Critical key audiences identified for more intensive analysis were health workers, religious, public health, and societal (political and community) leaders.

Area 1. Major research needs and priorities:

- Investigation of the role of different communication channels and communication formats for different target audiences in a pandemic, particularly for groups that are ''hard-to-reach.''
- Determining effects of perceptions related to pandemic influenza (severity, susceptibility, response efficacy, self efficacy, perceived social norms) on protective behaviours in different groups.
- Understanding audience in terms of their knowledge, preventive activities, and reasons why engaged/not engaged.
- Developing mechanisms to synergies between risk communication and behavior oriented approaches in the pandemic and inter-pandemic phases.
- Determining social, economic, cultural, and religious factors which support behaviours to limit spread and minimize impact in different settings.
- Identification of the key predictors/factors that influence people's behavior among different groups and populations vis-à-vis pandemic flu behaviors.
- Identification of elements that contribute to trust among populations and in different settings (country, public, professional, community), particularly where trust was previously compromised.
- Understanding psychology of different groups regarding their response to uncertainty, and finding the best way to communicate uncertainty.

Area 2. Designing communication interventions

The research questions in this section relate to the planning, development, and evaluation of tools that can be quickly accessed and used in a pandemic situation. These may include communication materials and channels; the setting up of key stakeholder and champion communication networks; research protocols that are ready for rapid assessment during a pandemic or new communication tools.

The use and understanding of terminology and language by both lay and professional groups and communities in planning for and/or reacting to a pandemic are important areas of research. Acute examples, such as the naming of the viruses or the use of the word ''pandemic,'' illustrate this need well.

Area 2. Major research needs and priorities:

- Development and evaluation of communication to facilitate vaccination uptake in a) health care personnel, b) key public service personnel, c) risk groups, and d) the general public.
- Development and evaluation of strategies to communicate the use of modeling for decision-making in a pandemic situation to decision makers and the general public.

- Develop a social mobilisation data base and evaluate internal and external experiences.
- Identify models of communication on the human-animal interactions to limit the risk of emergence of pandemic influenza.

Other areas of research interest:

- Development and evaluation of communication interventions to facilitate and optimize distribution and use of antivirals.
- Research to improve the cost effectiveness of community dialogue and engagement models.
- Research on tools that can be used to track the bloggershpere.

Area 3. Implementation and evaluation research

The research focus of this area is to look at lessons learned from the A(H1N1) 2009 pandemic and to document and evaluate case studies, both looking at best practices, challenges, and barriers that were experienced. Different communication strategies need to be evaluated and models to be built not only in terms of reach, but also in terms of impact on thinking, emotional response, and behavioural modification. A key question was how to prepare communication for a pandemic and how can the pandemic communication contribute to longer term ''behavioural change.'' Mathematical modelling on gauging outcomes of such ''behaviour change'' would provide strategic approaches in risk communication.

Area 3. Major research needs and priorities:

- Monitoring the implementation of pandemic communication campaigns to identify barriers and enablers with regard to specific settings and specific target groups.
- Mapping of networks that can be rapidly activated during a pandemic.
- Review political influence on pandemics influenza communication strategies.
- Determine what kind of expertise is needed at a country level.
- Evaluate existing interventions and develop multi-component strategies for communication to promote protective behaviors, such as hand-washing, coughing and sneezing etiquette, environmental hygiene, etc.

Area 4. Research methods

This section aims to answer the question whether the modeling, mapping, and scenario planning are actually useful in the pandemic situation. The expert group agreed that the research on the above issues should use a variety of methods and engage a number of disciplines. This would include literature reviews, case studies, trials, ethnographic studies, modelling, surveys, network analysis, as well as any other useful methodology.

Area 4. Major research needs and priorities:

- Develop and evaluate modeling methods to study the potential impact of strategic communication interventions on protective behaviour *vis-à-vis* different scenarios and variables.
- Review and evaluate the validity of ''rapid assessment'' monitoring devices.
- Research on the predictive validity of hypothetical ''scenario-research'' in an inter-pandemic situation for actual behaviour under pandemic conditions.
- Study the synergies and develop priority research topics on strategic/risk communication for influenza under inter-pandemic situations that includes zoonotic and seasonal infections.

Discussion

The *WHO Public Health Research Agenda for Influenza* initiated and facilitated a multi-disciplinary discussion for communication during pandemic and inter-pandemic situations. It focused on both theoretical and practical issues to improve practice and ensure the health of the public for influenza. Critical areas for research were identified to build evidence in this field. It was recognized that there are extensive bodies of knowledge in a number of disciplines,[2,3] such as health promotion, behavioural psychology, social sciences, social and behaviour change communication, social marketing, and communication for development relating to these questions, and that these should be explored.

Outcomes of these research activities are expected to widen the evidence base which will support developing communication strategies for influenza by countries, institutions, and individuals and will, consequently, help to improve public health world-wide.

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The role of innate sensing receptors in H5N1 associated hyper-induction of cytokines

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Keywords Cytokines, H5N1, innate sensing receptors, RIG-I, TLR3.

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Abstract

Background: Cytokine dysregulation contributes to the unusual severity of H5N1 (Reviewed in[1]). Previously, we demonstrated that interferon regulatory factor 3 (IRF3) and p38 MAP kinase (p38) signaling pathways separately contribute to the induction of pro-inflammatory cytokines and chemokines in H5N1-infected cells.[2] Here we investigate the role of innate sensing receptors in the induction of these cytokines and chemokines in response to H5N1 and seasonal H1N1 infection. Materials and methods: Human macrophages derived from peripheral blood monocytes were infected with H5N1 (483/97) or seasonal H1N1 (54/98) viruses. The role of innate sensing receptors in cytokine and chemokine induction by H5N1 virus was investigated using transient knock-down of these receptors with siRNAs. The expression of innate sensing receptors in infected cells, and as a result of paracrine activation (by virus free supernatants of infected cells) of adjacent uninfected cells were also monitored by real-time PCR and/or Western Blotting. The involvement of Janus kinase (JAK) signaling pathways in these autocrine/paracrine cascades was investigated using a JAK inhibitor. Results: We previously showed that TNF-alpha, IFN-beta, and IFN-lambda 1 are the key mediators directly induced by the H5N1 virus in primary human macrophages with other cytokines and chemokines being induced as part of a secondary autocrine and paracrine cascade. Here we demonstrated that retinoic-acid-inducible gene I (RIG-I) rather than Toll-like receptor 3 (TLR3) plays the predominant role in H5N1-induced cytokines and chemokines in human macrophages via the regulation of IRF3 and NF-kB nuclear translocation. In addition to the effects on virus infected cells, paracrine interactions between macrophages and alveolar epithelial cells contributed to cytokine cascades via modulation of JAK signaling and by the upregulation of sensing receptors. Conclusions: H5N1 directly induced TNF-alpha and IFN-beta mainly via RIG-I signaling, and the subsequent activation and nuclear translocation of IRF3 and NF-kB in human macrophages. In addition to the effects on cytokine signaling, the innate immune sensing regulators themselves were also up-regulated by H5N1 infection, much more so than by seasonal influenza infection, via JAK signaling. The up-regulation of innate sensing receptors was not limited to the infected cells, but was also found in adjacent uninfected cells through paracrine feedback mechanisms. This may lead to broadened and amplified cytokine signals within the microenvironment of the infected lung. A more precise understanding of the signaling pathways triggered by H5N1 virus leading to cytokine induction may provide novel options for the design of therapeutic strategies for severe human H5N1 influenza and also for treating other causes of acute respiratory disease syndrome.

Introduction

Human H5N1 infection is associated with a mortality rate of more than 60%. The basis for the unusual severity of H5N1 disease has not been fully explained. Cytokine dysregulation has been suggested to contribute to the disease severity of H5N1 (Reviewed in[1]). However, signaling pathways involved in the cytokine induction by H5N1 virus are not fully understood. Previously, we demonstrated that IRF3 and p38 MAP kinase (p38) are separate signaling pathways which contribute to the induction of pro-inflammatory cytokines and chemokines in H5N1-infected cells.[2]

RIG-I and melanoma differentiation-associated gene 5 (MDA5) are important cytosolic sensors of nucleic acid of pathogens, while TLR3 and TLR8 also recognize nucleic acid species of pathogens, but they are localized at the endosomal membrane.[3] RIG-I was found to be responsible for the recognition of influenza A virus infection,[4] and the transfection of vRNPs induces IFN-beta expression.[5] While many studies have shown the role of RIG-I in the induction of IFN-beta by influenza virus infection, the majority of these studies used either immortalized cell lines or

mouse embryonic fibroblasts. There is a lack of data on the role of these innate sensing receptors in highly pathogenic avian influenza H5N1 infection in primary human cells *in vitro*, which are more physiologically relevant. Furthermore, there is little data on the autocrine and paracrine up-regulation of these innate immune sensors following virus infection.

Materials and methods

Human macrophages were obtained from peripheral blood monocytes by adhesion and differentiation in vitro for 14 days in RPMI medium supplemented with 10% autologous plasma. The cells were infected with H5N1 (483/97) or seasonal H1N1 (54/98) viruses at a MOI of 2·0. A549 cells were obtained from ATCC and cultured in MEM medium supplemented with 10% FCS and 1% penicillin and streptomycin. The role of innate sensing receptors in cytokine induction by H5N1 and H1N1 viruses was investigated using transient knock down of these receptors with siRNAs in human macrophages as previously described[2] using specific siRNAs purchased from Qiagen. Immunofluorescence staining assay of IRF3 and NF-κB was employed to detect the nuclear translocation of these transcription factors after H5N1 infection. Rabbit polyclonal antibodies against human IRF3 and and NF-kB were obtained from Santa Cruz Biotechnology. Goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 was a product of Molecular Probes.

For investigation of paracrine effects on RIG-I and TLR3 expression, culture supernatants collected from mock, 54/98 or 483/97 infected human macrophages were used to treat uninfected cells. The supernatants were first passed through a filter with 100-kDa cut-off. Virus particles as well as molecules with a molecular weight higher than 100 kDa were retained and removed, while the filtrate was collected for treatment of uninfected cells.

The expression of innate sensing receptors in infected cells and in adjacent uninfected cells following paracrine activation by virus free supernatants of infected cells was monitored by real-time PCR. The involvement of JAK signaling pathways in these paracrine cascades was investigated using a JAK inhibitor (Calbiochem).

Results

We previously showed that TNF-alpha, IFN-beta, and IFN-lambda 1 are the key mediators directly induced by the H5N1 virus in primary human macrophages with others being induced as part of a secondary autocrine and paracrine cascade.[2] In this study, we demonstrate that knockdown of RIG-I or TLR3 led to the reduction of IFN-beta

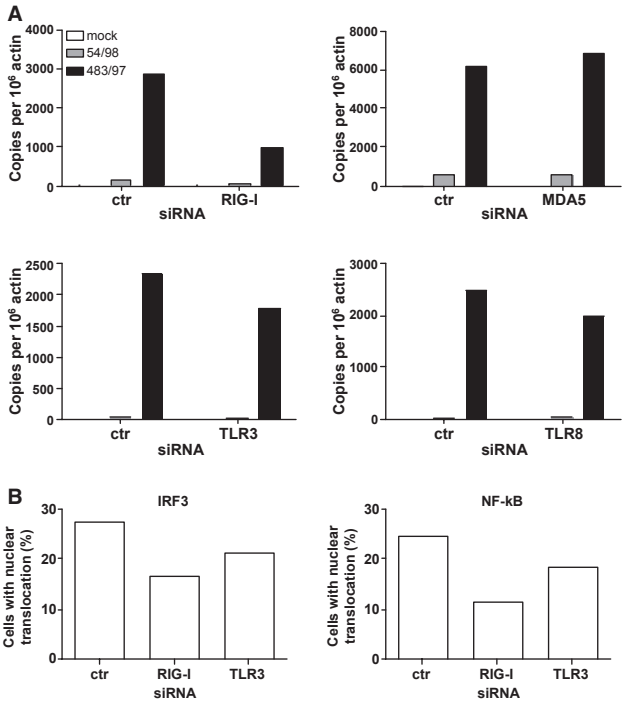

Figure 1. (A) IFN-beta mRNA expression in human macrophages transfected with non-targeting control (ctr), retinoic-acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), Toll-like receptor 3 (TLR3), or TLR8 siRNA. 72 hours later, the cells were mock, 54/98, or 483/97 infected at MOI of 2. (B) Nuclear translocation of interferon regulatory factor 3 and NF-κB in human macrophages transfected with non-targeting control (ctr), RIG-I, or TLR3 siRNAs following 483/97 infection.

and TNF-alpha in human macrophages by both 54/98 (H1N1) and 483/97 (H5N1) infection. As shown in Figure 1A, 483/97 virus induced higher level of IFN-beta mRNA expression than 54/98 infection. Cells transfected with RIG-I or TLR3 siRNA significantly reduced the expression of IFN-beta after 483/97 infection, by 63% and 29%, respectively. RIG-I silencing also significantly reduced the IFN-beta expression in 54/98 infected cells by 52%. In contrast, silencing of MDA5 or TLR8 did not suppress the induction of IFN-beta by either 54/98 or 483/97 infection; in fact, there was a slight (15%) increase of IFN-beta in cells transfected with MDA5 siRNA. Based on these results we conclude that while both RIG-I and TLR3 contribute to H5N1-induced interferon-beta induction in human macrophages, RIG-I plays the dominant role.

In order to investigate the relationship between these innate sensing receptors and the activation of transcription factors IRF3 and NF-κB, we next measured the nuclear translocation of IRF3 and NF-κB in cells with RIG-I or TLR3 silencing after H5N1 infection. Immunofluorescence staining assay on IRF3 and NF-κB was performed and the

number of cells with nuclear translocation was quantitated. The percentages of cells with nuclear translocation were plotted in Figure 1B. We demonstrated that RIG-I knockdown led to a significant reduction of IRF3 nuclear translocation after 483/97 infection, whereas the nuclear translocation of NF-κB after 483/97 infection was significantly suppressed by RIG-I or TLR3 silencing. These results suggest that the involvement of RIG-I and TLR3 in the cytokine induction by 483/97 was via the regulation of IRF3 and NF-κB nuclear translocation.

Since RIG-I and TLR3 are important in influenza A virus-induced cytokine expression, we next explored the expression of these innate receptors in neighboring uninfected human macrophages by treating the uninfected macrophages with the filtered culture supernatants collected from mock, 54/98, or 483/97 infected macrophages. As shown in Figure 2A, 483/97 supernatant differentially induced the mRNA expression of RIG-I, MDA5, and TLR3 compared to 54/98 supernatant treated human macrophages. The induction of RIG-I was higher than the induction of MDA5 and TLR3. In the presence of 1 μm of JAK inhibitor, the up-regulation of all three innate sensing receptors was significantly reduced showing their induction was dependent on JAK activity.

Human lung epithelial A549 cells were also treated with the supernatants collected from macrophages infected with mock, 54/98, or 483/97 virus. Differential induction of RIG-I, MDA5 and TLR3 by 483/97 supernatant compared to 54/98 supernatant treated cells was observed (Figure 2B). 483/97 supernatant dramatically induced all three innate sensing receptors, while 54/98 supernatant only marginally induced RIG-I and MDA5, but not TLR3. As in human macrophages, treatment with 1 μm of JAK inhibitor caused a significant suppression of 483/97 supernatant-induced RIG-I, MDA5, and TLR3 expression in A549 cells.

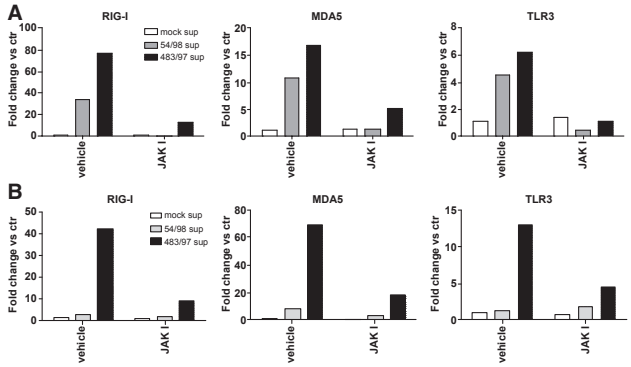

Figure 2. Retinoic-acid-inducible gene I, melanoma differentiation-associated gene 5, and Toll-like receptor 3 mRNA expression in (A) human macrophages or (B) human lung epithelial cell line A549 cells, treated with culture supernatants collected from mock, 54/98, or 483/97 infected macrophages with or without the presence of Janus kinase inhibitor (JAK I).

These results, taken together with the direct effects on virus infected cells, suggest that paracrine interactions between macrophages and alveolar epithelial cells contributed to cytokine cascades via modulation of JAK signaling and by the up-regulation of innate sensing receptors.

Discussion

H5N1 directly induced IFN-beta (Figure 1) and TNF-alpha (data not shown) mainly via RIG-I signaling and the consequent activation and nuclear translocation of IRF3 and NF-kB in human macrophages. These results were consistent with a previous study using BEAS-2B cells showing the essential role of RIG-I in IFN-beta reporter activity by H3N2 influenza virus infection.[4] While TLR3 also played a role in induction of IFN-beta and the activation of IRF3 and NF-kB, it plays a less important role compared to RIG-I. The reduction of IRF3 and NF-kB activation was also confirmed with the study by Le Goffic[4] showing differential regulation of IRF3 and NF-kB by RIG-I and NF-kB can also be regulated by TLR3.

In addition to the direct role of RIG-I and TLR3 in sensing and signaling the presence of influenza virus, the innate immune sensing regulators were themselves also highly up-regulated in both infected (data not shown) and adjacent uninfected cells by influenza virus infection. Compared with seasonal H1N1 virus, the H5N1 viruses had a much more dramatic effect on inducing innate sensing receptors via JAK signaling pathways activated by autocrine and paracrine mediators. The up-regulation of RIG-I, MDA5, and TLR3 was markedly induced by virus free culture supernatants from H5N1-infected macrophages, while supernatant from 54/98-infected cells induced the expression of these receptors only to a lesser degree. The soluble mediators in the virus infected cell supernatant caused paracrine upregulation of RIG-I, MDA5, and TLR3 in uninfected macrophages as well as human lung epithelial cells. These effects may lead to broadened and amplified cytokine signals within the microenvironment of the infected lung. Taken together these results provide, at least, part of the explanation on the hyper-induction of cytokines in H5N1 infection. A more precise identification of the signaling pathways triggered by H5N1 virus leading to cytokine induction may provide novel options for the design of therapeutic strategies for severe human H5N1 influenza and also for treating other causes of acute respiratory disease syndrome.

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H9N2 influenza virus acquires intravenous pathogenicity on the introduction of a pair of di-basic amino acid residues at the cleavage site of the hemagglutinin and consecutive passages in chickens

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Keywords Chicken, H9N2, HA cleavage site, passage, pathogenicity.

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Introduction

Avian influenza (AI) outbreaks caused by low pathogenic H9N2 viruses have occurred in poultry, resulting in serious economic losses in Asia and the Middle East.[1–9] Eradication of this virus is still difficult because of its low pathogenicity, frequently causing unapparent infection. It is important for the control of AI to assess whether the H9N2 virus is capable of becoming pathogenic like H5 and H7 highly pathogenic avian influenza viruses (HPAIVs). In the present study, we investigated whether a non-pathogenic virus with H9 HA, A/chicken/Yokohama/aq-55/2001 (Y55) (H9N2), becomes pathogenic to chickens when a pair of di-basic amino acid residues are introduced at the cleavage site of its HA molecule.

Materials and methods

We generated mutants of Y55 (H9N2) and A/duck/Hokkaido/Vac-1/2004 (Vac1) (H5N1) which have basic amino acid residues at their HA cleavage sites by site-directed-mutagenesis and reverse genetics. The amino acid sequences at the cleavage sites are as follows: Y55sub (H9N2) (PARKKR/G), Vac1sub (H5N1) (PRRKKR/G), and Vac1ins (H5N1) (PQRERRKKR/G). These mutant viruses were then consecutively passaged in the air sacs of 3-day-old chicks. Passaged viruses were identified by their parental strains, mutation (substitution or insertion), and number of passages. Pathogenicity of the viruses was evaluated by inoculating into 4-week-old chickens intravenously (1:10 diluted allantoic fluid, 200 μl) or intranasally ($10^{6.5}$ EID_{50}/100 μl).

Results

Generation and characterization of mutant viruses

rgY55sub (H9N2), rgVac1sub (H5N1), and rgVac1ins (H5N1), which have a serial basic amino acid residues at their HA cleavage sites were generated by site-directed-mutagenesis and reverse genetics. rgY55sub (H9N2) and rgVac1ins (H5N1) required trypsin to replicate in MDCK cells, and showed similar levels of growth to their parental viruses (Table 1). Chickens intravenously inoculated with rgY55sub (H9N2) or rgVac1ins (H5N1) did not show any signs of disease. rgVac1sub (H5N1) replicated in MDCK cells without exogenous trypsin, and one of the eight chickens inoculated with the virus showed slight depression at 1 day post-infection.

Consecutive passages of the viruses in the air sacs of chicks

The H9 and H5 mutant viruses were serially passaged in the air sacs of chicks to assess their ability to acquire pathogenicity. Plaque formation in MDCK cells and pathogenicity in 3-day-old chicks and 4-week-old chickens are shown in Table 1. rgY55sub (H9N2) replicated in MDCK

Table 1. Replicability in MDCK cells and pathogenicity for chicken of each virus

Viruses	Plaque formation (log PFU/ml)		Virulence (no. of dead/no. of sick/total no.) against	
	With trypsin	Without trypsin	3-day-old chicks (air sac inoculation)	4-week-old chickens (intravenous inoculation)
rgY55 (H9N2)	8·1	–*	NT**	0/0/8
rgY55sub (H9N2)	8·0	–	0/0/3	0/0/8
rgY55sub-P5 (H9N2)	7·6	–	0/0/4	0/0/8
rgY55sub-P6 (H9N2)	7·2	6·7	3/3/3	0/1/8
rgY55sub-P7 (H9N2)	7·8	7·5	3/3/3	0/5/8
rgY55sub-P8 (H9N2)	7·6	7·6	3/3/3	2/7/8 (4·0)***
rgY55sub-P9 (H9N2)	7·2	6·9	3/3/3	1/8/8 (10·0)
rgY55sub-P10 (H9N2)	6·5	6·1	3/3/3	6/8/8 (1·8)
rgVac1 (H5N1)	7·6	–	NT	0/0/8
rgVac1sub (H5N1)	7·6	7·8	0/0/3	0/1/8
rgVac1sub-P1 (H5N1)	6·8	6·8	3/3/3	3/5/8 (5·3)
rgVac1sub-P2 (H5N1)	6·4	6·5	6/6/6	8/8/8 (2·6)
rgVac1ins (H5N1)	7·3	–	0/0/3	0/0/8
rgVac1ins-P1 (H5N1)	7·8	7·1	3/3/3	6/7/8 (6·8)
rgVac1ins-P2 (H5N1)	7·1	7·1	4/4/4	8/8/8 (4·4)

*No plaques.
**Not tested.
***Mean time to death (days) is shown in parentheses.

cells in the absence of trypsin and killed all of the chicks after six consecutive passages. Two of the eight-four-week-old chickens inoculated intravenously with rgY55sub-P8 (H9N2) died within 5 days. Eventually, over 75% of the chickens intravenously infected with rgY55sub-P10 (H9N2) died by 2 days post inoculation, and its pathogenicity was comparable to that of HPAIVs.[10] rgVac1sub-P1 (H5N1) was pathogenic to both chicks and 4-week-old chickens, and mortality increased after one more passage. rgVac1ins-P1 (H5N1) replicated in MDCK cells in the absence of trypsin, killed all of the chicks, and caused 75% mortality among 4-week-old chickens. The lethal effect of rgVac1ins-P1 (H5N1) on chickens increased with one additional passage in the air sacs of chicks, as in the case of rgVac1sub (H5N1).

Pathogenicity of the viruses on intranasal infection in chickens

To examine whether the pathogenicity of each virus via the natural route of infection correlated with that by intravenous infection or not, three 4-week-old chickens were challenged intranasally with the viruses at an EID_{50} of $10^{6.5}$ and observed for clinical signs until day 14 post-infection (data not shown). All chickens inoculated with rgY55sub-P10 (H9N2) or its parental viruses survived without showing any clinical signs, and serum antibody responses were detected in the HI test. On the other hand, rgVac1sub-P2 (H5N1) and rgVac1ins-P2 (H5N1) were pathogenic as in the intravenous experiment, killing two of three chickens by day 11 post-inoculation.

Additional passages of the Vac1-based viruses in chickens

One of three chickens were not infected with rgVac1sub-P2 (H5N1) or rgVac1ins-P2 (H5N1) via intranasal route (data not shown), indicating these P2 viruses had not been completely adapted to the host. To investigate the possibility of these P2 viruses to acquire further pathogenicity for chicken, rgVac1sub-P3 (H5N1) and rgVac1ins-P3 (H5N1) were obtained from the brain homogenates of the chickens that died on 11 days post intranasal inoculation with the P2 viruses. Although mortality rate of chickens inoculated with the P3 viruses was equal to that with P2 viruses, enhancement of pathogenicity was observed in intranasal inoculation study; all of the chickens inoculated with rgVac1sub-P3 (H5N1) were infected, and time to death was shortened to 4–6 days post inoculation in chickens with rgVac1ins-P3 (H5N1) (data not shown).

Table 2. Virus recovery from the chickens intranasally inoculated with each virus

Inoculated viruses	No. of chickens	Days p.i. (health status)	Virus recovery (log EID_{50}/g)						
			Brain	Trachea	Lung	Liver	Kidney	Colon	Blood*
rgY55 (H9N2)	3	3 (sacrificed)	-,-,-**	-, -, 1·7≥	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -
rgY55sub (H9N2)	3	3 (sacrificed)	-, -, -	5·5, 5·7, 6·5	-, 2·7, 6·7	-, -, 2·7	-, -, 2·5	-, -, -	-, -, -
rgY55sub-P10 (H9N2)	3	3 (sacrificed)	-, -, -	-, -, 3·3	-, 3·7, 6·0	-, -, 2·5	-, 4·3, 4·5	-, 3·3, 4·5	-, -, -
rgVac1 (H5N1)	3	3 (sacrificed)	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -	-, -, -
rgVac1sub (H5N1)	3	3 (sacrificed)	-, -, 2·7	-, -, -	-, -, 2·5	-, -, -	-, -, -	-, -, 2·0≥	-, -, -
rgVac1sub-P2 (H5N1)	2	3 (sacrificed)	5·0	3·8	5·0	5·2	7·5	5·2	4·3
	1***	3 (sacrificed)							
rgVac1sub-P3 (H5N1)	2***	3 (dead)	5·7, 6·7	5·3, 6·5	4·7, 8·2	3·5, 6·0	7·3, 9·0	5·5, 6·5	NA†
	1***	3 (sacrificed)	6·3	6·5	6·7	6·5	6·5	6·5	5·8
rgVac1ins (H5N1)	3	3 (sacrificed)	-, -, 2·6≥	-, -, 3·5	-,3·3, 3·7	-, -, 3·5	-, 2·7, 3·0	-, 2·5, 3·0	-, 1·6 ≥ , 2·8
rgVac1ins-P2 (H5N1)	1***	2 (dead)	3·5	3·4	4·7	3·7	4·8	4·7	NA
	1***	3 (sacrificed)	3·8	3·7	3·0	2·0 ≥	4·7	2·0 ≥	–
	1	3(sacrificed)	–	–	–	–	–	–	–
rgVac1ins-P3 (H5N1)	3***	3 (sacrificed)	3·4, 4·7, 5·5	3·5, 4·2, 4·7	5·2, 5·5, 6·7	4·3, 4·5, 5·7	4·5, 4·7, 5·2	4·5, 5·3, 5·3	2·5, 3·0, 3·5

*log EID_{50}/ml.
**1·5≥ (0·5≥ for blood samples).
***Each chicken showed depression.
†Not applicable.

Replication of the viruses intranasally inoculated in chickens

To investigate whether tissue tropism of the viruses was involved in their pathogenicity, we determined viral titers in the tissue and blood samples from 4-week-old chickens intranasally inoculated with each virus on 3 days post infection (Table 2).

rgY55 (H9N2) and rgVac1 (H5N1) were scarcely recovered from the samples, and the mutant strains before passage showed broader tissue tropism than the parental viruses. None of the chickens inoculated with rgY55sub-P10 (H9N2) showed any signs of disease, and viruses were recovered from each of the samples except the brain and the blood. One chicken inoculated with rgVac1sub-P2 (H5N1) showed clinical signs such as depression, and viruses were recovered from virtually all of its organs and blood samples. Two of three chickens inoculated with rgVac1ins-P2 (H5N1) showed disease signs, and one died 2 days post inoculation. The viruses were recovered from almost all samples of the two chickens showing signs of disease. P3 viruses were efficiently replicated in systemic organs of the chickens as compared with P2 viruses. Throughout the study, the viruses were recovered from the brains of all of the chickens showing clinical signs.

Discussion

Here, we demonstrated that the H9 influenza virus acquired intravenous pathogenicity after a pair of di-basic amino acid residues was introduced into the cleavage site of the HA and passaged in chicks. rgY55sub-P10 (H9N2) killed 75% of chickens infected intravenously, and its pathogenicity was comparable to that of HPAIVs (Table 1). However, chickens intranasally inoculated with rgY55sub-P10 (H9N2) did not show any clinical signs of disease (data not shown). These results are consistent with a previous study in chickens that found some H10 influenza viruses did not show intranasal pathogenicity although their intravenous pathogenicity index was over 1·2, classified as HPAIV according to the definition by European Union.[11] Ohuchi *et al.*[12] reported that the insertion of additional basic amino acids into the H3 HA cleavage site resulted in intracellular proteolytic cleavage. Other groups reported that H3 and H6 HAs tolerated amino acid mutations into their cleavage sites, and the viruses with the mutated HAs replicated in MDCK and/or QT6 cells in the absence of trypsin.[13,14] The results in the present study is in agreement with these, namely, cleavage-based activation by a ubiquitous protease is not restricted to the H5 and H7 HAs.

The intranasal pathogenicity of the H9 and H5 mutants were different (data not shown), although these viruses similarly replicated in MDCK cells in the absence of trypsin and killed chickens by intravenous inoculation (Table 1). The viruses were recovered from the brain and the blood of some chickens infected with rgVac1 mutants (H5N1), and morbidity was closely associated with viral titers in the brain (Table 2). On the other hand, no viruses were recovered from the brain of chickens infected with rgY55 mutants (H9N2), explaining why rgY55sub-P10 (H9N2) did not show intranasal pathogenicity. All the viruses passaged in the air sacs of chicks killed chicken embryos by 48 hours post allantoic inoculation (data not shown). rgVac1sub-P3 (H5N1) and rgVac1ins-P3 (H5N1) were more pathogenic to chicken embryos than rgY55sub-P10 (H9N2); the allantoic fluid obtained from the embryonated eggs inoculated with the H5 viruses passaged in air sacs was turbid. It has been reported that infection of a highly pathogenic H7 virus were strictly confined to endotherial cells in chicken embryos or chickens.[15,16] Therefore, it is suggested that endotheliotropism differed between the H9 and H5 viruses passaged in air sacs and affected their intranasal pathogenicity. Taken together, it is assumed that rgVac1sub-P3 (H5N1) and rgVac1ins-P3 (H5N1) showed marked intranasal pathogenicity with high levels of viremia caused by replication in vascular endothelial cells, leading to invasion of the brain. In the intravenous experiment, rgY55sub-P10 (H9N2) easily reached systemic organs, including the brain hematogenously, replicated through the cleavage of HA by a ubiquitous protease, and then exerted its pathogenicity. Further study including a pathological analysis is currently underway to test this hypothesis.

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Activation of the highly pathogenic avian influenza virus replication by membrane-bound proteases MSPL and TMPRSS13 and its inhibition by the protease inhibitors

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Keywords Highly pathogenic avian influenza virus, processing protease, type II transmembrane serine protease.

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Abstract

MSPL and TMPRSS13 are unique type II membrane-bound serine proteases, which preferentially cleaved at multi-basic cleavage site motifs. Hemagglutinin (HA) of highly pathogenic avian influenza (HPAI) viruses have two consensus multi-basic cleavage motifs of K-X-K/R-R and R-X-K/R-R. MSPL/TMPRSS13 efficiently cleave both motifs, although furin and pro-protein convertase in the trans-Golgi network cleave only R-X-K/R-R motif. In the present study, we report HA cleavage and multiplication of HPAI virus H5N1 with K-X-K/R-R motif in human endothelial cells expressing MSPL/TMPRSS13, but not furin, and HA cleavage and multiplication of H5N1 virus with R-X-K/R-R motif in the cells expressing MSPL/TMPRSS13 and/or furin. Furthermore, we demonstrated that membrane fusion activity of HA molecules of diverse HPAI viruses, particularly the virus with K-X-K/R-R motif, were strongly and selectively suppressed by inhibitors against MSPL/TMPRSS13. These results suggest that MSPL and TMPRSS13 are novel drug targets for the treatment of diverse HPAI virus infection.

Introduction

For all HPAI viruses of subtypes H5 and H7 known to date, the cleavage of HA occurs at the C-terminal R residue in the consensus multibasic motifs, such as R-X-K/R-R with R at position P4 and K-K/R-K/T-R with K at P4, and leads to a systemic infection. Early studies demonstrated that the ubiquitously expressed furin and PCs are activating proteases of HPAI viruses.[1] Furin and PCs cleave the consensus multi-basic motif R-X-K/R/X-R with R at position P4.[2] However, replacement of P4 R by K and a nonbasic amino acid significantly suppresses the processing activities of furin and PCs.[2] Most of the type II transmembrane serine protease identified so far recognize a single R at position P1, but the newly isolated MSPL and its transcript variant TMPRSS13 preferentially recognize paired basic residue, particularly R and K at position P4, at the cleavage

site.[3–5] Thus, MSPL and TMPRSS13 can activate various bioactive polypeptides with multibasic residue motifs, including fusogenic viral envelope glycoproteins. The present study was designed to characterize the proteolytic processing of the HPAI virus HA by MSPL and TMPRSS13 in comparison with furin.

Materials and methods

HPAI virus A/crow/Kyoto/53/2004 (H5N1)[6] was isolated from embryonated eggs inoculated with tracheal homogenates from dead crows. Then, the mutant HA sequence was constructed by changing R residue to K residue (N'-RKKR-C' to N'-KKKR-C') at the HA cleavage site by site-directed mutagenic PCR as described.[7] We used human cell line ECV304, which expresses MSPL and TMPRSS13 at levels below detection, and established the cells stably expressing MSPL and TMPRSS13, such as ECV304-MSPL and ECV304-TMPRSS13.[7] To determine the cleavage specificities of MSPL/TMPRSS13 and furin, peptides (20 μg each) were incubated with 0·25 mU MSPL/TMPRSS13 for 1 hour and furin for 8 hours at 37°C, respectively. After incubation, the samples were separated by reverse-phase-high-performance liquid chromatography (RP-HPLC) with the use of a C_{18} column. The elution samples were then identified by amino acid sequence analysis and by MALDI-TOF-MS.

Results

Cleavage of HA peptides by MSPL/TMPRSS13 and furin

We analyzed the cleavability of 14-residue synthetic peptides derived from HA cleavage sites of HPAI strains, such as A/chick/Penn/1370/83 (H5N2)[8] and A/FPV/Rostock/34 (H7N1),[9] and low pathogenic strain A/Aich/2/58 (H3N2). After incubation with human MSPL or human furin, the digested samples were separated by RP-HPLC, and peptide fragments were characterized by mass-spectrometry and protein sequencing. In contrast to the low cleavage efficiencies of the H3 HA peptide with a single R at the cleavage site (Figure 1A), both the H5 HA peptide with the K-K-K-R motif (Figure 1B) and the H7 HA peptide with the R-K-K-R motif (Figure 1C) were fully processed at the correct positions by MSPL within 1 hour. In the case of H7 HA peptide with multiple basic residues, MSPL cleaved two carboxyl-terminal sides of R in the cleavage site sequence of N'-K-K-R↓-K-K-R↓-G-C', while furin cleaved only at a single site of R with R at position P4, N'-K-K-R-K-K-R↓-G-C' in the presence of 1 mm $CaCl_2$. These cleavage site specificities of furin were consistent with that reported for the H5 HA peptide of HPAI virus A/Hong

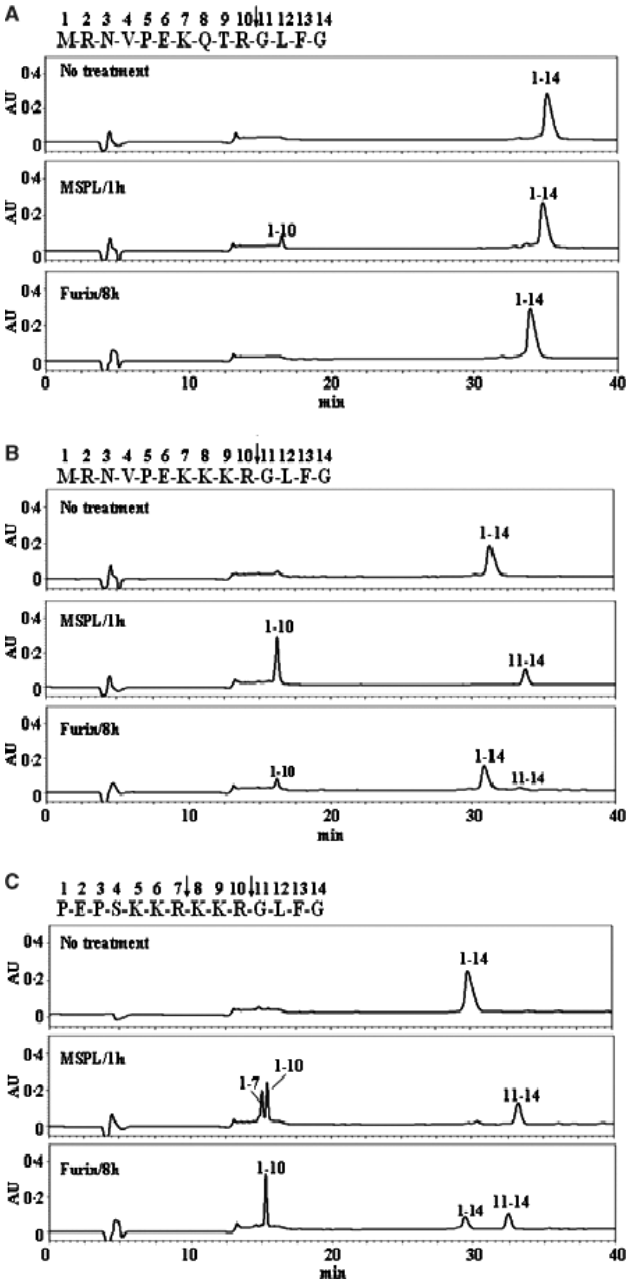

Figure 1. Processing by MSPL and furin of synthetic hemagglutinin peptides with a single basic R in the cleavage motif (A), and with multibasic cleavage motifs (B and C). Synthetic 14-residue peptides (20 μg each) indicated at the top of each panel [A; derived from A/Aichi/2/68 (H3N2), B; derived from A/chick/Penn/1370/83 (H5N2), C; derived from A/FPV/Rostock/34 (H7N1)], were incubated with or without MSPL for 1 hour at 37°C or furin for 8 hours at 37°C. After incubation, the samples were separated by reverse-phase-high-performance liquid chromatography. The intensity of the separated peaks, monitored by absorbance at 215 nm (AU, arbitrary unit), was recorded. Peaks in C with retention times of 15·2 and 15·5 minutes were identified as the N-terminal 1–7 residue peptide and the N-terminal 1–10 residue peptide, respectively.

Kong/156/97 (H5N1) with R-K-K-R motif.[1] However, the H5 HA peptide with K at position P4 (Figure 1B) was hardly cleaved by furin under the same experimental conditions. TMPRSS13 showed similar results (data not shown). These findings suggest that MSPL and TMPRSS13 cover diverse cleavage specificities, including non-susceptible specificity to furin.

Full length recombinant HA of HPAI virus with KKKR cleavage motif was converted to mature HA subunits with membrane-fused giant cell formation in MSPL or TMPRSS13 transfectant cells.[7]

Role of MSPL/TMPRSS13 in live HPAI virus infection and multiplication

To confirm the involvement of MSPL/TMPRSS13 in HPAI HA cleavage and infection, ECV304-WT and ECV304-MSPL cells were infected with the HPAI virus A/Crow/Kyoto/53/2004 (H5N1) (HA: N'-R-R-K-K-R↓G -C') and the genetically modified mutant recombinant virus (HA: N'-R-K-K-K-R↓G -C') (Figure 2). Although HA processing of mutant recombinant virus (N'-K-K-K-R-C') in ECV-WT cells was hardly detected, conversion of HA0 to the mature form was detected in ECV-MSPL cells. In addition, this conversion was suppressed by Bowman-Birk trypsin inhibitor, a membrane non-permeable high-molecular mass inhibitor against MSPL/TMPRSS13. To test for the generation of infective virus, the conditioned media of 1-day culture of ECV304-WT and ECV304-MSPL cells infected with WT and mutant HPAI H5N1 viruses were inoculated into newly prepared cells and cultured for 24 hours. Although spreading of WT virus infection with HA cleavage motif of R-K-K-R was detected from the conditioned medium of both ECV304-WT and ECV304-MSPL cells, that of mutant virus with HA cleavage motif of K-K-K-R was only detected from the condition medium of ECV304-MSPL cells. These results strongly suggest that the expression of MSPL, but not furin, potentiates multicycles of HPAI virus with K-K-K-R HA cleavage motif.

Discussion

Seasonal human influenza A virus HAs have consensus monobasic cleavage site sequence, N'-Q/E-X-R↓-G-C', and all HPAI virus HAs have two types of cleavage site sequences with multiple basic amino acids, N'-R-K/R-K/R/X-R↓-G-C' with R at position P4 in a large number of HPAI viruses and N'-K-K/R-K/T-R↓-G-C' with K at position P4 in a small number of HPAI viruses. Figure 1 shows furin efficiently cleaved synthetic HPAI A/Hong Kong/156/97 (H5N1) HA cleavage site peptide with the R-K-K-R motif, but hardly cleaved the HPAI virus

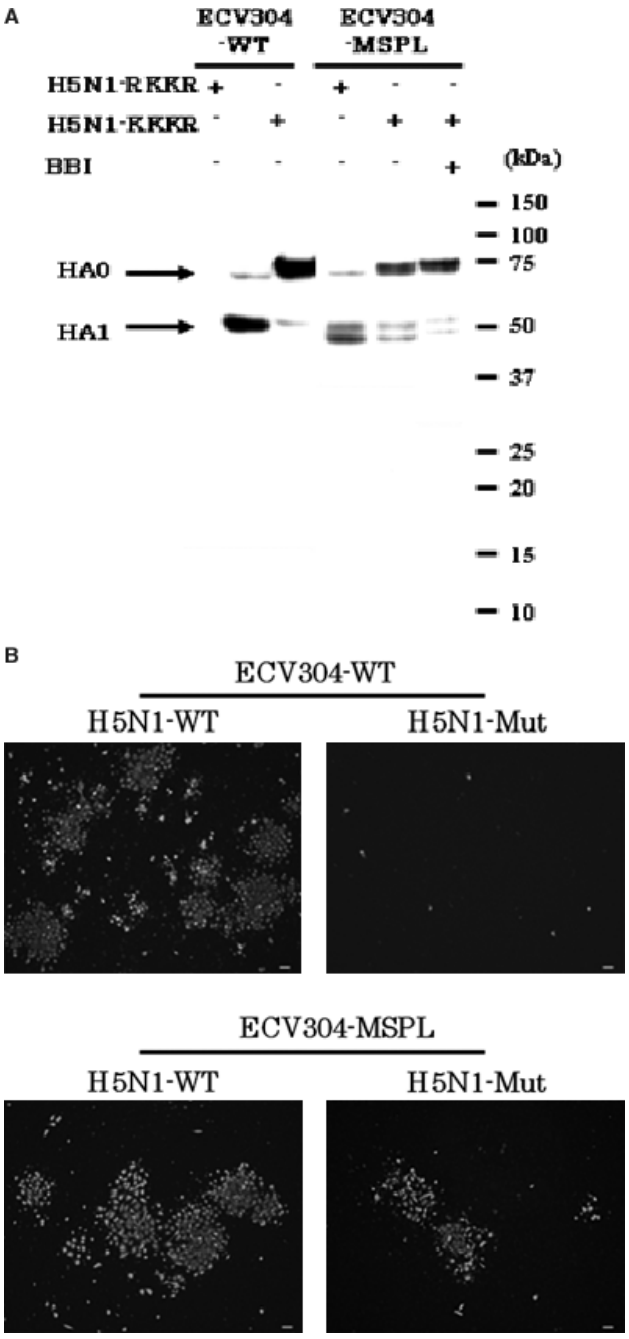

Figure 2. Cleavage and multiple infections of highly pathogenic avian influenza (HPAI) WT and mutant viruses A/Crow/Kyoto/53/2004 (H5N1) by MSPL. (A) ECV304-MSPL and ECV304-WT cells were infected with HPAI virus A/Crow/Kyoto/53/2004 (H5N1) (H5N1-WT) and genetically-modified recombinant virus (H5N1-Mut) for 24 hours, and cell lysates were subjected to SDS-PAGE and analyzed by western immunoblotting with anti-H5N1 monoclonal antibody (4C12). Molecular weight markers are on the right. (B) Immunofluorescence assay was performed as described previously.[10] Wild-type virus infectivity for ECV304-WT cells, mutant recombinant virus infectivity for ECV304-WT cells (top), wild-type virus infectivity for ECV304-MSPL cells, and mutant recombinant virus infectivity for ECV304-MSPL cells (bottom). Bar, 50 μm.

A/chick/Penn/1370/83 HA cleavage site peptide with the K-K-K-R motif. Furthermore, cleavage of the full-length HA of HPAI virus with R-K-K-R motif was detected, but cleavage of HPAI virus HA with K-K-K-R motif was hardly detected in ECV304-WT cells containing furin (Figure 2). These substrate specificities of furin suggest that proteases other than furin and PC5/6 play a role in the processing of HAs of HPAI virus with K-K/R-K/T-R cleavage motif.

MSPL and TMPRSS13 show unique cleavage site specificities of the double basic residues at the cleavage site, and R or K at position P4 greatly enhanced the efficiency, which none of the other TTSPs have shown similar substrate specificities so far. Furthermore, infectious and multicycle viral replication along with HA processing was also noted in genetically modified mutant recombinant live HPAI virus A/Crow/Kyoto/53/2004 (H5N1) with K-K-K-R cleavage motif in ECV304-MSPL cells (Figure 2). These results were supported by the data of two cleaved peptides by MSPL in Figure 1C. These findings suggest that MSPL has diverse cleavage specificities and may cleave HA at least two sites, although multiplicity of the mutant HPAI virus was observed under the conditions. These results also suggest that MSPL and TMPRSS13 in the membrane might potently activate the HA membrane fusion activity of HPAI viruses and promote their spread.

Highly pathogenic avian influenza viruses replicate in various organs in birds, and the HA processing proteases might be widely distributed in these organs. Indeed, TMPRSS13 and MSPL are ubiquitously expressed in almost all human organs tested and are highly expressed in lungs, leukocytes, pancreas, spleen, and placenta.[4,5] In addition, MSPL and TMPRSS13 are strictly localized in the plasma membranes, suggesting that proteolytic activation of HPAI virus HA occurs not only through the *trans*-Golgi network by furin and PC5/6, but also on the cell surface by MSPL and TMPRSS13.

Acknowledgements

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Pro-inflammatory effects of H5N1 and 20th century pandemic influenza virus PB1-F2 proteins are mediated by macrophages

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Keywords Apoptosis, inflammation, macrophage, pandemic, PB1-F2.

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Abstract

Understanding molecular signatures of virulence of influenza A viruses (IAVs) is important in the context of pandemic planning. The IAV protein PB1-F2 enhances the pathogenesis of both primary viral and secondary bacterial infections. We hypothesized that macrophage mediated effects of the IAV PB1-F2 protein were important for increased pathogenicity of pandemic viruses. We demonstrate that PB1-F2 proteins from all three pandemic strains from the 20th century, as well as an H5N1 strain, increase the numbers of macrophages in the lung, enhancing inflammation and pathology. Recently circulating seasonal IAV strains have lost this immunostimulatory activity through truncation or mutation of the PB1-F2. Of the proteins tested, only the PB1-F2 from the laboratory PR8 strain induced cell death. We conclude that enhancement of virulence of pandemic strains by PB1-F2 is mediated by inflammation, not apoptosis. Since the novel H1N1 pandemic virus does not encode a full-length PB1-F2, acquisition of a functional, pro-inflammatory PB1-F2 by reassortment poses a threat for greatly enhanced virulence in the future.

Introduction

The PB1-F2 protein, which is translated from the +1 reading frame of the PB1 gene segment, has been linked to the pathogenesis of both primary viral and secondary bacterial infections in a mouse model.[1–3] A mitochondrial targeting sequence is located in the C-terminal portion of the PB1-F2 open reading frame, and expression of full length PB1-F2 has been associated with mitochondrial targeting and apoptosis in a monocyte dependent manner.[1,4] It has been theorized that enhanced virulence could result from mitochondrial disruption with subsequent cell death mediated by PB1-F2.[4,5] A suggested second function of the PB1-F2 protein is that it enhances immunopathology by triggering the inflammatory response.[3,6] In earlier studies from our group, the pro-inflammatory phenotype was markedly upregulated when the PB1-F2 from the 1918 pandemic strain was expressed, arguing that this protein may be an important virulence factor for highly pathogenic pandemic viruses.[3,6]

In this report we analyze the PB1-F2 protein's contribution to pathogenesis in a mouse model, examining both inflammation and cell death. PB1-F2 proteins from a variety of epidemiologically important IAV strains including all pandemic strains from the 20th century, a highly pathogenic avian influenza virus of the H5N1 subtype, and representative seasonal strains were utilized to determine the relevance to pandemic disease. We demonstrate that macrophage mediated immunopathology, but not apoptosis, are relevant functions of PB1-F2 proteins from past or potential pandemic influenza viruses.

Materials and methods

Peptide generation

Using the predicted amino acid sequences of the PB1-F2 proteins from PR8, A/Brevig Mission/1/1918, 1/Singapore/1/1957, A/Hong Kong/1/1968, A/Wuhan/359/1995, and A/Vietnam/1203/2004, peptides from the C-terminal end were synthesized as described.[3] An additional N-terminal peptide was synthesized from the PR8 sequence as a positive control (MGQEQDTPWILSTGHISTQK) as described.[3]

Generation of plasmids and viruses

A panel of viruses were reverse engineered as described [7,8] and included laboratory strain PR8, a virus unable to express PB1-F2 (ΔPB1-F2/PR8), or expressing the PB1-F2 of the 1918 pandemic strain (1918 PB1-F2/PR8) or the truncated 1956 H1N1 strain (Beij PB1-F2/PR8).[3,7] In addition, 7:1 reassortants encoding PB1 gene segments from a

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2004 highly pathogenic avian influenza of the H5N1 subtype (H5N1 PB1/PR8), or from a 1995 human H3N2 strain (H3N2 PB1/PR8) were utilized along with their isogenic deletion mutants for PB1-F2 (H5N1 ΔPB1-F2/PR8 and H3N2 ΔPB1-F2/PR8).

Cell lines and cell death assays

RAW264.7 cells were grown under conditions as described.[9] Cells were infected with one multiplicity of infection (MOI) of virus for 2–12 hours, or exposed to 50 μm (final concentration) of peptides derived from the C-terminal portion of PB1-F2 for 1 hour. Cells from the supernatant and monolayers were harvested, washed, and stained with Annexin (APC) and Propidium Iodide (PI) (Becton Dickinson, San Jose, CA, USA), then analysed for cell death as described.[3]

Animal model

Six- to eight week old female Balb/cJ mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained in a Biosafety Level 2 facility in the Animal Resource Center and procedures approved by the Animal Care and Use Committee at SJCRH. Infectious agents and peptides were diluted in sterile PBS and administered intranasally to anesthetized mice (n = 6–10) in a volume of 100 μl (50 μl per nare) and monitored for overt signs of illness and weight loss daily. Following euthanasia by CO_2 inhalation, the trachea was exposed and cannulated with a 21 gauge plastic catheter (BD Insyte; Becton Dickinson, Sandy, UT, USA). Bronchoalveolar lavage fluid (BALF) was collected, red blood cell depleted, and cellular content analyzed via flow cytometry as described.[3]

Statistical analysis

One way analysis of variance (anova) was used for multiple comparisons of cell death and cellularity of BALF. A P-value of <0·05 was considered significant for these comparisons. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was utilized for all statistical analyses.

Results

PB1-F2 enhances the influx of macrophages into BALF

To assess the contribution of PB1-F2 to inflammation, we utilized a panel of previously described reverse engineered viruses in the mouse infection model.[3,7] The effect of PB1-F2 expression was observed clearly in the inflammatory infiltrate in response to infection in the lungs. Deleting PB1-F2 from PR8 or expression of the C-terminally truncated Beij PB1-F2 had a significantly reduced influx of

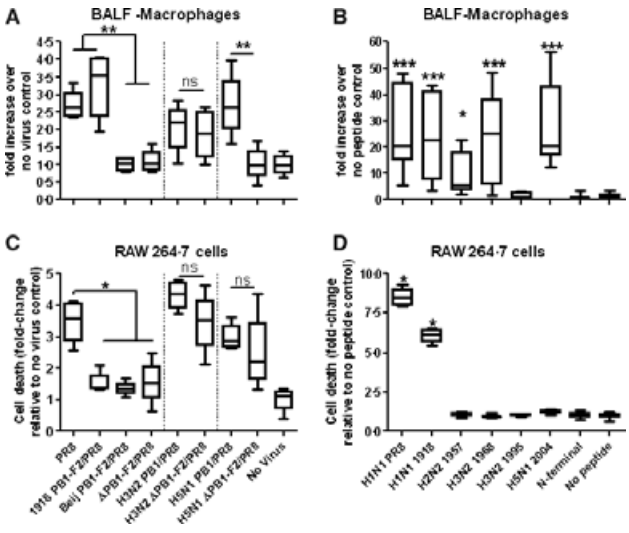

Figure 1. PB1-F2 enhances inflammation in the lung. (A) Mice (n = 6–10) were infected with 100TCID$_{50}$ of the panel of viruses and euthanized 72 hours post-infection. BALF from the animals was assayed for the mean number of macrophages and compared to unchallenged controls.[10] Asterisks (**) indicates a significant difference compared to the deletion or truncation mutant of PB1-F2 and no virus control. (B) Mice (n = 6–10) were exposed to the panel of peptides (100 mm final concentration) and euthanized 24 hours later and BALF analysed. An asterisk (*) indicates a significant difference compared to the 1995 H3N2 peptide and controls by anova ($P < 0.05$). A double asterisk (**) indicates a significant difference compared to the controls by anova ($P < 0.01$). (C and D) Induction of cell death was determined in mouse macrophage RAW264.7 cell lines by detection of Annexin$^+$PI$^+$ events after (C) 8 hours post-infection with the panel of viruses at 1.0 multiplicity of infection; and (D) 1 hour exposure to the panel of 27-mer C-terminal peptides of the PB1-F2 protein, a N-terminal 27-mer peptide derived from PR8 (final concentration 50 μm for each peptide), or no peptide. An asterisk (*) indicates a significant increase compared to (C) all viruses with a PB1 gene segment derived from an H1N1 virus and the no virus control; and (D) all peptides and controls except PR8 and 1918, by anova ($P < 0.05$). Error bars indicate the standard deviation from the mean.

macrophages (Figure 1A). Expression of the 1918 PB1-F2 caused similar inflammatory effects as the PR8 virus. Disruption of PB1-F2 expression the virus containing the H5N1 PB1 gene segment in a PR8 background also significantly decreased the inflammatory response compared to the virus maintaining the ability to express full length PB1-F2 (Figure 1A). However, no differences were seen that could be attributed to the 1995 H3N2 derived PB1-F2.

The lungs of mice infected with the panel of PB1-F2 variant viruses were examined at 72 hours. Pathologic changes typical of PR8 viral infection were observed in all lungs. These typical findings included perivascular inflammation, airway necrosis, hemorrhage, and deposition of cellular debris (Figure 2). In the lungs of mice infected with PR8 or 1918 PB1-F2/PR8, however, significantly more perivascular cuffing was noted, with a prominent increase in numbers of macrophages (Figure 2A, C). The overall number of

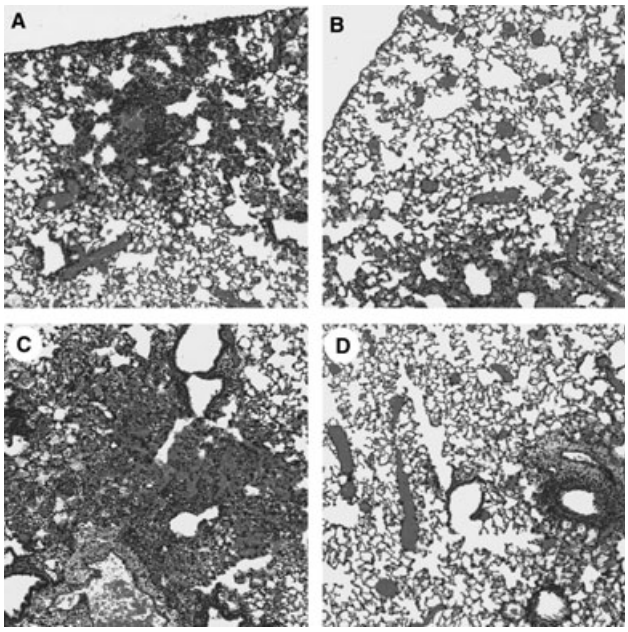

Figure 2. Mice (n = 6–10) were infected with 100$TCID_{50}$ of the panel of viruses and euthanized 72 hours post-infection. Whole lungs were removed, sufflated, sectioned, stained with hematoxylin and eosin, and examined for histopathology. Panels show representative sections of tissue taken at 20 × magnification of mice infected with (A) PR8, (B) ΔPB1-F2/PR8, (C) 1918 PB1-F2/PR8, and (D) Beij PB1-F2/PR8.

inflammatory cells throughout the lungs, including both airways and alveoli, was quantitatively greater in these mice than in mice infected with ΔPB1-F2/PR8 or Beij PB1-F2/PR8 (Figure 2B, D).

As the function and influence of PB1-F2 protein on normal viral function is not currently understood, and given the abrogation of enhanced inflammation induced by the truncated PB1-F2 Beij/PR8 virus, we sought to elucidate whether the C-terminal domain of PB1-F2 could alone induce this inflammatory response. Mice were exposed to a panel of peptides and were euthanized 24 hours later for collection of BALF. Significant influxes of macrophages into the BALF were seen following exposure to C-terminal PB1-F2 peptides derived from PR8, the pandemic strains from 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2), and the 2004 H5N1 virus compared to controls (Figure 1B). Similar effects were not seen with the peptide derived from a more recent H3N2 strain, A/Wuhan/359/1995. When peptide exposed mice were followed for morbidity for 7 days, peptides proven to induce a heightened inflammatory response correlated strongly with overt clinical signs of illness (data not shown). Thus, the ability to cause lung inflammation appears to be a property of PB1-F2 proteins of viruses containing PB1 gene segments reassorted directly from the avian reservoir.

PB1-F2 induction of cell death is virus strain specific

The PB1-F2 protein may contribute to virulence by rendering the host cellular immune response ineffective through inducing apoptosis.[5] We sought to determine whether this was an epidemiologically important function for combating the host immune response to infection by testing the ability of PB1-F2 proteins from several different IAV strains to cause cell death. We therefore infected RAW264.7 cells with the panel of recombinant viruses at an MOI of 1 for 2–12 hours. As has been demonstrated previously,[1,4,5] PR8 virus induces significant cell death compared to uninfected controls (Figure 1C). When RAW264.7 cells were infected with PR8 virus, necrotic death peaked 8 hours after infection. Viruses lacking the C-terminal portion of PB1-F2, including the ΔPB1-F2/PR8 and the Beij PB1-F2/PR8 were unable to cause cell death (Figure 1C). In addition, expression of the 1918 PB1-F2 also did not cause significant increases in cell death over controls. Expression of PB1-F2 or deletion of PB1-F2 in either an H3N2 or H5N1 PB1 gene segment background similarly did not alter the cell death phenotype.

To examine additional strains for which we did not have isogenic virus pairs, we next exposed the BalbcJ mouse derived macrophage cell line RAW264.7 to the panel of PB1-F2 peptides derived from PR8, the pandemic strains from 1918 (H1N1), 1957 (H2N2) and 1968 (H3N2), and the 2004 H5N1 for 1 hours. Cell death in RAW264.7 cells was caused only by the peptides derived from the laboratory strain PR8 and the peptide derived from the 1918 pandemic strain (Figure 1D). Viability was not affected by exposure of RAW264.7 to peptides derived from other virus strains. We conclude from these data that the mechanism by which PB1-F2 contributes to the pathogenicity of pandemic influenza is unlikely to be through its reported ability to cause cell death.

Discussion

These data presented here demonstrate that the lung inflammatory response is enhanced by the influenza A virus PB1-F2 protein in a mouse model. This inflammatory response was characterized by increased cellular infiltration of macrophages into the interstitial and alveolar spaces of the lungs, as well as enhanced perivascular inflammation, airway necrosis, hemorrhage, and deposition of cellular debris. This augmentation was shown to be induced by PB1-F2 proteins only from those strains contributing to the formation of all pandemic strains of the 20th century and from the currently circulating, highly virulent H5N1 strains that constitute an imminent pandemic threat. The IAV H1N1 strains circulating in humans since around 1950

code for a truncated PB1-F2. These viruses may lack the C-terminal residues responsible for the inflammatory effects demonstrated in this publication. Additionally, recently circulating H3N2 strains, in contrast to their pandemic forbear from 1968, have lost the capacity to cause PB1-F2 mediated inflammation through mutation of the C-terminus of this protein.

In 2009 a novel H1N1 IAV emerged from an animal reservoir and caused a human pandemic. Disease burden from this strain has been considered mild [10] in contrast to the three pandemics of the 20th century.[11] The reasons for this disparity in pathogenesis are unclear. An examination of the origins of the three 20th century pandemics shows that only 2 the hemagglutinin (HA) and PB1 gene segments were reassorted directly from the avian reservoir in every case, suggesting gene products of one or both of these may be important.[12] The HA surface glycoprotein provided the antigenic novelty required for the each virus to achieve pandemic status. However, the significance of inclusion of a novel PB1 gene segment in each of the 20th century pandemics is not yet understood. We show here that the PB1-F2 of these pandemic strains contributes to virulence through induction of inflammatory responses. Thus PB1-F2 may serve as a marker of the pathogenicity of pandemic strains. Since the 2009 H1N1 strain codes a truncated PB1-F2 of only 11 predicted amino acids, the lack of PB1-F2 mediated inflammation may account in part for its relatively lower virulence.[13,14]

Of the panel of PB1-F2 proteins studied, only that from the laboratory strain PR8 was capable of rendering responding host-immune cells ineffective by induction of cell death. We therefore hypothesize that molecular signatures specific to induction of apoptosis may have been lost through genetic mutation of the PB1-F2 gene throughout the evolution of the IAVs. Our findings suggest that this apoptotic function is unlikely to be important for the virulence of any of the known pandemics. Rather, the inflammatory phenotype appears to be the dominant contribution of PB1-F2 to pandemic disease.

Acknowledgements

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Influenza virus-cytokine-protease cycles are principal mechanisms of multi-organ failure in severe influenza and therapeutic approaches

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Keywords Hemagglutinin-processing protease, influenza A virus, influenza-associated encephalopathy, matrix metalloprotease, multi-organ failure, pro-inflammatory cytokines, trypsin.

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Abstract

Severe influenza is characterized by cytokine storm and multi-organ failure (MOF). However, the relationship amongst factors, such as cytokines, hyper-vascular permeability, host-cellular factors, and metabolic disorders in severe influenza remain unclear. The aim of the present study was to define the pathogenic impact of cytokine storm in influenza A virus (IAV) infection, and the effects of transcriptional inhibitors on cytokine, trypsin, and matrix metalloprotease (MMP-9) expressions and viral replication were determined. Since up-regulation of these cytokines and host-cellular proteases is associated with activation of transcription factors nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1), we treated mice with transcriptional inhibitors of NF-κB [pyrrolidine dithiocarbamate (PDTC), N-acetyl-L-cysteine (NAC)] and AP-1 [nordihydroguaiaretic (NDGA)]. Here, we report that treatment with PDTC, NAC, and NDGA suppressed viral multiplication and induction of cytokines, trypsin, and MMP-9, with improved animal survival.

Introduction

Influenza A virus is the most common infectious pathogen in humans, causing significant morbidity and mortality, particularly in infants and the elderly. MOF with severe edema is observed in the advanced stage of influenza pneumonia.[1] However, the relationships amongst factors that induce vascular hyper-permeability in severe influenza remain unclear. It is reported that significant increases in levels of pro-inflammatory cytokine levels, such as TNF-α, IL-6, and IL-1β, affect host survival both positively and negatively.[2] The inflammatory response affects cell adhesion, permeability, apoptosis, and mitochondrial reactive oxygen species, potentially resulting in vascular dysfunction and MOF.[3] In addition, IAV infection up-regulates several cellular proteases including ectopic trypsin[4] and MMP-9.[5] Up-regulated ectopic trypsin mediates the post-translational proteolytic cleavage of viral envelope hemagglutinin (HA),[6] which is crucial for viral entry and replication[7] and the subsequent tissue damage in various organs.[8]

The aim of the this study was to define the pathogenic impact of cytokine storm in IAV infection and the molecular mechanisms by which pro-inflammatory cytokines and proteases cause vascular dysfunction in animal model.

Materials and methods

Weanling female mice aged 3 weeks (C57BL/6CrSlc) were infected with IAV/WSN/33 (250 PFU) with and without treatment of PDTC (2.5 mg/kg), NAC (10 mg/kg), and NDGA (10 mg/kg). These inhibitors were administrated once daily for 4 days after infection. The levels of cytokines in tissue homogenates were measured by ELISA kits. The effect of inhibitors on viral replications was determined by real-time PCR. Gelatin zymography and Western blotting were conducted as reported previously.[9]

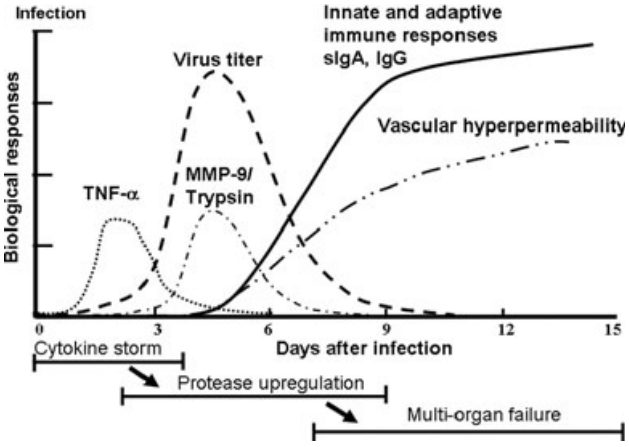

Figure 1. Schematic diagram of host cellular responses in the mouse airway after influenza A virus infection.

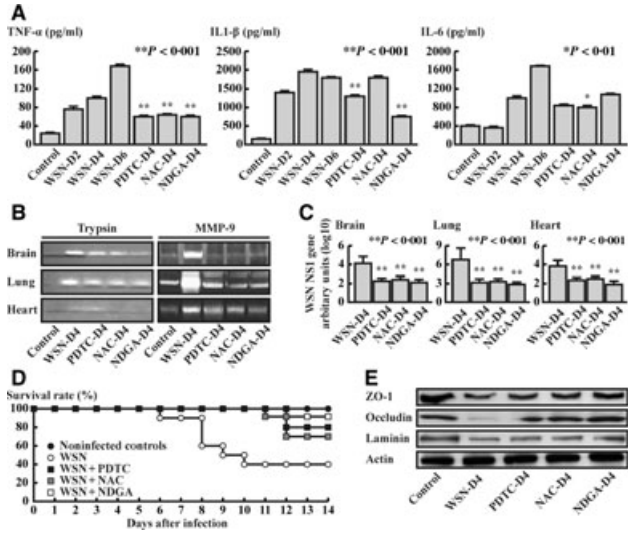

Figure 2. Suppression of influenza A virus -induced upregulated cytokines, trypsin, matrix metalloprotease (MMP-9) and viral RNA replication by inhibitors of nuclear factor-kappa B and AP-1. (A) Mice were infected with 250 PFU of WSN virus with and without treatment with pyrrolidine dithiocarbamate (PDTC), N-acetyl-L-cysteine (NAC), and nordihydroguaiaretic (NDGA). Levels of TNF-α, IL-6, and IL-1β in lung homogenates ($n = 3$) were analyzed before (Control) and at day 2 (WSN-D2), day 4 (WSN-D4), and day 6 (WSN-D6) post-infection. PDTC, NAC, and NDGA were treated once daily for 4 days. (B) Trypsin and MMP-9 activities analyzed by gelatin zymography. (C) Quantitative analysis of viral NS1 RNA copies normalized by β-actin at day 4 post-infection conducted by real-time PCR ($n = 3$). (D) Each group ($n = 10$) of animals infected with WSN were treated with PDTC, NAC, and NDGA once daily for 4 days and the survival rates of the different groups were compared. (E) Loss of tight-junction proteins, zonula occludens-1, occludin, and laminin in the brain were analyzed by Western blot and its restoration by PDTC, NAC, and NDGA treatments. The levels before infection are shown as control.

Results

Host cellular responses in the airway after IAV infection

Figure 1 shows schematic view of typical biological responses in the airway of mice after IAV infection. An initial response before viral proliferation is significant increases in pro-inflammatory cytokine levels. Immediately after cytokine inductions, there is a marked up-regulation of ectopic trypsin along with an increase in virus titer in the airway, lung, and brain.[4] Ectopic trypsin mediates the post-translational proteolytic cleavage of IAV HA, which is crucial for viral entry and replication and the subsequent tissue damage in various organs. We also found that IAV infection markedly induces MMP-9 and matrix degradation.[5] Just after the peak of viral proliferation, the innate and adaptive immune responses of protective immunity are induced for defense and recovery, or oppositely on rare occasions, MOF with vascular hyper-permeability is started into the advanced stage of influenza.

Suppression of IAV-induced upregulated cytokines, trypsin, MMP-9, and viral RNA replication by inhibitors of NF-κB and AP-1

The levels of TNF-α and IL-6 in the lungs were increased persistently for 6 days after IAV WSN infection, and that of IL-1β peaked at days 4–6 post-infection (Figure 2A). Since these cytokine responses are associated with activation of NF-κB and AP-1, we treated mice once daily for 4 days with anti-oxidant inhibitors: PDTC and NAC against NF-κB activation, and NDGA against AP-1 activation. PDTC and NDGA significantly suppressed the up-regulation of TNF-α and IL-1β ($P < 0.001$), and NAC suppressed TNF-α ($P < 0.001$), and IL-6 ($P < 0.01$) at day 4 post-infection. Gelatin zymography showed up-regulation of ectopic trypsin and MMP-9 in mice lung, brain, and heart during infection for 4 days (Figure 2B). Trypsin and MMP-9 induction was inhibited by treatment with PDTC, NAC, and NDGA, probably via blockade of NF-κB and AP-1 binding in the promoter region of the genes. Viral RNA replication in various organs at day 4 post-infection was suppressed by more than one order of magnitude by PDTC, NAC, and NDGA (Figure 2C). Suppression of viral multiplication and induction of cellular factors by PDTC, NAC, and NDGA, significantly improved the survival of mice at day 14 post-infection, the late stage of infection (Figure 2D). To elucidate the mechanisms underlying brain vascular dysfunction of influenza-associated encephalopathy, changes in the levels of tight-junction proteins, intracellular zonula occludens-1 (ZO-1) and transmembrane occludin, and the matrix protein laminin, were analyzed by western blotting. Marked reductions in the expression levels of tight-junction constituents were detected at day 4 post-infection, which were partly rescued by PDTC, NAC, or NDGA (Figure 2E).[9] No other tight-junction protein, claudin-5 or matrix fibronectin and type IV collagen, were affected.

Discussion

The present study reports several new observations: (i) proinflammatory cytokines, TNF-α, IL-1β, and IL-6, when up-regulated by IAV infection, induce trypsin and MMP-9 expression in various organs in mice; (ii) inhibitors of NF-κB and AP-1 effectively suppress the up-regulation of pro-inflammatory cytokines, trypsin, and MMP-9 and improve survival rates of infected mice. Based on these results, we propose the 'influenza virus-cytokine-protease cycle' hypothesis as one of the mechanisms of vascular dysfunction in MOF with cytokine storm in severe influenza and influenza-associated encephalopathy.[9]

The significance of pro-inflammatory hyper-cytokinemia, or 'cytokine storm,' in the pathogenesis of IAV infection remains unclear. On the positive effects, cytokines promote

lymphocyte activation and infiltration at the sites of infection and exert direct antiviral effects. However, on the negative effects of excess cytokines, the hyper inflammatory process evoked by viral infection may become harmful through intracellular activation of NF-κB, AP-1, and the Janus kinase-signal transducers and activators of transcription signaling pathways.[3,10–12] The *in vivo* experiments presented here showed that NF-κB and AP-1 inhibitors markedly suppress the expression of cytokines, trypsin, MMP-9, and viral replication, resulting in a significant increase in the survival of infected mice. Furthermore, cytokines interact with mitochondria to increase the production of reactive oxygen species, resulting in the production/activation of vasodilatory mediators such as nitric oxide and bradykinin, and subsequent endothelial dysfunction and edema in various organs.[3]

The molecular mechanisms underlying tight-junction disruption in endothelial cells and vascular hyper-permeability following the 'cytokine storm' remain unclear. TNF-α up-regulation alters the cellular redox state, reduces the expression of four complex I subunits by increasing mitochondrial O_2^- production and depleting ATP synthesis, decreases oxygen consumption thereby resulting in mitochondrial damage,[3,13] and increases $[Ca^{2+}]_i$[14] ATP depletion dissociates ZO-1 from the actin cytoskeleton and thereby increases junctional permeability.[15]

Endothelial dysfunction induced by 'influenza virus-cytokine-protease cycle' in the early stage of severe influenza may further affect various circulating factors, coagulation factors and complement systems, and vascular interacting cells, such as neutrophils, macrophages and lymphocytes. MOF is the final outcome of metabolic and mitochondrial fuel disorder, immunosuppression, endocrine disorder, and tissue injury followed by endothelial dysfunction in many organs. Another key pathway of acute lung injury in the highly pathogenic avian influenza virus H5N1and acute respiratory syndrome-corona virus infection reported recently involves oxidative stress and formation of oxidized phospholipids, which induce lung injury via Toll-like receptor 4 signaling pathway.[16] In addition to these data, up-regulated trypsin and pro-inflammatory cytokines may also affect tissue destruction and immunosuppression in the late stage of IAV infection. Further studies are required on the role of the 'influenza virus-cytokine-protease cycle' in the pathogenesis of MOF, particularly in the late stage of viral infection.

Acknowledgements

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Replication and innate host response of influenza A virus in lung microvascular endothelial cells: new insights into systemic infection and pathogenesis

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Keywords Avian H5N1 virus, cytokine, human seasonal H1N1 virus, microvascular endothelial cells, pandemic H1N1pdm virus, polarity, systematic infection.

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Introduction

Though influenza A virus replication kinetics and host responses have been previously studied in umbilical vein endothelial cell or transformed endothelial cell lines, the tropism of influenza A virus including H5N1 and pandemic H1N1pdm for primary human lung microvascular endothelial cell has not been well defined.[1] In this study we employed primary human lung microvascular endothelial cells, which are more physiologically relevant for understanding pathogenesis of influenza in the lung as to obtain a better understanding of the links of endothelial cell infection to systematic virus dissemination and multiple organ involvement in severe human influenza.

Materials and methods

Human swine-origin pandemic H1N1pdm virus (A/HK/415742/09), human seasonal influenza H1N1 virus (A/HK/54/98), human seasonal influenza H3N2 virus (A/OK/1992/05), and two virus isolates of highly pathogenic avian influenza (HPAI) H5N1 subtype (A/HK/483/97 and A/VN/3046/04) were studied. Primary human lung microvascular endothelial cells were maintained in endothelial cell growth-medium with antibiotics. Endothelial cells were infected at a multiplicity of infection (MOI) of 0·01 and 2 at 37°C for 1 hour. Cells were washed twice with PBS and replenished with endothelial cell serum-free medium with 5 ng/ml of TPCK-trypsin. At 3, 6, 8, and 24 hours post infection, the cell free supernatant was collected for virus titration in cells infected at MOI = 0·01. Supernatants of cells infected at MOI of two were collected for cytokine protein assays, and total RNA was extracted for gene expression analysis using qPCR.

Results

We found that seasonal influenza H1N1 and H3N2 viruses initiated viral gene transcription and viral protein expres-

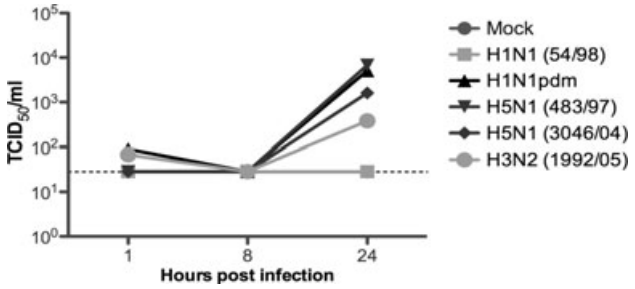

Figure 1. Replication kinetic of influenza A viruses in human lung microvascular endothelial cells *in vitro* by virus titration expressed in $TCID_{50}$/ml. The chart shows the mean of the virus titer pooled from two independent experiments.

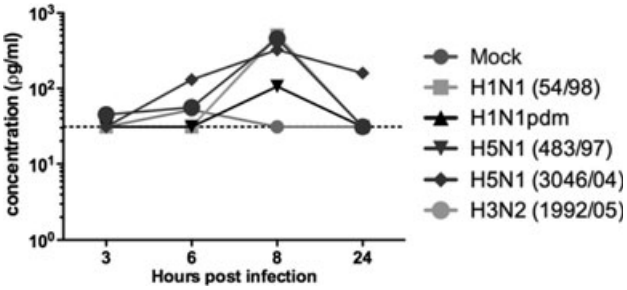

Figure 2. Cytokine protein expression in human lung microvascular endothelial cells after influenza A virus infection. IP-10 protein secretion in lung microvascular endothelial cells infected with influenza A viruses at1, 8, and 24 h post infection. The graph shows the mean from two independent experiments.

sion, but did not produce infectious progeny, while the highly pathogenic avian influenza H5N1 and the pandemic influenza H1N1pdm virus could replicate even with the absence of exogenous protease (Figure 1). Furthermore, when compared to seasonal H1N1 and H3N2, the H5N1 virus was a more potent inducer of cytokine and chemokine including IFN-β, MCP-1, RANTES, IP-10 (Figure 2), and IL-6, in virus infected endothelial cells, whereas H1N1pdm induced intermediate levels of cytokine and chemokine.

Discussion

Avian influenza H5N1 and pandemic H1N1pdm virus (but not the seasonal H1N1 and H3N2 virus) can productively replicate in human lung microvascular endothelial cells. This is likely to be of relevant to pathogenesis and provides a possible explanation for the extra-pulmonary infection seen in animal infection models. This extra-pulmonary spread may support the previous speculation and anecdotal evidence that H5N1 and H1N1pdm virus can infect the gastrointestinal tract through the virus dissemination from the infected respiratory tract as the first target cells for influenza infection.[2–4] In addition, the release of proinflammatory cytokine and chemokine induced by influenza H5N1 and H1N1pdm virus infection in lung microvascular endothelial cells may be important contributors to the pathogenesis of severe human influenza disease leading to endothelial cell dysfunction that contributes to severe pulmonary disease symptoms.

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Identification of human annexin A6 as a novel cellular interactant of influenza A M2 protein: implications for influenza life cycle

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Background

During its replication, influenza virus utilizes the host cellular machinery for many aspects of its life cycle. Characterization of such virus-host protein-protein interactions is a must to identify determinants of pathogenesis. The M2 ion channel protein plays a crucial role during the entry and late stages of the viral life cycle where its C-terminal domain, well conserved among influenza A viruses, is accessible to cellular machinery after fusion with endosomal membrane and during its trafficking along the secretory pathway prior to assembly and budding.[1] The aim of the study is to identify cellular interactants of M2 that play important regulatory roles during influenza infection.

Materials and methods

To identify cellular partners of M2 we performed a genome-wide yeast-two-hybrid (Y2H) screening approach[2] using the cytosolic domain of M2 as bait and a human placenta random primed cDNA library as prey and tested more than 60 million interactions.

Results

From the Y2H screening, an interesting interaction with the human annexin A6 (ANXA6) protein,[3] a member of annexin family proteins that binds to phospholipds in a Ca^{2+}-dependent manner, was identified. Co-immunopre-

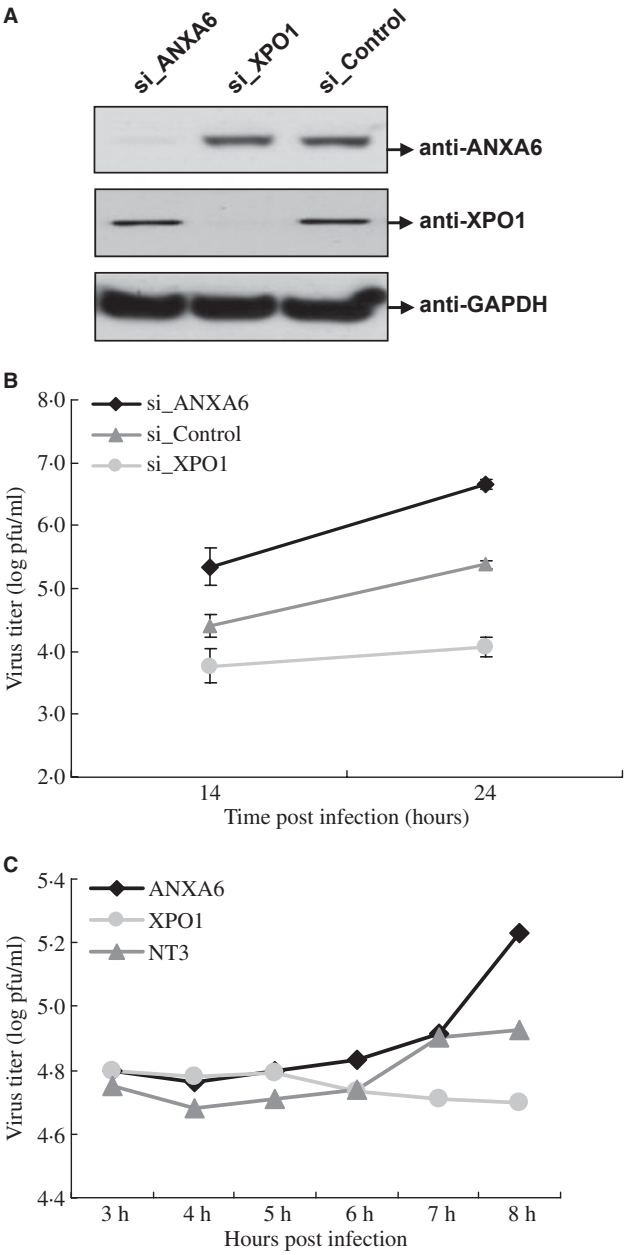

Figure 1. The knockdown of ANXA6 increases the propagation of influenza virus in the multi-cycle and single cycle growth kinetics study. (A) A549 cells were transfected with ANXA6 specific siRNA (si_ANXA6) for 60 hours, the siRNA for exportin-1 (si_XPO1) was used as positive control,[4] while the non-specific siRNA (si_Control) was used as experiment control. (B)The knockdown efficiency of the above siRNA was checked by western blot. After 60 hours post transfection, the cells were infected with A/WSN/33/H1N1 influenza virus at 0·1 MOI, and the supernatant was collected for virus titration by plaque assay at 14 hours and 24 hours post infection to study the effect of ANXA6 knockdown on the multi-cycle replication of influenza virus. (C) For single cycle study, the siRNA treated cells were infected with this virus at 5 MOI and the supernatant was collected every 1 hour from 3 hours post infection to 8 hours post infection and the virus titer was determined by plaque assay.

cipitation of myc-tagged ANXA6 and viral M2 proteins co-expressed in HEK293T cells after transfection and infection confirmed the direct interaction between ANXA6 and M2. We further investigated whether this interaction had any functional significance with regards to influenza life cycle. Using a RNA interference strategy to silence the ANXA6 gene in human lung epithelial A549 cells, we observed increased progeny virus titers either in a single or multiple viral growth kinetics study, suggesting a negative regulatory role for ANAX6 during viral infection (Figure 1).

Conclusions

A novel interaction between M2 and ANXA6 was identified. More functional studies are in progress to define precisely the potential negative regulatory role of this interaction during viral infection. A systematic dissection of the viral life cycle will be performed to identify the step(s) affected by the ANXA6 cellular factor using specific assays such as real-time quantitative RT-PCR in a single or multiple viral growth kinetics study, cell transduction with HA- and M2-pseudotyped lentiviral particles, virion attachment and internalization assay, immunofluorescence staining of NP protein as a marker of viral ribonucleoproteins localization, viral polymerase activity measurement, and viral budding observation by electron microscopy.

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Co-amplified quantitative real-time PCR: an objective comparison of H1N1 viral load between patients with mild, moderate, and severe disease

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Keywords Clinical severity, H1N1, quantitative polymerase chain reaction, swine influenza, viral load.

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Abstract

Dual-primer PCR using quantitative measurement of swine 'flu and human pyruvate dehydrogenase (PDH) was performed on 100 nasopharyngeal swab (NPS) specimens to attain values of viral genome copy per human epithelial cell. Forty-two patients had accessible clinical details, and a study was made of the correlation of viral genome concentration with severity of clinical illness and biochemical markers of inflammation. No statistically significant correlations were found, although trends were observed with higher concentration values in mild illness. This may represent high numbers of virions prevented from migrating to the lower respiratory tract of patients with only mild disease.

Introduction

The 'Dublin' PCR assay[1] has proved highly sensitive and specific for detection of swine flu in NPS specimens. There is a yet underdeveloped domain of this technology: formal quantification of viral loads and their relevance to disease prediction and treatment. Considering the ubiquitous nature of the influenza virus during pandemic eras, an obvious question is what correlation is there between viral load of swine influenza and clinical severity?

Materials and methods

Respiratory specimens

RNA extraction was achieved by QIAgen BioRobot EZ1 prior to respiratory multiplex PCR analysis. What remained of the extracted material of each specimen was stored by refrigeration at 4°C. Electronic patient records were searched for parameters, such as C-reactive protein (CRP), white cell count (WCC), length of admission in days, and patient co-morbidities.

Patient classification

Patients were divided into three groups according to clinical severity: mild, moderate, and severe. The 'mild' group comprised of those admitted for three days or fewer, or not admitted at all. The 'moderate' group comprised those who required admission to hospital for more than 3 days as a result of swine flu, but who did not require admission to an intensive care unit (ITU). The 'severe' group comprised those who had required ITU admission.

Swine flu probe and primers ('Dublin' array)

Primer Swine 'flu Taq 1: 5′ TGT GCC ACT TGT GAA CAG ATT G 3′
Primer Swine 'flu Taq 2: 5′ CTG ATT AGT GGA TTG GTG GTA GTA GC 3′
Swine 'flu probe: 5′ TGA TTC ACA GCA TCG GTC TCA CAG ACA G 3′

PDH probe and primers

Primer PDH Taq1: 5′ TGA AAG TTA TAC AAA ATT GAG GTC ACT GTT
Primer PDH Taq2: 5′ TCC ACA GCC CTC GAC TAA CC
Probe PDHTaqPR: 5′ JOE-CCC CCA GAT ACA CTT AAG GGA TCA ACT CTT AAT TGT TAMRA

Enzyme

SuperScript III/RT Platinum Taq mix (Invitrogen one-step PCR).

'Reaction mix'

Invitrogen '2× Reaction Mix': 0·4 mm of each dNTP + 6 mm Magnesium sulphate.

Primer/probe mix recipe

For *N* number of reactions, quantities for polymerase mix (μl): 2 N Primer/probe mix + 0·5 N enzyme + 12·5 N

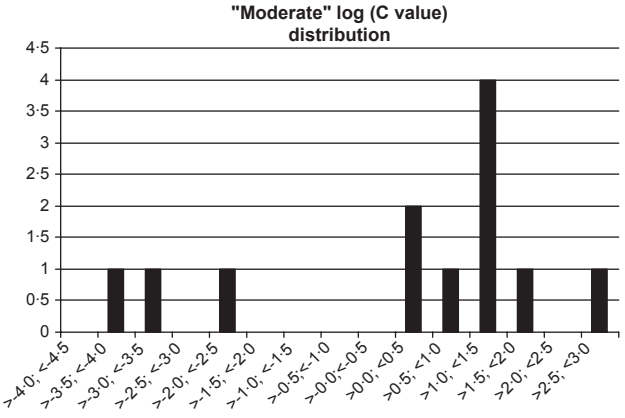

Figure 1. log of mean average *C* values for severity groups.

Reaction Mix (consisting of primers and probes swine flu and PDH, diluted in RNase-free water). Per reaction: 15 μl polymerase array mix + 10 μl extracted RNA aliquot.

PCR hardware

Applied Biosystems 7500 Fast real-time PCR system, 'respiratory multiplex' program. Well content 25 μl; thermocycler initial stage 50·0°C for 15 minutes, then 95°C for 2 minutes. Subsequent cycles of 95·0°C for 15 seconds followed by 60°C for 33 seconds for 45 cycles.

PCR analysis software

Sequence Detection Software version 1.4 (Applied Biosystems).

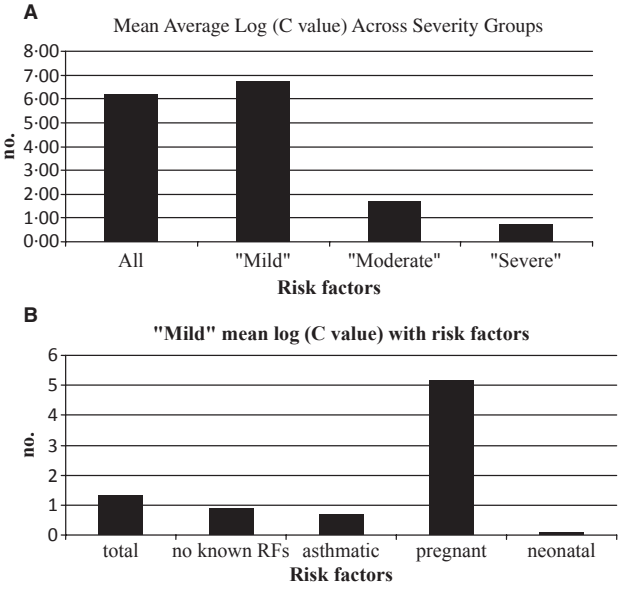

Figure 2. Mean average (log C values) of clinical groups.

Results

Copy thresholds (CT)

Of 126 clinical isolates analyzed, all samples produced amplification of PDH material; 100 produced amplification of both swine flu and PDH material.

Quantification controls

Human male DNA (lot no. 36048611039 at 10 ng/μ, Applied Biosystems) at concentration calculated at 16 666·6 cells/μl was diluted from 10^{-1} to 10^{-4}, yielding mean average CT values of respectively 30·10, 33·60, 37·78, and 37·22. Plotting log of cell number versus CT gave a $y = mx + c$ line from which CT could be interpolated into cell numbers.

For swine flu quantification, a sample of swine flu CT 19·28 was diluted through 10^{-1} to 10^{-10}. It must be noted that due to variability in resultant swine flu CT values, repetitions at these dilutions were done using an RNA carrier (1350 μg/l, Qiagen; Cat no. 1017794) in place of RNAse-free water. The 10^{-4} concentration was positive in nine out of 15 assays; this fraction was used in the calculation described by Simmonds[2] to obtain a copy number of targets per reaction by the equation copy value = $-\ln(f)$, where f is decimal fraction of failure rate. Here, $f = 6/15 = 0{\cdot}4$; $-\ln 0{\cdot}4 = 0{\cdot}916$ copies. A control curve was generated with CT values of 26·73, 30·02, 33·67, and 37·23 giving copy values of 916, 91·6, 9·16, and 0·916, respectively.

Using Excel (Microsoft Office, 2010), these control series were adapted into formulae to convert swine flu and PDH CT values into copy numbers of these elements per reaction. Simple division derived a value for swine flu copy per PDH copy, but this was chosen to be expressed as swine flu copy number per 100 human cells. This will be referred to as the '*C*' value.

Patient statistics

Forty-two patients had known clinical details; average age was 29·74, female to male ratio 63:37, and average admission length of 7 days. Of the mild group ($n = 26$), nine cases were not admitted to hospital. Of the remainder, the mean average admission length was 2·5 days.

C values

Mean average *C* value for all samples was $1{\cdot}49 \times 10^6$, with a standard deviation of $1{\cdot}49 \times 10^7$; geometric mean was 4·28, and median average was 6·45. Log(mean average *C* value) is shown for each severity group and for identified risk factors in the 'mild' severity group (Figures 2A, B respectively). In each case variation was too great to yield statistical significance. Figure 1 shows the range of C values observed in the 'moderate' severity group.

Discussion

In a study by Duchamp *et al.*,[3] no significant correlation was observed between viral CT value and presence or absence of cardiaorespiratory disease, myalgia, digestive symptoms, or upper or lower respiratory tract infection (although a trend was observed towards patients presenting with signs of upper respiratory tract infection). To our knowledge, no other study has used a dual PCR for analysis of respiratory virus concentrations, and no study has attempted to correlate biochemical markers with respiratory virus concentration.

The data exhibited a spectrum of *C* values, from values $<5 \times 10^{-5}$ to over 1×10^{8}. The three severity group standard deviations all overlapped with each other, preventing statistical significance.

Analysis of co-morbidities showed a high mean average *C* value when asthma was present ($6{\cdot}05 \times 10^{7}$), but again this was associated with an excessive standard deviation. Whereas the median average *C* value in the presence of asthma was higher than the overall average *C* value (9·09 versus 5·85), it was significantly lower than the median *C* value when no co-morbidity was documented (15·55).

There are multiple caveats that may be the cause of such variety of *C* values obtained.

The duration between initial RNA extraction and study PCR had a range of 21 to 285 days, with mean average delay of 211 days. The degradation of viral RNA is an important contributor to assay variance and failure; RNA degradation in clinical samples has been studied.[4–6] Degradation of human DNA in clinical samples may have occurred. Several studies have chartered degradation of stored human DNA.[2,7] With regards to sampling, the clinical collection of throat swabs is naturally variable according to the method of the collector. A small number of bronchoalveolar lavage samples were analyzed, yet did not amplify, presumably due to RNA degradation.

The upper respiratory tract may be only a physical stepping stone for the virus, and take no further role in pathogenesis of severe disease (although undoubtedly is crucial for transmission). Interestingly, a ferret study of pathogenesis observed that swine flu yields from the upper respiratory tract were greater than those given by ordinary seasonal H1N1, with consequently increased shedding.[8] The review by Mansfield[9] cites significant findings regarding influenza pathogenesis, including the predilection of H5N1 strains for type II pneumocyte cells and alveolar macrophages. It also highlights the limitation of knowledge through dearth of human autopsy studies; an exception is the recognition of haematophagocytic syndrome in severe cases. It is known that specific immunoglobulin is effective against establishment of infection in the upper respiratory tract, whereas specific cytotoxic T lymphocytes (CTLs) are necessary for clearance of the virus from the lower respiratory tract.[10] It is also suggestive that a gap of two whole days transpires between initial infection and instigation of a specific immune response.[11] It is plausible that in the healthy individual, virus progression is confounded by efficient natural mucosal immunity, in part through good secretory immunoglobulin levels.

Airway inflammation associated with asthma exacerbation is known to increase both risk of respiratory viral infection and poorer outcome. It is unproven but likely that the local inflammatory processes give rise to increased virion burdens in the upper airways; however, the same effect is conceivable for epithelial cell turnover.

There will likely be variance within each clinical category due to patient circumstances and clinicians' judgment of required admission. Unfortunately, the duration of symptoms prior to swab collection was often omitted in the clinical notes. Finally, stratification of patient group by receipt of antiviral treatment was not studied.

Conclusions

No correlations were observed with *C* values and CRP, WCC or admission length. Trends were observed towards higher *C* values in 'mild' cases, but without statistical significance. The relative small study size, coupled with the intrinsic variability of the parameters studied, warrants larger, better controlled, prospective studies to elucidate clinical use of the *C* value for influenza illness prediction and management.

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Genetic and antiviral drug susceptibility profiles of pandemic A(H1N1)v influenza virus circulating in Portugal

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Keywords A(H1N1)2009, influenza, antiviral drug susceptibility, genetic characterization, Portugal.

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Abstract

Global A(H1N1)2009 genetic characterization, molecular evolution dynamics, antiviral susceptibility profiles, and inference of public health implications require nation and region wide systematic analysis of circulating virus. In this study we analysed the genetic and antiviral drug susceptibility profiles of pandemic A(H1N1)2009 influenza virus circulating in Portugal. Genetic profile analysis was performed in 37 isolates to the hemagglutinin (HA), neuraminidase (NA) and MP genes, and in six of these isolates the PB1, PB2, PA, NP and NS genes were also analysed. Antiviral drug susceptibility profile was analysed for 96 isolates, phenotypically and genotypically to neuraminidase inhibitors (NAI) and genotypically to amantadine. The point mutations identified in HA, NA, and MP genes of different strains do not seem to evidence an evolutionary trend. This is in agreement with the genetic and antigenic homogeneity that has being described for A(H1N1) 2009 virus. All analysed strains were found to be resistant to amantadine, and five of these strains exhibited a reduced susceptibility profile to NAI, three only for oseltamivir and two for both inhibitors.

Introduction

In mid-April 2009 a novel variant of A(H1N1) influenza virus began to spread rapidly throughout the world, causing the first pandemic of the 21st century. The majority of the cases associated with this new virus show to be mild, but severe and fatal cases have been reported. Molecular markers associated with severity have already been identified, as is the case of the mutation D222G.[1] Resistant viruses to antiviral drugs have also been identified, highlighting the importance of rapid determination of the antiviral drug profile.

Global A(H1N1) 2009 genetic characterization, molecular evolution dynamics, antiviral susceptibility profiles, and inference of public health implications require nation and region wide systematic analysis of circulating virus.

The objective of this ongoing research study was, primarily, to thoroughly characterize the genetic profile and evolution of the emergent influenza A(H1N1) 2009 virus circulating in Portugal and its phenotypic expression on antiviral drugs susceptibility.

Materials and methods

The cases considered in this study were obtained from the community and from two collaborating hospitals in Lisbon – a reference hospital for adults (Hospital de Curry Cabral) and a reference hospital for children (Hospital Dona Estefânia).

The CDC real-time PCR protocol, recommended by World Health Organization (WHO), was the method used to confirmed all influenza A(H1N1) 2009 cases. From a total of 577 A(H1N1) 2009 positive cases diagnosed and confirmed, 163 were selected for this study, taking in consideration that they should cover the period of epidemic activity in Portugal and include cases from persons belonging to risk groups and cases associated with more severe clinical features. Ninety-six A(H1N1) 2009 strains were isolated in MDCK-SIAT1 cells, from combined naso-oropharyngeal swabs.

For the evaluation of the genetic profile of A(H1N1) 2009 virus circulating in Portugal, 37 of the 96 isolates

were characterized by genetic analysis of the HA, NA, and MP genes. The remaining five gene segments (PB1, PB2, PA, NS, and NP) were also sequenced for six of this 37 isolates. Briefly, sequencing was performed according to the protocol developed by CDC and recommended by WHO,[2] using BigDye Terminator V.1.1 technology. Nucleotide sequences were determined in a DNA automatic sequencer ABI PRISM 3130XL Genetic Analyzer. For each genomic segment, genetic analysis was performed with Lasergene V.4.05 software (DNASTAR Inc, USA) using an average of 4–6 overlapping readings, including sense and antisense, for precise nucleotide and amino acid sequence determination. Genetic mutation and phylogenetic analysis were performed by neighbor-joining method, using MEGA4.0 software, against published sequences from the vaccine strain (A/California/7/2009) and from selected A(H1N1) 2009 strains available on GISAID EpiFlu Database. All mutations were identified with reference to the vaccine strain genome sequence.

Antiviral drug susceptibility profile of A(H1N1) 2009 influenza virus circulating in Portugal was evaluated both phenotypically and genotypically for NAIs and genotypically for amantadine.

Phenotypic evaluation to NAIs, oseltamivir and zanamivir, was performed for all 96 isolates by IC_{50} determination through MUNANA fluorescence assays.[3] Genotypic evaluation was performed by searching for mutations associated with resistance to NAIs in all 37 NA gene sequences. Amantadine susceptibility profile was performed for all 96 isolates by searching on M2 sequence for the 5 molecular markers associated with resistance to this antiviral drug (L26F/I; V27A/D; A30T; S31N; G34E).

Results

Genetic characterisation of the HA1 subunit of HA reveals point mutations in different strains. All 37 analysed strains present P83S and I321V mutations, which distinguish them from the vaccine strain (Figure 1A). Thirty-three of the 37 sequenced strains group in the S203T branch. This mutation is referred in the literature as being associated with the putative antigenic site Ca.[4] Most of these strains (19) further subgroup in the D222E branch, this mutation being associated with one loop of the receptor-binding site.[1]

From the early to the late epidemic period, an increased circulation of virus carrying the mutation S203T was observed. This is in agreement with the association between this mutation and an enhanced viral fitness that is described in the literature.[5] Additional mutations were also observed in a small number of virus, of which we highlight: (i) mutation A73T in A/Portugal/03/2009 and mutation S185N in A/Portugal/14/2009, associated with the putative

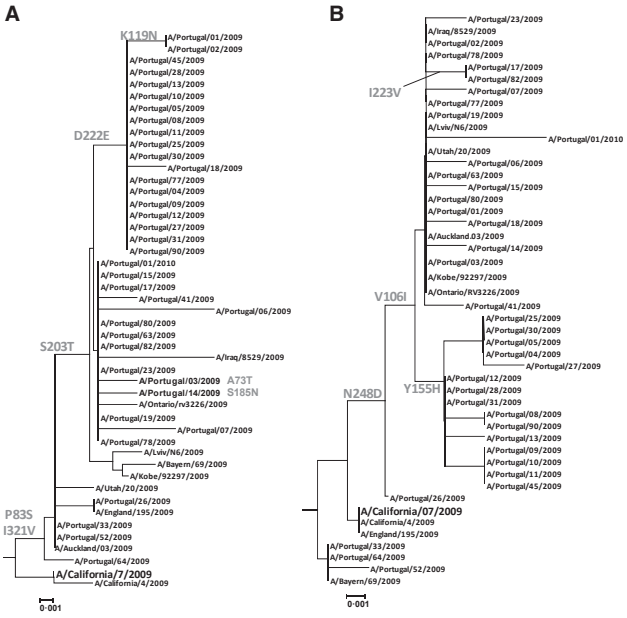

Figure 1. Phylogenetic tree of the amino acid sequences of HA1 subunit of hemagglutinin (A) and neuraminidase (B) genes of Influenza A(H1N1)2009 strains.

antigenic sites Cb and Sb respectively,[4] and (ii) mutation K119N in A/Portugal/01/2009 and A/Portugal/02/2009 that can result in a new N-glycosilation site, as it originates an N-X-S motif in the aminoacid sequence.

Regarding the genetic characterisation of NA, the majority of strains analysed (34 of 37) presents the mutations N248D and V106I (Figure 1B). As mutation S203T in HA gene, these two NA mutations are described in the literature as associated with enhanced viral fitness.[5] The few strains not carrying these mutations have circulated in the beginning of the epidemic period. Fifteen of the 37 analysed strains further subgroup in Y155H branch. Additionally, mutation I223V was identified in two strains.

The analysis of the complete MP gene of 37 strains revealed three mutations in the M1 protein (M128L in A/Portugal/45/2009, E201D in A/Portugal/26/2009, and M203I in A/Portugal/77/2009) and three in the M2 protein (P10H in A/Portugal/26/2009, R18K in A/Portugal/03/2009, and E66K in A/Portugal/28/2009). However, no reference to these mutations was found in the literature.

For the remaining gene segments available for the six analysed strains, the observations include: (i) no previously described virulence markers in PB2, PB1-F2, and NS1 were detected; (ii) PB1-F2 protein is present in the truncated form of 11 amino acids; (iii) the presence of mutations I123V and L122Q in NS1 and V100I in NP; (iv) the described association of mutation I123V in NS1 and V100I in NP genes with viral fitness.[5]

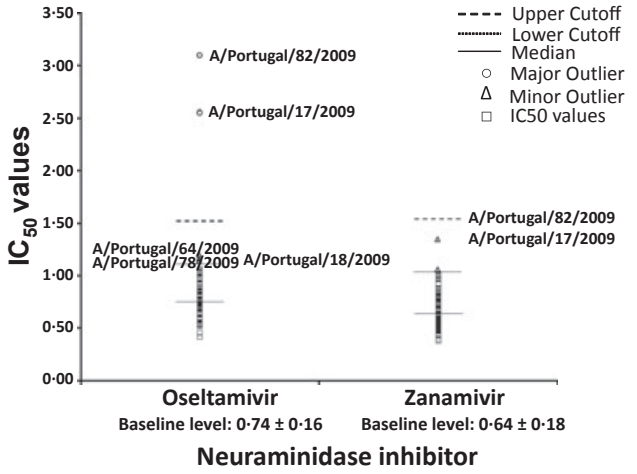

Figure 2. IC_{50} values obtained by fluorescence assay for influenza A(H1N1)2009.

Phenotypic evaluation of NAIs susceptibility revealed the existence of three minor and two major outliers to oseltamivir (Figure 2). The two minor outliers exhibited a reduction of approximately twofold in the susceptibility to this antiviral drug, comparing to the baseline level, while the reduction exhibited by the two major outliers was of approximately three- and fourfold.

Regarding zanamivir, two minor outliers were identified with a reduction of approximately twofold in the susceptibility, compared to the baseline level. These two minor outliers (A/Portugal/17/2009 and A/Portugal/82/2009) correspond to the two major outliers identified for oseltamivir.

Genetic analysis revealed the presence of the mutation I223V in the NA sequence of these two strains. The contribution of this mutation for the profile of reduced susceptibility identified for both NAIs is not known, but a mutation in the same NA position (I223R) has been referred to as being associated with a reduction in NAIs susceptibility.[6] Full genome sequence analysis of these strains shows that both strains also present the V480I mutation in PB2 gene. However, no association of this mutation with antiviral drug susceptibility is referred in the literature.

Concerning genetic evaluation of susceptibility to amantadine, all 96 analysed strains present a Serine in position 31, which is a molecular marker of resistance to M2 inhibitors.

Discussion

These preliminary results allow us to discuss several points. However, the additional data that is being obtained through this ongoing study will be essential for a more complete analysis. For example, more information is needed to determine if the mutations found alter the biology and the fitness of the virus or if there are associated with an increased prevalence of the virus.

The majority of the mutations identified in HA1 subunit have been detected in A(H1N1)2009 strains distributed throughout the epidemic curve, not evidencing a specific evolutionary trend. This is in agreement with the genetic and antigenic homogeneity that has being described for A(H1N1)2009 virus.[7]

The occurrence of mutations in the position 222 of the HA1 subunit of A(H1N1)2009 virus have been described. However, more studies are needed to clarify the outcome of these mutations, as for example in patients with severe complications. It could also be relevant to investigate the presence of single and mixed variants in viruses and in clinical specimens and the possibility of these mutations affecting the binding specificity.

Regarding the susceptibility of A(H1N1)2009 pandemic viruses to antiviral drugs, all analysed strains were found to be resistant to amantadine. This resistant profile was not unexpected since the MP gene from this new variant had originated in the Eurasian swine lineage, which is characterised by being resistant to this antiviral drug.[8]

The majority of the A(H1N1)2009 strains analysed revealed to be susceptible to both NAIs, with only five strains exhibiting a profile of reduced susceptibility, three to oseltamivir and two to both NAIs. For these last two, the presence of the I223V mutation in the NA sequence could explain the reduction observed, but a more complete analysis is needed to confirm this.

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A(H1N1)2009 pandemic in France: epidemiological features based on virological surveillance

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Abstract

The French national pandemic plan includes an early containment phase followed by a limitation phase. The efficacy of such a plan depends on pre-existing surveillance and laboratory networks. The GROG community surveillance network and the hospital lab networks organized by the two French NICs carried out the virological monitoring of the A(H1N1)2009 pandemic. The NICs set up and distributed the RT-PCR tools to the lab networks early May 2009. During the containment phase, all suspected and virologically confirmed cases were hospitalized and declared to InVS. During the limitation phase, the clustered cases were monitored, and GROG swabs were collected by practitioners in general population. During the pandemic, the NICs carried out additional testing for the monitoring of antiviral resistance and of genetic changes involved in virus adaptation (PB2) and virulence (HA). The first imported A(H1N1)2009 influenza cases were detected at the end of April 2009. Local transmission was observed at the end of May. Clusters were observed in schools in June and in summer camps during summer. Sporadic cases were reported up until October when the pandemic wave started. One single 10 week-long pandemic wave was observed between mid-October and the end of December. Overall, 103 352 samples have been tested with 24 279 positives. The weekly positive rate ranged from 0% to 48% with a peak week 48 (3877 positives). Phylogenetic and antigenic analyses did not show any emerging genetic or antigenic variants. Eight cases had a D222G mutation in the HA. Eleven cases had an oseltamivir-resistant virus (H275Y); one harboured a reduced sensitivity to zanamivir (additional I223R mutation). All but one resistant virus were detected in treated immunocompromised patients. According to the profile of hospitalized cases, A(H1N1)2009 was more virulent than seasonal viruses. Even if the mortality was limited (312 cases), the age distribution of the deceased patients was different as compared to seasonal influenza (75% mortality in <65 years of age). The virological monitoring of the pandemic was achieved by the preexisting seasonal influenza networks.

Introduction

The French national pandemic plan, prepared in response to the A(H5N1) pandemic threat, has been implemented in France since 2003 with updates in 2006 and 2008. This plan describes the response to a pandemic with an early containment phase to delay the virus circulation in the population, followed by a limitation phase to contain as much as possible virus circulation and avoid excess hospitalisations and deaths. These phases should use mitigation measures, antivirals, and vaccination, if available.

The efficacy of such plan depends on pre-existing influenza surveillance and laboratory networks. In France, the community surveillance is carried through the GROG surveillance network. In addition, surveillance is also carried out in hospitals by the RENAL network. This RENAL network is divided in two sub-networks: the so-called H5-labs network, activated during the containment phase and the Extended RENAL Lab network activated in the limitation

phase. The H5-labs have BSL-3 facilities that can be used for diagnosis purposes.

As part of the national influenza surveillance system led by the French Institute for Public Health Surveillance (InVS), the GROG community surveillance network and the lab networks linked to the two French NICs carried out the virological monitoring of the A(H1N1)2009 pandemic from the early containment phase up until the end of the pandemic phase.

Materials and methods

During the containment phase, all suspected cases were hospitalized and declared to InVS. Each patient was tested on the same day by specific virological diagnosis. Hospital admission was not mandatory during the limitation phase, (i) the clustered cases were monitored to study transmission chains, and (ii) the circulation of the virus in the community was monitored through GROG swabs collected by practitioners.

The NICs organized the influenza surveillance to fulfill several objectives according to the epidemiological situation. First, RT-PCR tools (Influenza A M gene RT-PCR and A(H1N1)2009 specific H1 and N1 genes RT-PCRs) were developped and distributed to the lab networks on the 14th of May 2009.[1] From the early phase, the NICs and the H5-Lab network analyzed all the samples collected from hospitalized and community patients. During the early phase of the limitation phase, an increasing number of labs were performing the specific assays. When the pandemic wave started, all hospital labs could do the testing. Results were centralised by NIC and reported on a weekly basis.

In addition, NICs carried out the monitoring of antiviral resistance emergence (NA pyrosequencing, specific H275Y RT-PCR, and phenotypic assays), and real-time surveillance of genetic changes involved in virus adaptation (PB2) virulence factors or antigenic variations (HA). This sequencing was carried out by the PF8 sequencing platform of the Institut Pasteur.

Results

Epidemiological data

The first imported A(H1N1)2009 influenza cases were observed from the 28th of April 2009. A limited number of cases have been reported in May. Local transmission could be detected end of May. Clusters were observed in schools in June and in summer camps during summer. As opposed to the epidemiology of the A(H1N1)2009 virus in other European countries, no summer wave was observed in France. Only a limited number of sporadic cases were reported up until October. Early September, a significant

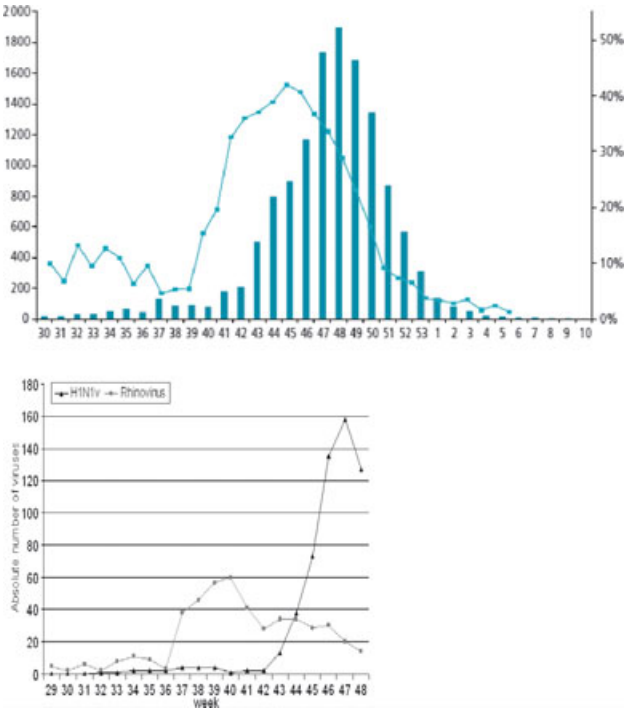

Figure 1. Specimens and positives collected during the pandemic. In the upper panel, the solid line shows the number of specimens tested weekly by the networks, the bars shows the percentage of positives. The weeks are numbered as usual. In the lower panel, the rate of detection of rhinoviruses and influenza A(H1N1) viruses are showed, emphasizing the counfounding factor the rhinovirus was for determination of the real impact of influenza using clinical surveillance only.

number of cases presenting with influenza-like illness was reported (Figure 1). The virological investigation of these cases showed high prevalence of rhinovirus infection. This circulation of rhinovirus was a counfounding factor of the pandemic. The pandemic wave lasted 11 weeks between mid-October and the end of December (week 43 to week 53, Figure 1). The pandemic wave started week 42–43 in the Ile-de-France area, and only week 44–45 in the rest of France. The peak was recorded week 48 (Figure 1). The impact of the pandemic was mainly observed in the 5–15 years group of age. Overall, 1334 severe cases have been admitted to the hospital, and 308 deaths have been recorded by the end of the pandemic wave. The major impact was observed in the 15–65 years group of age (66% of deaths recorded). Amongst the severe cases and the deceased cases, 20% and 16% of cases had no risk factor, respectively.

Virological data

Overall, 103 352 samples have been tested between the 27th of April 2009 and end of March 2010 (Figure 1). Amongst

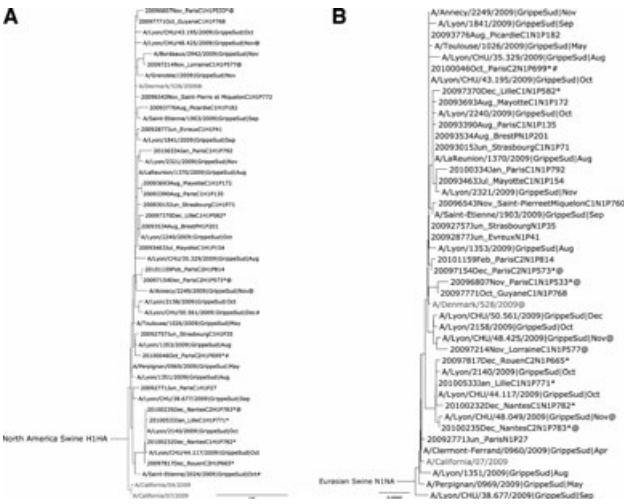

Figure 2. Phylogenetic analysis of representatives isolates collected during the different periods of the pandemic in France [HA in (A) Na in (B)]. Isolates in grey are reference strains. @ = H275Y oseltamivir resistant viruses; # = D222G Mutant viruses; * = Severe cases.

these specimens, 24 279 were positives for H1N1, representing 99·7% of total influenza virus detections. Only nine Brisbane-like H1N1, 62 Brisbane-like H3N2, and eight B viruses have been detected in the same period of time. The weekly positive rate ranged from 0% to 48%. Phylogenetic and antigenic analyses of the viruses collected during the pandemic wave did not show any emerging genetic or antigenic variants (Figure 2A,B). Eight patients, all among cases presenting with severe illness, were infected by a virus harbouring the D222G mutation in the HA.[2] Amongst the virus tested for antiviral susceptibility or screened for the H275Y mutation by or specific RT-PCR, only 11 oseltamivir-resistant viruses related to the NA H275Y mutation have been detected. One of these cases also had an I223R mutation associated to a reduced sensitivity to zanamivir. All but one resistant virus were detected in treated immunocompromised patients. Overall, eight patients presented a virus with the D222G mutation in the HA. All these patients had a severe infection; one of these had also a H275Y mutation in the NA asociated to oseltamivir resistance.

Discussion

The pandemic started by the end of April 2009. Although the first cases recorded were as early as the 28th of April, the epidemic wave associated with a widespread spread of the virus was only recorded in October. The french population did not have to face a summer wave, as observed in North America and in numerous European countries.[3,4] It is difficult to speculate the reasons for the lack of summer wave; the specimens collected were negative for influenza. Moreover, during September, it was anticipated that school openings would be the trigger for the beginning of the pandemic wave. As a matter of fact, a significant increase of influenza-like syndromes were observed at that time, but the virological investigation carried out by the laboratories showed thta is was related to a very large epidemic of rhinovirus.[5] The epidemic circulation of other respiratory viruses can be counfounding factors for the surveillance of the influenza epidemic clinical when the survellance is only based on collection of clinical information. The starting of the pandemic wave was heterogeneous in France. The Ile-de-France region (Paris and its suburbean area), where the population is dense, experienced an early start as compared to the rest of France. However, once the pandemic started in the rest of the county, the epidemic curves were quite similar. The peak was reached at identical times, although it may have been delayed in some remote places in France. Overall, we estimate that 10% of the French population consulted for an ILI presentation. The impact was mainly observed in the 5–15 years groupe of. However, this age groupe represented only a limited number of severe cases and deaths. On the other hand, the 15–65 years groupe of age, where the prevalence was not high, was the age group where the majority of severe cases and deaths was recorded (74% and 66%, respectively).[6] This data is consistent with the observational data reported by numerous other countries.[7]

According to the profile of hospitalized cases, A(H1N1)2009 was more aggressive than seasonal viruses. The number of admission to the hospital was ten-fold that observed during a normal influenza epidemic. Even if the mortality was limited (312 cases), the age distribution of the deceased patients was different as compared to seasonal influenza (75% mortality in <65 years of age). The lack of recordeable excess mortality has been interpreted to be the consequence of a very mild pandemic, milder than some seasonal epidemics. However, the median age of the fatal cases was much younger than those observed during the seasonal flu, leading to a mis-interpretation of the real impact of the pandemic. When the impact is measurered in loss of years of life, the impact of this pandemic is larger than seen with seasonal influenza, and is quite comparable to these of the two last pandemics.[8] The pandemic preparadness of numerous countries, the develoment of new intensive care techniques and equipment, and the large use of antivirals have reduced the overall impact of this pandemic. These are new factors that should be taken into account when evaluating the real impact of the 2009 H1N1 virus.[8]

The virological monitoring of the pandemic was achieved by the community-based and hospital-based sea-

sonal influenza networks, reminding the importance of maintining such networks. The diagnosis of influenza in most of the patients was carried out by molecular techniques. It has been clearly stated from the beginning of the pandemic that near-patient tests were lacking of susceptibility and could not be used for patient management. The distribution of a set of validated and comprehensive techniques by the two NIC was very helpfull for the monitoring of the pandemic and the patients. However, this diagnostic procedure change should not preclude maintaining virus isolation that is necessary for whole genome analysis, monitoring of antigenic changes, and phenotypic testing for antiviral testing. Some of the mutants that have been recorded, including viruses with antiviral resistance phenotype or genotype, could be analysed from grown virus strains. It is striking that despite a large antiviral usage, only a limited number of isolates had mutations associated to resistance. However, the frequent isolation of such resistant virus was observed in immunocompromised patients that presented severe infections and long virus shedding.[9]

The impact of the pandemic is still under evaluation. Sero-epidemiological analysis will be performed to asses for the real attack rate of the 2009 pandemic virus. As in other countries, it has been recorded that asymptomatic infections could be observed frequently.[10] It is quite unlikely that the impact of the pandemic was reduced by the vaccination campaign, although this vaccination started on the 12th of November, just when the pandemic started in France. It is estimated that 6 millions received the vaccination.

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Patterns of viral shedding in pandemic and seasonal influenza virus infections in a community setting

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Abstract

Introduction: The dynamics of pandemic influenza A/H1N1 compared to seasonal strains of influenza is not clearly understood. It is important to understand the patterns of viral shedding and symptoms over time in community-based infections.

Materials and methods: Household infections were followed-up in two large community-based studies. Patterns of viral shedding, symptoms and signs, and tympanic temperature were plotted over time and grouped according to strain for analysis.

Results: The patterns of viral shedding, symptoms and signs, and tympanic temperature in three influenza A strains (pandemic A/H1N1, seasonal A/H1N1, and seasonal A/H3N2) were comparable. Peak viral shedding occurred close to the onset of symptoms and resolved after 6–7 days. Patterns of viral shedding in influenza B virus infections differed.

Discussion: The patterns of viral shedding and clinical course of pandemic influenza A/H1N1 infections were broadly similar to seasonal influenza A/H1N1 and A/H3N2. Only the clinical course of seasonal influenza B infections was similar to pandemic influenza A/H1N1. The dynamics of pandemic influenza A/H1N1 were observed to be largely alike to the dynamics of seasonal influenza A/H1N1 and A/H3N2.

Introduction

Pandemic strains of the influenza virus sporadically emerge, deviating from the regular endemic strains of seasonal influenza. In April 2009, a novel pandemic influenza virus A/H1N1 emerged, swiftly spreading across the world. Immediately, domestic and international public health agencies were forced to develop containment and mitigation strategies in response to the pandemic. However, the dynamics and transmission patterns of this novel virus are yet to be fully understood. Simultaneously, seasonal strains of influenza (A/H1N1, A/H3N2, and B) continued to circulate in many nations. Both pandemic and seasonal variants of influenza are responsible for significant morbidity and mortality.[1] To characterize the dynamics of this disease and the variation within strains, a more detailed understanding of the patterns in viral shedding during natural infection is required.

The majority of data on the patterns of viral shedding during influenza infection are a result of volunteer challenge studies.[2] In these studies, volunteers are commonly screened for pre-existing immunity against the challenge strain and are of a certain demographic and age. Information on the patterns of viral shedding in natural influenza infections, pandemic or seasonal, is limited but should provide greater generalizability. We describe the trends of viral shedding and clinical illness in community acquired cases of pandemic and seasonal strains of influenza.

Materials and methods

In 2008, a community-based study was conducted to analyse the effectiveness of non-pharmaceutical interventions to prevent the spread of influenza in households.[3] In 2009, a similar community-based study was initiated to collect comparative data from individuals infected with seasonal and pandemic influenza.[4] Both studies were conducted with very similar protocols, involving 617 households in total. The specimens and symptom data required for this study all arise from secondary infections ascertained in these two community-based studies.

The recruitment process in both studies was essentially identical. Index cases were first recruited from their healthcare provider if they presented with influenza-like illness

(ILI). This individual would be included in the follow-up if he/she tested positive for influenza virus infection by rapid antigen test (QuickVue) and was the first person in his/her household that showed signs of ILI in the previous 2 weeks. Follow-up consisted of three home visits that spanned approximately 7–10 days. At each home visit, nasal and throat swab (NTS) specimens were collected from all household members, regardless of the presence or absence of symptoms. Symptoms were recorded in daily symptom diaries provided for every household member, and digital thermometers were provided to record daily tympanic temperature. The symptoms recorded were fever ≥37·8°C, headache, myalgia, cough, sore throat, runny nose, and phlegm.

Influenza virus infection and subtype was identified by reverse transcription polymerase chain reaction (RT-PCR) on the NTS specimens. Viral shedding was quantified from the same specimens by RT-PCR to determine viral loads, as well as by quantitative viral dilutions to determine median tissue culture infectious dose ($TCID_{50}$). The details concerning laboratory methods have been described in a previous study.[5]

All analyses in this study focus exclusively on secondary cases; these are household contacts of recruited index cases who acquire influenza virus infection following the initial home visit. Index cases generally presented with a certain threshold of illness severity requiring medical attention, whereas infections among household contacts can vary from asymptomatic to severe representing naturally acquired influenza infections. These secondary cases must be negative for influenza for their first NTS specimen, and subsequently tested positive.

We analysed mean viral loads measured by RT-PCR and quantitative culture by plotting by day since acute respiratory illness (ARI) onset according to strain of influenza (pandemic A/H1N1, seasonal A/H1N1, seasonal A/H3N2, and seasonal B). ARI is the reference time point, because the day of infection is unknown and is defined as the presence of ≥2 of the symptoms mentioned above. Average symptom scores were also plotted according to ARI onset and grouped into upper respiratory symptoms (sore throat and runny nose), lower respiratory symptoms (cough and phlegm), and systemic signs and symptoms (fever ≥ 37·8°C, headache, and myalgia). Mean daily tympanic temperatures were also plotted since date of ARI onset and according to strain of influenza virus. All analyses were conducted using r software (version 2.10.1; R Development Core Team).[6]

Results

A total of 617 households and 2499 individuals were followed-up in the two studies. Of 1887 household contacts tested by RT-PCR, 153 were found to be influenza positive. Among these influenza infections, 13 (8·5%) were asymptomatic (RT-PCR positive plus 0 symptoms recorded), 20 were subclinical (RT-PCR positive plus 1 symptom recorded), and 88 presented with an onset of ARI during the follow-up period. From the cases with ARI onset, seven pandemic A/H1N1, 40 seasonal A/H1N1, 22 seasonal A/H3N2, and 19 seasonal B influenza virus infections were identified.

Table 1. Characteristics and symptoms of RT-PCR influenza positive household contacts according to type and subtype of influenza virus

	Pandemic H1N1 (*n* = 7)	Seasonal H1N1 (*n* = 40)	Seasonal H3N2 (*n* = 22)	B (*n* = 19)
Age group (%)				
≤5 years	1 (14)	7 (18)	2 (9)	2 (11)
6–15 years	1 (14)	4 (10)	6 (27)	7 (37)
16–30 years	2 (29)	5 (13)	3 (14)	1 (5)
31–50 years	3 (43)	23 (58)	11 (50)	11 (58)
50+ years	0 (0)	1 (3)	4 (18)	2 (11)
Unknown	0 (0)	0 (0)	1 (5)	1 (5)
Male (%)	1 (14)	22 (55)	8 (36)	8 (42)
Mean family size	4·1	4·4	4·0	4·3
Mean flat size (sq. ft.)	492	851	900	826
Symptoms (%)				
Cough	6 (86)	37 (93)	20 (91)	17 (89)
Sore throat	5 (71)	27 (68)	17 (77)	10 (53)
Runny nose	4 (57)	38 (95)	21 (95)	16 (84)
Phlegm	4 (57)	24 (60)	20 (91)	14 (74)
Fever (≥37·8°C)	4 (57)	20 (50)	12 (55)	9 (47)
Myalgia	3 (43)	14 (35)	13 (59)	9 (47)
Headache	2 (29)	18 (45)	17 (77)	8 (42)

The age distribution among secondary cases was observed to be largely comparable across the four strains of interest (Table 1). There were a lower proportion of males who acquired pandemic A/H1N1 compared to the seasonal strains of the virus. Cough was the most commonly reported symptoms during follow-up in cases of pandemic A/H1N1 and seasonal B, whereas runny nose was most common in seasonal A/H1N1 and A/H3N2 cases. Cumulatively, fever (≥37·8°C) was reported in approximately half (51%) of the secondary cases.

Patterns of viral shedding were analysed in a subset of 88 influenza positive individuals who recorded an onset of ARI in their symptoms diaries (Figure 1). Household contacts that were asymptomatic, subclinical, or did not have an ARI onset were excluded from the analysis. Viral shedding in all three influenza A strains were recorded to occur on the day of ARI onset or 1 day post-ARI onset. Follow-

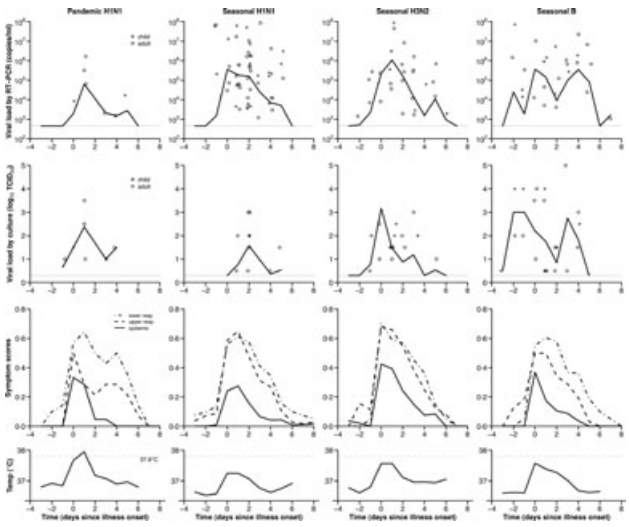

Figure 1. Patterns of viral shedding, symptoms scores, and tympanic temperature in household contacts with influenza virus infection.

ing the peak, measured levels of viral shedding declined steadily to undetectable levels over 5–6 days. The trend of viral shedding in influenza B infected individuals rose 2 days before ARI onset, fluctuated for around 6 days before eventually resolving. The patterns of viral shedding over time measured by quantitative viral culture were generally similar to the patterns measured by RT-PCR. The patterns of symptoms and signs were comparable in the four strains of influenza included in this study, peaking on the day or 1 day post-ARI onset, and gradually declining over a period of 5–7 days. In all strains, systemic symptoms and signs were observed to resolved faster than upper and lower respiratory symptoms. The trend of tympanic temperature in each influenza strain was comparable to the respective symptom pattern.

Discussion

Patterns of viral shedding observed in influenza A strain infections (pandemic A/H1N1, seasonal A/H1N1, and seasonal A/H3N2) were broadly similar. The pattern differed from the observed pattern of viral shedding in seasonal influenza B infections. The majority of viral shedding in influenza A strains occurred at and near ARI onset, whereas there were variable amounts of viral shedding pre- and post-ARI onset for those with influenza B. The biological reason for this difference is yet to be clarified. These differences are consistently observed regardless of laboratory method used to quantify the viral loads. It was observed that viral shedding measured by $TCID_{50}$ resolved more quickly than when measured by RT-PCR, suggesting that RT-PCR is more sensitive, but it could be detecting inactivated fragments of RNA instead of active virus. The trends observed for the seasonal strains of influenza in this study were similar to those reported in literature.[2]

The patterns of symptoms and signs as well as tympanic temperature in the four different strains of interest in this study were found to be comparable. These patterns closely resemble the patterns of viral shedding observed in the influenza A virus strains, but not in the influenza B virus strain. The trends of viral shedding, symptom scores, and tympanic temperature for pandemic A/H1N1 were similar to trends observed for seasonal A/H1N1 and seasonal A/H3N2 infections, suggesting that the dynamics of these viruses are largely the same. The clinical course of infection with pandemic A/H1N1 influenza virus appeared to be similar to the seasonal B influenza virus, but the patterns of viral shedding over time diverges. In general, our results suggest that the dynamics of the pandemic A/H1N1 virus were similar to the seasonal A/H1N1 and A/H3N2 viruses, and clinically similar to the seasonal B virus.

This study faced sample size limitations; very few cases of pandemic A/H1N1 were detected and the secondary attack rate in general was low, though a total of 617 households were followed up. This lack of power led to the inability to analyse the differences between adult and children and other characteristics that could be correlated with amount of viral shedding. There are also biases that must be factored in during recruitment. The eligibility criteria of only healthy households could select for households with higher innate immunity. On the other hand, recruitment at health care providers can be biased towards index cases that had more severe illness that required medical attention. The strength of the study is the broad generalizability of the results due to the strict classification of secondary cases. The infections reported in this study were all community-based and should represent true natural infections.

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Study of transmissibility of wild type and cold-adapted influenza viruses in guinea pigs

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Keywords Cold-adapted influenza viruses, influenza virus, transmission.

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Abstract

The declaration of the 2009 influenza pandemic raised an important key question regarding the efficiency with which the causative strain transmits between human hosts. The goal of this study was to model the transmission of pandemic, potential pandemic, and cold-adapted influenza viruses. Novel influenza A H1N1 and H5N1 viruses may be transmitted among guinea pigs by direct contact of non-infected with infected individuals. Cold-adapted influenza strains did not transmit between animals and prevented transmission of *wt* viruses. Our results highlight the weakness of yet unsubstantiated fears that widespread vaccination with live attenuated influenza vaccine (LAIV) in the presence of circulating influenza viruses could lead to generation of reassortants possessing an unpredictable level of pathogenicity.

Introduction

Pandemic potency of the influenza virus is largely determined by its transmissibility. The first objective of this study was to model the transmission of influenza H1N1 and H5N1 viruses. At present, vaccination with LAIV has been used as a widespread, effective public health measure for influenza prophylaxis. Some unsubstantiated concerns have been raised about a potential possibility of reassortment of circulating influenza viruses with LAIV viruses following vaccination with LAIV. Thus, another objective of this study was to assess the probability of pig-to-pig transmission of cold-adapted viruses and their potential reassortment with *wt* influenza strains.

Materials and methods

Viruses

A/California/7/2009 (H1N1) pandemic virus. A/Leningrad/134/17/57 (H2N2), MDV for Russian LAIV. A/17/California/2009/38 (H1N1) pandemic LAIV candidate. Reassortants for inactivated vaccine subtype H5N1 (NIBRG-23, Indo/5, VN1203) prepared from A/turkey/Turkey/1/05, A/Indonesia/05/2005, and A/Vietnam/1203/2004 avian influenza viruses with PR8 strain as a donor of internal genes. Viruses were obtained from CDC (Atlanta, GA) and propagated in 10 day old hen eggs.

Animal study

Female albino guinea pigs weighing 300–350 g were inoculated intranasally with 10^5 EID_{50} of virus without anaesthesia. Transmission studies were then performed 24 hours after inoculation. Inoculated animals were housed at 25% relative humidity and 22°C in the same cage with non-infected guinea pigs or in cages placed 3 m away from non-infected pigs. Virus replication was determined by virus isolation in hen eggs and by PCR. Sera were collected at 0 and 28 days post inoculation. Seroconversions were assessed by routine HAI test.

Genome composition and phenotype analysis

Genome composition of reassortants was monitored by RFLP analysis. Capacity of the viruses to grow at optimum, low, and elevated temperatures (*ca/ts* phenotype) was evaluated, and virus growth properties were observed following virus titration in hen eggs.

Results

Direct transmission of influenza viruses

When infected pigs were co-caged with non-infected (naïve) individuals, VN1203, Indo/5, A/California/7/2009, and NIBRG-23 were isolated in 0%, 25% 83·3%, and 100% of contact animals, respectively. Serological confirmation of virus transmission was higher than virological data (25%, 100%, 100%, and 100%, respectively). In addition, it was shown that when pigs inoculated with A/California/7/2009 were co-caged with animals inoculated with NIBRG-23, they got infected with both viruses (Table 1).

The ability of direct transmission of cold-adapted viruses was also investigated. Data show that the A/17/California/

Table 1. Transmission of influenza viruses between infected and naïve guinea pigs, which were co-caged or housed at a distance

	Confirmation of virus replication									
		Contact naïve guinea pigs, no. (%)								
	Infected guinea pigs, no. (%)	Co-caged			Distance of 3 m					
Virus	No.	PCR (%)	HAI (%)	No.	PCR (%)	HAI (%)	No.	PCR (%)	HAI (%)	Efficiency of transmission
NIBRG-23*	15	15 (100)	15 (100)	15	15 (100)	15 (100)	4	4 (100)	4 (100)	++++
Indo/5**	4	4 (100)	4 (100)	4	1 (25)	4 (100)	6	0	3 (50)	+++
H1N1 2009***	7	7 (100)	7 (100)	6	5 (83·3)	6 (100)	3	0	0	++
VN1203†	4	2 (50)	4 (100)	4	0	1 (25)	6	0	0	+
MDV††	5	4 (80)	5 (100)	3	0	0	4	0	0	None
H1N1/38†††	8	7 (87·5)	8 (100)	5	0	0	nd	nd	nd	None

*NIBRG-23 (H5N1) reassortant virus.
**Indo/05 (H5N1) reassortant virus.
***A/California/7/2009 (H1N1) virus.
†VN1203 (H5N1) reassortant virus.
††A/Leningrad/134/17/57 (H2N2) MDV.
†††A/17/California/2009/38 (H1N1) pandemic live attenuated influenza vaccine (LAIV) candidate.

2009/38 LAIV candidate was detected in the upper respiratory tract of 87·5% vaccinated pigs. The MDV was identified in 100% of infected animals. However, neither group of contact pigs, co-housed with the vaccinate pigs, had evidence of infection with cold-adapted viruses. In addition, none of the contact pigs had any evidence of seroconversion to the cold-adapted viruses as determined by HAI assay. It was also most interesting to note that pig-to-pig transmission of the highly transmittable NIBRG-23 reassortant virus was not seen when pigs, vaccinated with MDV, were co-caged with animals infected with NIBRG-23 virus (Table 1). This strongly implies a form of interference or protection from transmissibility that was provided by the cold-adapted virus.

Transmission of influenza viruses at a distance of 3 m

The results show that NIBRG-23 and Indo/5 viruses were able to spread between cages over the 3 m distance (100% and 50% naïve animals were successfully infected, respectively). A/California/7/2009 influenza and VN1203 viruses did not transmit between infected and non-infected guinea pigs housed in separated cages (Table 1). Pigs with confirmed A/California/7/2009 virus replication were also infected with NIBRG-23 virus if H1N1- and H5N1-infected animals were separated by a space. Thus, influenza virus transmission from H5N1- to H1N1-infected pigs has been shown, but the reverse pattern did not occur.

Transmission of NIBRG-23 or A/California/7/2009 viruses was not observed when contact pigs were first vaccinated with the MDV and housed at a 3 m distance (Table 2). It was also shown that efficiency of transmission of NIBRG-23 was much higher than of other studied H5N1 viruses; it can be transmitted between naïve guinea pigs separated from infected animals at a distance of 4–5 m (data not shown).

Reassortment of A/California/7/2009 virus with NIBRG-23

Five reassortants were isolated from animals which were infected with A/California/7/2009 virus and co-caged with pigs inoculated with NIBRG-23. Two reassortants possessed different combinations of PR8, NIBRG-23, and A/California/7/2009 genes and demonstrated the *non-ca/non-ts* phenotype typical of *wt* viruses. Unexpectedly, two other reassortants inherited HA gene from NIBRG-23, NA gene from A/California/7/2009, and other genes from PR8 became *ca* and *ts*. 7:1 *non-ts* reassortant inherited PA gene from PR8 and seven other genes from A/California/7/2009, gained *ca* properties.

Reassortment of MDV with NIBRG-23

In spite of aforesaid experimental data, we cannot exclude the theoretical possibility of simultaneous infection of

Table 2. Reciprocal transmission of influenza viruses between infected guinea pigs, which were co-caged or housed at a distance

No. animals	Treatment group	Virus isolation (PCR), No. (%)		Serological confirmation (HAI), No. (%)		Confirmed transmission
Direct transmission of influenza viruses between co-caged infected animals						
		MDV*	TUR**	MDV	TUR	
3	MDV	3 (100)	0	3 (100)	0	No
4	TUR	0	4 (100)	0	4 (100)	No
		SW***	TUR	SW	TUR	
3	SW	3 (100)	3 (100)	3 (100)	3 (100)	No
2	TUR	0	2 (100)	0	2 (100)	Yes
Transmission of influenza viruses between infected animals housed at a distance of 3 m						
		MDV	SW	MDV	SW	
10	MDV	8 (80·0)	0	9 (90·0)	0	No
4	SW	0	3 (75·0)	0	4 (100)	No
		MDV	TUR	MDV	TUR	
9	MDV	8 (88·9)	0	9 (100)	0	No
4	TUR	0	4 (100)	0	4 (100)	No
		SW	TUR	SW	TUR	
2	SW	2 (100)	2 (100)	2 (100)	2 (100)	No
2	TUR	0	2 (100)	0	2 (100)	Yes

*A/Leningrad/134/17/57 (H2N2) MDV.
**NIBRG-23 (H5N1) reassortant virus.
***A/California/7/2009 (H1N1) virus.

human host with cold-adapted and *wt* influenza viruses. To better understand possible consequences of such a reassortment event, we co-infected guinea pigs with a mixture of MDV and NIBRG-23 viruses. Nasal washes were collected and cloned by limited dilutions in hen eggs in the presence or absence of immune serum to the MDV. Cloning of nasal washes without antiserum led to isolation of over 100 clones, which were all identical to the MDV (data not shown). When nasal washes were cloned in the presence of antiserum, only nine clones were isolated. Genome composition analysis showed that all isolates were triple reassortants, which had inherited PB2 and NA genes from MDV, PA gene from PR8, and HA gene from NIBRG-23. The origin of the other gene segments (PB1, NP, M, NS) in the genome of guinea pig-derived reassortants varied. Reassuringly, all reassortants generated *in vivo* had the phenotype typical of the MDV.

Discussion

The severity of influenza outbreaks is partly determined by efficient spreading of the causative virus strain between human hosts. However, little is known about mechanisms underlying influenza virus transmission in humans.

Guinea pigs have been shown to be a suitable model for influenza transmission studies.[1] Our *in vivo* study showed that influenza A viruses vary in their transmissibility. NIBRG-23 and Indo/5 viruses were able to transmit to naïve animals caged distantly from infected animals. In contrast, cold-adapted viruses, the same as those used for licensed LAIVs, showed no signs of transmission from one guinea pig to another. Our study also provided evidence of a lower level of transmissibility of the novel pandemic H1N1 virus compared to the NIBRG-23 and Indo/5 H5N1 strains evaluated.

Benefits of vaccination with LAIV to aid in the control of influenza outbreaks are acknowledged by the WHO.[2] In our study, the MDV inoculated into guinea pigs appeared to interfere with and even offer protection from transmission of the highly transmissible NIBRG-23 virus. The ability to immunize with the LAIV and subsequently block the spread of a homologous H3N2 subtype and a heterologous H3N2 subtype influenza virus between guinea pigs has been shown.[3] Interference between cold-adapted and wild-type influenza virus infection was the most likely explanation for the data observed in our study. The MDV inoculated into guinea pigs might in some way interfere with transmission of highly transmissible influenza viruses.

It is believed by some that widespread use of LAIV could increase the potential risk of reassortment of the vaccine strain with circulating influenza viruses immediately following vaccination. However, it was shown that any such potential reassortments would most likely lead to yet attenuated viruses.[4] Our *in vivo* studies have shown that introduction of MDV genes into the genome of NIB-RG-23 virus led to the generation of triple reassortants inherited PB2 and NA genes of MDV and HA gene of H5N1 virus. All isolates possessed phenotypical markers associated with attenuation of MDV. Our data suggest that even if a reassortment event of such rare occurrence between a LAIV strain and a circulating virus were to occur, it would most likely lead to a reassortant that would retain highly attenuated phenotypic properties of the vaccine strain.

Our data strongly support the safety of LAIVs, especially those developed against highly transmissible H5N1 and H1N1 pandemic influenza viruses. This information builds upon databases that have clearly shown the low likelihood of transmitting an LAIV, as well as the high likelihood of any field reassortment of LAIV with a circulating influenza virus to retain important properties of the cold-adapted, temperature-sensitive vaccine master composition. Very interestingly, we also present data that show the potential of a LAIV to prevent the transmission of highly infectious influenza viruses, perhaps identifying a broader role for LAIV in the overall scheme of influenza virus prophylactic use.

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Schlieren imaging: a real-time, non-invasive method to visualize human exhaled airflows to assist aerosol infection control

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Keywords Aerosol, airborne, airflow, imaging, infection control, influenza, non-invasive, real-time, Schlieren, transmission, visualization.

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Abstract

Background: Schlieren imaging is a non-invasive, real-time airflow visualization technique that relies on differences in air temperatures (and the resulting changes in the refractive index) to allow exhaled human airflows to be seen clearly against the background of more-stationary, ambient air. Recently, this technique, well-known to engineers, has been applied to better understand and characterize airflow behaviors associated with everyday, as well as healthcare-related, human respiratory activities.

Materials and methods: As a surrogate marker for the behavior of airborne infectious agents, Schlieren imaging was used to visualize the airflow patterns produced by adult human volunteers of different ages while coughing with and without the wearing of standard surgical and N95 masks.

Results: The cough plumes were generally similar in shape and range for all the adult volunteers used in this

study. Although both the surgical and N95 masks decelerated and blocked some of the forward momentum of the coughed airflows, much of the cough plume was redirected and escaped around the top, bottom, and side edges of the masks to merge with the volunteer's natural, vertically-moving thermal plume.

Conclusions: Schlieren imaging is a safe technique for visualizing exhaled airflows from human volunteers without the need for potentially-irritant or toxic particle tracers. Findings from these Schlieren imaging experiments will assist the development of more effective aerosol infection control guidelines in healthcare premises where patients infected with potentially airborne infectious agents (e.g., influenza and tuberculosis) are present. These infectious agents may be transmitted to healthcare workers, other patients, and their visitors by way of exhaled airflows.

Introduction

With the recent influenza pandemic[1,2] and the ongoing concerns about human cases of avian influenza H5N1 infections,[3] there is now a very real concern about the potential for the aerosol transmission of respiratory pathogens. Such concerns amongst staff and patients in healthcare environments have led to a greater emphasis on the understanding and control of infectious airflows.[4,5] Previous visualization techniques have used potentially-toxic or irritant gas or particulate tracers with hazardous laser light sources that have precluded the use of human volunteers as subjects. Instead, various forms of lung models that simulate human respiratory patterns with such particulate tracers have been used.[6,7]

Schlieren imaging is a technique familiar to engineers and offers a non-invasive (i.e., no tracer required) airflow visualization method that depends only on differences in the refractive index of the warmer, human-exhaled air and the cooler ambient air.[8] The use of a simple incandescent or light-emitting diode (i.e., non-laser) light source is safe and allows human volunteers to be used as experimental subjects, where their exhaled airflows are then observed using a large, precise spherical or parabolic telescopic mirror and a camera, and are recorded for later analysis and presentation.[9–11]

The analysis of these patterns of 'real-life' human airflows will be useful in optimizing aerosol infection control guidelines, which aim to reduce the transmission of airborne infectious agents to other healthcare personnel, patients, or their visitors.

Materials and methods

The images and analysis presented here have all been obtained from the large 1 m diameter parabolic mirror (Figure 1) situated at the Gas Dynamics Laboratory of Penn State (directed by Gary S. Settles). This large Schlieren imaging system has been in use for over 30 years to obtain high quality Schlieren images for various engineering applications. It has only recently been applied to clinically-relevant imaging.

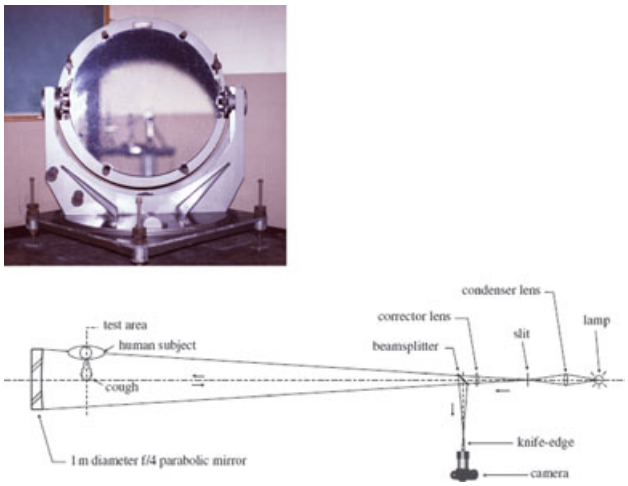

Figure 1. Large, 1-m diameter Schlieren mirror imaging system at the Gas Dynamics Laboratory, Pennsylvania State University. (Photograph by Gary S. Settles).

The objective of this paper is to augment and expand upon the details of the methods and results presented in an earlier study using this same Schlieren imaging system.[10] The aim of this series of studies is to visualize and capture a series of airflow images produced by coughing from adult human volunteers of different ages (24–80 years old). These included males (three of 26 years, one of 80 years of age) and females (one of 24 years, one of 30–40 years, and one of 40–50 years of age). Each volunteer was tested with and without wearing either a standard surgical mask or N95 mask. More specifically, the aim was to visualize the extent and direction of leakage around the mask whilst each subject was coughing. Penn State Institutional Approval for experiments involving human subjects was also obtained.

Each volunteer was asked to stand approximately 1 m in front of the Schlieren mirror, facing across the surface of the mirror on one side, and to cough several times as the real-time, color image and video footage was recorded by the operator (using a Nikon D90 camera; Nikon Inc. Melville, NY, USA). This process was repeated whilst each volunteer was wearing a standard surgical mask then an N95 mask (supplied by 3M™, St Paul, MN, USA).

Results

Some of the Schlieren images obtained from some of these volunteers have been published previously: for a 26-year old male,[9] the 24 year-old female and a 26-year old male,[10] and the 40–50 year-old female.[11] This article completes this series of Schlieren images obtained from these experiments

Figure 2. Examples of Schlieren images of an 80 year-old man, whilst coughing without wearing any mask (A), whilst wearing a surgical mask (B) and whilst wearing an N95 mask (C). (All images by Thomas J Liebner and Gary S. Settles).

by including the images recorded for the older, 80 year-old man.

Generally, it was found that the shape of the cough plumes (shown in the figure as darker shadows emanating from the subject's mouth) produced by adult humans of different ages was relatively similar. Cough plumes are roughly conical in shape and very turbulent, usually passing beyond the extent of the 1 m mirror (Figure 2A). A previous detailed study of one of these images measured a maximum airflow velocity of 8 m/second for an adult cough.[9] Similarly, the effects of wearing surgical and N95 masks can be generalized across different ages. Wearing a surgical mask allows leakage of the coughed air from the sides, top, and bottom of the mask (Figure 2B). There is also some leakage through the mask, as indicated by the darker patches of air directly in front of the mask (Figure 2B, C).

The useful effect of the mask appears to be a deceleration and redirection of this coughed (and potentially infectious) air into the natural, upward-rising human thermal plume, which captures it and carries it upwards where it is diluted and less likely to transmit infection to others. The effects of the N95 mask are similar (i.e., deceleration and redirection), yet due to its tighter (mask-fitted) face seal, more of the coughed air appears to penetrate the front of the mask (Figure 2C). This penetrating air is, however, also decelerated sufficiently to allow the wearer's natural thermal plume to carry it upwards.[10,11]

Discussion

From these series of Schlieren images presented in this and other related studies,[9–11] it is clear that Schlieren imaging offers a safe, non-invasive, real-time technique to visualize human exhaled airflows for all age groups.

It is apparent that, at least where airflow patterns are an acceptable surrogate marker for airborne transmission risks, there are beneficial effects of wearing either type of mask, even when the mask fit is relatively poor. This is often the case when N95-style masks are purchased and used by the general public – in contrast to the situation with healthcare workers, who are often accurately fit-tested for this type of mask. The immediate significance of this can be seen when masks are bought by parents for their children. Often, these will not be of pediatric size and the mask-fit will be loose. Children are well-known to be major sources of infection in the community because of their relatively poor immunity to many types of infectious agents due to their young age and, therefore, limited past-exposure history.[12]

These images allow infection control teams to literally see how far and how fast potentially-infectious human exhaled airflows can travel from an individual. This may have significant implications for guidance on the wearing of masks for infected staff and patients, on ward bed-spacing, as well as for the types of masks to be used in different situations. The important practical potential lies in the non-intrusive visualization of airflows associated with human volunteers, to assist in heightening the awareness amongst healthcare workers of the risks and potential for the airborne transmission of infectious agents, as well as the development of more effective aerosol infection control policies.

Schlieren images can be analysed more quantitatively, e.g., with the 'Schlieren-PIV' technique,[9,13] though this additional quantitative data is probably more of research interest than being of immediate practical use to everyday hospital infection control teams.

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Relationship of subtype influenza A pandemic strains to virucidal activity of a quaternary ammonium disinfectant

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Keywords Disinfection susceptibility, plaque assay, TCID50.

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Introduction

Spread of influenza infection is mitigated not only through antiviral agents and vaccination, but also by treatment of environmental surfaces with surface disruptive chemical germicides. Little data is available on the comparative susceptibility of pandemic strains of Influenza A to chemical agents. We have embarked on a systematic study of the effect of various germicides on strains of influenza. The present work deals with BAC, a dual active antimicrobial agent accepted world-wide as both a disinfectant and antiseptic and various subtypes of Influenza A.[1] The major antigenic changes[2] in the influenza genome over the past 50 years have involved hemagglutinins H1, H2, and H3 producing the pandemics of 1957 (A/Japan/305/57/H2N2), 1968 (A/Hong Kong/8/68/H3N2), and the novel swine flu pandemic of 2009 (A/California/04/2009/H1N1). These are the subtypes that we have studied. Clearly, the question arises as to whether the changes in antigenicity are coupled with changes in germicide susceptibility.

Materials and methods

We have employed a modified log-reduction method[3] in a cell culture system employing MDCK cells[4] in serum-free Ex-Cell™[5] medium supplemented with trypsin. Microscopic examination of CPE was the marker for infectivity together with plaque assay. We confirmed antiviral potency by using specific subtype Influenza identification subtype technology, Quidel QuickVue® Influenza A + B Test.

Results

The log inactivation and percent inactivation by BAC after a 60 second contact time for the H1, H2, and H3 pandemic strains are as follows: A/Swine/Iowa/12/30 H1N1, 3·5 log/99·97%; A/Swine/Cal/2009 H1N1, 4·8 logs/99·998%; A/J305/57/H2N2, 5 logs/99·999%; and A/Hong Kong 8/68 H3N2, 5·0 logs/99·999% (Table 1).

Discussion

Comparable results of Antiviral efficacy are obtained with the $TCID_{50}$ and Plaque assays against all subtypes studied. When performing the plaque assay the sensitivity of virus recovery was better in the vessel with a larger surface area and overall recovery was in agreement with the potency determined by $TCID_{50}$ assay.

In our plaque assay, we inoculated A/Hong Kong/8/68 virus dilutions into two different vessels with 2 hours adsorption time: 6-well plate and T-25 flask, 9 ml inoculum per replicate. Virus titers obtained were: $1{\cdot}4 \times 10^6$ pfu/ml from 6-well plate and $2{\cdot}2 \times 10^6$ pfu/ml from T-25 flask (Table 2). The discrepancy on virus potency can possibly be explained as: the binding of virus to host cell occurs only when virus gets a chance to interact with the cell on the monolayer during adsorption time. The percentage of virus population in the inoculum that has the opportunity to bind to the cell mainly depends on the surface area where this interaction takes place. Therefore, in our experiment the plaque assay in the T-25 flask gave

Table 1. Efficacy of BAC against four strains of Influenza A using high titer viral pools

Antiviral = BAC 60 second contact time		H1N1 A/Swine/Iowa/15/30				H1N1 A/Swine/Cal/04/09				H2N2 A2/J305				H3N2 A2/Hong Kong/8/68				Representative inactivity all subtypes			
Viral Infectivity Assay	10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	T	T	T	T
	10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	T	T	T	T
	10^{-3}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
	10^{-4}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
	10^{-5}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
	10^{-6}	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
	10^{-7}	0	0	0	0	+	+	+	0	0	0	0	0	+	+	+	+	0	0	0	0
	Cell control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$TCID_{50}$		$10^{6\cdot0}$				$10^{7\cdot3}$				$10^{6\cdot5}$				$10^{7\cdot5}$				$\leq10^{2\cdot5}$			
Δ Log		≥3·5				≥4·8				≥4·0				≥5·0				Legend: 0 = no virus; T = cytotoxic; + = virus			

Table 2. Comparison of viral titer obtained in different vessels using quantal $TCID_{50}$ and plaque assay methods

Virus recovery			
Plaque assay		**$TCID_{50}$ assay**	
T-25 ($25\ cm^2$)	**6-well plate ($9\ cm^2$)**	**$TCID_{50}$/ml**	**TCID/ml**
$2{\cdot}2 \times 10^6$ pfu/ml	$1{\cdot}4 \times 10^6$ pfu/ml	5×10^6	$2{\cdot}5 \times 10^6$

higher virus recovery 2·2 10^6 versus $1{\cdot}4 \times 10^6$ pfu/ml. The increased virus recovery can translate into better sensitivity of the test system for disinfectant and antiviral agents. The potency of the virus used in this study was determined by $TCID_{50}$ was 5×10^6 $TCID_{50}$/ml.

Rapid diagnostic testing for influenza (QuickVue® Influenza A + B Test, Quidel) for AJ305 versus BAC was studied. The presence of Influenza viral nucleoprotein A determined by QuickVue kit correlated 100% with the viral infection based on by CPE in viral culture. Interestingly, the inactivation of viral nucleoprotein was able to be revealed with diagnostic kit in the dilutions of virus/BAC reaction mixture, which possessed prominent cytotoxic effect for the host cells in viral culture system. This type of molecular testing method is useful for interpreting antiviral efficacy against a background of cytotoxicity.

These experiments are intended for the sponsor to substantiate to US FDA that their antiviral substances are safe and effective. The data shows that the three hemagglutinin subtypes were highly susceptible to the Quaternary Ammonium Compound in the short term *in vitro* experiment. The appearance of novel subtypes in the future can be met with the assurance that disinfectant and/or antiseptic resistance will be unlikely. Certainly, from the above data, although genetic reassortment of human and swine viruses may modulate influenza pathogenesis and limit existing vaccine benefit, it is not likely be a factor in control of viruses on environmental surfaces by Benzalkonium-type disinfectant/cleaning agents in community or health care environments.

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Outbreak of viral respiratory infections in an aged care facility in NSW – lessons about a pandemic virus

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Keywords Aged care facility, cross-reacting HAI antibody, outbreak, pandemic (H1N1) 2009, rhinovirus.

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Abstract

Influenza in aged care facilities (ACFs) is associated with an increased risk of poor health outcomes among residents, including death. In this paper we share our experience of managing an outbreak of viral respiratory infection in an ACF very early in the 2009 influenza pandemic and also describe some of the emerging issues relating to cross-reacting antibodies to the pandemic (H1N1) 2009 influenza virus in the very elderly.

The outbreak investigation was conducted as part of an urgent public health intervention initiated by the New South Wales (NSW) Department of Health during the early stages of the first southern hemisphere wave of the 2009 pandemic. Nose and throat swabs for nucleic acid testing (NAT) plus acute and convalescent serum samples (6 weeks apart) were collected from all the residents of an ACF where an influenza-like illness (ILI) outbreak occurred.

The investigation revealed dual outbreaks of pandemic (H1N1) 2009 influenza and rhinovirus infection. Out of 28 residents, three had laboratory confirmed influenza [two with pandemic (H1N1) 2009], and 10 had rhinovirus infection on NAT. Testing of acute sera collected from every subject found elevated (≥1:40) pandemic (H1N1) 2009 HAI antibody in 60% (9/15) subjects aged 85 years or more (born before 1925 and median age 88 years; Geometric Mean Titre-GMT 48·1) compared with none of the 13 residents aged under 85 years (born after 1924 and median age 79 years; GMT 10·1, $P = 0{\cdot}01$). The ACF was closed to visitors for 7 days. The symptomatic residents received treatment-dose oseltamivir, and all other residents were given oseltamivir prophylaxis.

More than one virus may be circulating in an ACF with an ILI outbreak at any one time in winter. A significant proportion of elderly residents had pre-existing cross reacting antibody to the pandemic (H1N1) 2009, which may explain the minimal clinical impact of pandemic (H1N1) 2009 in this elderly population.

Introduction

Influenza is one of the leading causes of infectious death in elderly people, principally due to co-morbidities and declining immune competence with age. It is the most important agent in outbreaks of respiratory illness.[1] Influenza in aged care facilities (ACFs) is associated with an increased risk of poor health outcomes among residents, including death.[2] The clinical presentation of influenza in residents of ACFs can be subtle, with a blunted febrile response and a non-specific decline in mental and functional status.[3] Residents commonly have underlying diseases that can be exacerbated by influenza infection, and in addition, they are at higher risk of serious influenza-related complications than community dwelling elderly people.[4]

People aged over 65 years are also at higher risk of influenza-related death, and more than 90% of annual influenza-related mortality is usually confined to this high risk group.[5] In Australia, influenza and pneumonia have sub-

stantial health impacts; recorded as being the underlying causes of death for 2623 persons in 2007.[6] Since the World Health Organization declared an influenza pandemic in June 2009, Australia has suffered one of the highest rates of confirmed infection during the first southern hemisphere wave. By late October 2009 there were 187 reported deaths due to pandemic influenza in Australia,[7] and to date there have been about 18 449 deaths reported worldwide.[8] Although disproportionately far fewer elderly people developed clinical influenza during the current pandemic than occurs with seasonal influenza, their case-fatality rate remained substantial.[9]

Early in the pandemic (June 2009), we investigated a suspected pandemic influenza outbreak in a rural ACF in the state of NSW, Australia. The epidemiology (including virulence and clinical outcome in the elderly) of the pandemic (H1N1) 2009 virus was mostly unknown at the time of investigation, and as time passed, this investigation provided clarity on some important issues of the influenza epidemiology in the elderly population. In this paper we share our experience of managing a dual outbreak of viral respiratory infections early in the pandemic, and also describe some of the emerging issues relating to the cross-reacting antibodies to pandemic influenza in the very elderly.

Materials and methods

The outbreak investigation was conducted as part of urgent public health intervention initiated by the NSW Department of Heath in conjunction with the local public health unit, the National Centre for Immunisation Research and Surveillance (NCIRS), and the Institute of Clinical Pathology and Medical Research (a WHO National Influenza Centre). To determine the extent and cause of the outbreak, a public health research doctor (GK) was dispatched from Sydney over a weekend to assist with outbreak investigation and control.

Time line of the outbreak and epidemiological investigation

On June 12th 2009, the Greater Southern Public Health Unit surveillance officer (BD) received a report of a possible pandemic (H1N1) 2009 outbreak in a local ACF. On investigation, it was discovered that 3 days earlier a 77 year old female resident had become generally unwell, but without specific symptoms of influenza like illness (ILI). Soon after, nine of the 27 co-residents (but no staff) had developed symptoms suggestive of influenza. One other resident had returned from a Melbourne (Victoria) hospital (where pandemic (H1N1) 2009 was known to be circulating) the previous week after surgery, but did not have ILI symptoms.

On June 10th, the 10 symptomatic residents had nasal swabs taken by the local doctor for influenza [including pandemic (H1N1) 2009] nucleic acid testing (NAT). There was rising concern due to reports of widespread pandemic (H1N1) 2009 influenza in a local Army camp just over the border in nearby Victoria, where pandemic (H1N1) 2009 influenza was known to be circulating widely. On June 12th, the 77 year old lady proved NAT positive for pandemic (H1N1) 2009, but none of the other samples were pandemic (H1N1) 2009 NAT positive. Concern arose that there might be an outbreak of pandemic (H1N1) 2009 in the facility, and that some of the swabs from other residents might be false negatives.

Between 14 and 15 June, after consent was obtained, directly or through next of kin in 13 demented residents, all 28 submitted to venipuncture for serology, 27 successfully, and the other 18 as yet un-swabbed residents were swabbed. Basic demographic data were collected from every resident with clinical information on co-morbidities and current medication use. Convalescent blood samples were collected after 4 weeks on 16th July 2009 from 23 of the 28 residents.

Laboratory investigation

Swabs were sent to ICPMR where NAT for influenza A [including pandemic (H1N1) 2009] and B was performed. The acute and convalescent serum samples were tested later (in December 2009), using haemagglutination inhibition assay (HAI) to detect pandemic (H1N1) 2009 antibody.[10,11]

Interventions

The ACF was closed to visitors from 12th until 18th June. Treatment of the positive case and the nine symptomatic residents, with twice daily oseltamivir, was begun on Saturday June 13th, and all other residents were started on once daily oseltamivir prophylaxis. The facility manager and local general practitioner (GP) monitored patient health on a daily basis, and none had to stop oseltamivir due to adverse events. One resident with ILI who was known to have moderately impaired renal function was given once daily rather than twice daily oseltamivir treatment.

Results

The age range of the residents was 58–97 years with a median of 85 years. All residents had underlying medical conditions, e.g., chronic cardiac and respiratory diseases (Table 1). All 28 residents had received seasonal influenza vaccine in early 2009. Out of 28 staff members, 27 also had been given seasonal influenza vaccine in April 2009.

There were 10 symptomatic residents (39·3%). Of the other 18 residents, none developed symptoms between June

Table 1. Demographic and clinical findings of the outbreak investigation

Age	Median 85 years (range 58–96 years)
Sex	Female 53·6% (15/28) male 46·4% (13/28)
Co-morbidities	
Chronic cardiac	64·3% (18/28)
Dementia	50·0% (14/28)
Osteoarthritis/osteoporosis	46·4% (13/28)
Respiratory	17·8% (5/28)
Diabetes	14·3% (4/28)
Number of co-morbidities	
4 or more	42·8% (12/28)
2 or more	64·3% (18/28)
any co-morbidity	100·0% (28/28)
Symptomatic residents	35·7% (10/28)
Cough	90·0% (9/10)
Coryza	90·0% (9/10)
Headache	70·0% (7/10)
Fatigue	60·0% (6/10)
Sore throat	50·0% (5/10)
Nausea/vomiting	30·0% (3/10)
Temperature/subjective fever	10·0% (1/10)

12th and 15th. The 'index' case of pandemic (H1N1) 2009 was confirmed on 12th June 2009 by NAT. Subsequently a second resident proved NAT positive for influenza A, but this was not sub-typed. Another case of pandemic (H1N1) 2009 was later confirmed on convalescent serum which showed a significant rise (from 10 to 80) in HAI titre.

Testing of acute sera collected from every subject found elevated (≥1:40) cross-reacting HAI antibody to the pandemic (H1N1) 2009 in 60% (9/15) of subjects aged 85 years or more (born before 1925 and median age 88 years; Geometric Mean Titre-GMT 48·1). However, the HAI titre was consistently <1:40 and significantly lower (GMT 10·1, $P = 0{\cdot}01$) in the 13 residents aged under 85 years (range 58–83 years, median 79 years) (Figure 1). The index case (NAT positive) did not show a significant raise in HAI level in convalescence (going from 20 to 40).

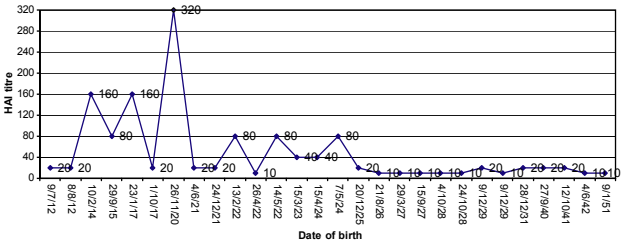

Figure 1. Pandemic influenza A (H1H1) 2009 cross-reacting HAI antibody according to birth year.

The pandemic (H1N1) 2009 case that was determined by serology was pandemic (H1N1) 2009 NAT negative.

To our surprise, seven of the other 18 asymptomatic residents had rhinovirus detected on extended NAT (reported on June 25th), despite being asymptomatic at time of swabbing and remaining so. The original nine influenza NAT negative samples were then tested and three of these were also NAT positive for rhinovirus; in total, ten proved NAT positive for rhinovirus (35·7%). The serologically confirmed pandemic (H1N1) 2009 case was also positive for rhinovirus infection. Of interest was that only one resident had a documented fever.

Discussion

This investigation illustrates some of the difficulties in managing and investigating possible influenza outbreaks in real time in the context of an influenza pandemic.[12] Finding a NAT positive case of pandemic (H1N1) 2009 influenza among many other symptomatic cases raised the possibility (although not the probability) that pandemic (H1N1) 2009 was the cause of the outbreak. Rhinovirus infection, however, was confirmed by NAT in ten residents. This outbreak illustrates that more than one virus (in this case 2 and perhaps 3) may be circulating in an ACF at any one time in winter. In ILI outbreaks in ACFs, broad laboratory testing is recommended; NAT is the most sensitive method of detecting influenza or other viruses in respiratory tract samples.[13]

Studies have found that the pandemic (H1N1) 2009 haemagglutinin (HA) gene is more closely related phylogenetically to the 1918 H1N1 virus and classical swine influenza A/H1N1 viruses than more recent seasonal human influenza A/H1N1 viruses.[14] It is antigenically similar to the 1918 H1N1 pandemic virus in terms of the immunodominant antibody response to haemagglutinin.[15–17] It is likely that individuals alive during the emergence and initial persistence of the 1918 pandemic virus would have higher levels of cross-reacting HAI antibodies to the pandemic (H1N1) 2009, which would contribute towards better clinical protection.[18] In our investigation, 60% of the residents born before 1925 (aged 85 years or above in 2009) had pre-existing cross-reacting HAI antibody to the pandemic (H1N1) 2009.

In elderly populations, severe illness may be associated with organisms typically considered to be mild, such as rhinovirus. However, studies have shown that nursing home residents may be susceptible to outbreaks of rhinovirus that may cause mild to severe respiratory illness, particularly in those with a history of lung disease. One rhinovirus outbreak in a nursing home in the USA caused 12 fatalities.[19] Another outbreak showed residents with underlying lung disease are more likely to have longer infection, require

antibiotics, develop bronchospasm, and have difficulty breathing; two residents with underlying lung disease required emergency treatment and one died.[20] A previous influenza outbreak in a NSW aged care facility in 2006 caused significant mortality and morbidity. That outbreak resulted in 14 hospital admissions and six deaths.[21] In our investigation we have found that 17% of the residents had chronic lung disease and 64% had chronic cardiac conditions both considered as high risk for severe complications of both rhinovirus and influenza infection. However, there were no hospitalisations or deaths in our outbreak investigation. Indeed only one resident developed fever, indicating that non-specific signs of illness (such as in our index case) may be the only, or early, indication of an ILI. Our own experience with managing other ILI outbreaks has also taught us that staff of ACFs may not be vigilant enough to detect fevers.

In this outbreak, the nursing home staff, local GP, public health unit and the outbreak investigation team and supporting laboratory staff acted quickly and in a coordinated way. Pre-existing cross-reacting antibody in the very elderly (aged ≥85 years) probably helped to limit the spread of the pandemic virus (compared to the circulation of rhinovirus) within the ACF. Exposure to the 1918 pandemic (or a close variant occurring before 1925) appears to be responsible for a high HAI titre in the very elderly, which contributed towards better clinical protection. However, wider testing early on would have alerted us more quickly to the main cause of the outbreak. Treatment and prophylactic use of oseltamivir may also have contributed to halting the spread of pandemic (H1N1) 2009 and also to symptom relief.

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Conflicts of interest

RB has received pharmaceutical industry support from CSL, Sanofi, GSK, Novartis, Roche and Wyeth, to attend/present at scientific meetings; if fees were offered, these were placed in a university research account. LH has received financial support from Novartis to attend/present at scientific meeting. The other authors declare that they have no conflict of interest in relation to this work.

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Detection and isolation of pandemic (H1N1) 2009 influenza virus from stool sample

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Keywords AH1pdm virus, binding specificity, gastrointestinal symptoms, stool, viral shedding.

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Introduction

Pandemic (H1N1) 2009 influenza virus (AH1pdm) has spread worldwide since March 2009. In a paper of AH1pdm, 25% of infected individuals have experienced gastrointestinal symptoms such as diarrhea and vomiting, which is higher than that of seasonal influenza. However, little is known whether viable virus shed from stool and replication of viruses are ongoing in the gastrointestinal tract.[1,2] Viral load and isolation of AH1pdm in cell culture in stool samples has been reported.[3]

Materials and methods

Stool specimens were collected from 35 patients suspected to have Pandemic (H1N1) 2009 infection from November 2009 through May 2010. Virus isolation was conducted in cell culture by using Madin–Darby canine kidney (MDCK) cells and TaqMan based RT-PCR from 10% (w/v) stool suspension in phosphate-buffered saline. TaqMan based RT-PCR was conducted by using primers, probes, and positive controls provided by NIID (National Institute of Infectious Diseases of Japan). To confirm presence of AH1pdm viral RNA, LAMP (Loop-Mediated Isothermal Amplification) was used as supplemental testing. Of patients, one child (Case 1) submitted one nasal swab and four stool samples, another one nasal swab and two stool samples, and the other one stool sample. Informed consent was obtained. Strand specific RT-nested PCR was performed for only case 1 by using only one primer at the RT reaction and also assayed Neu5Acα2-3Gal and Neu5Acα2-6Gal binding specificity about isolated strain derived from nasal swab and stool. Receptor binding specificity was performed using a solid-phase binding assay with the sialylglycopolymers (poly α-l-glutamic acid backbones containing Neu5Acα2-3Galβ1-4GlcNAcβ-pAP or Neu5Acα2-6Galβ1-4GlcNAcβ-pAP bond as described.[4]) Nucleotide sequences of the HA gene of AH1pdm viruses isolated from stool sample and nasal swab were analysed.

In order to exclude the possibility of contamination, the stool samples and nasal swabs were subjected to virus isolation separately. After getting the results on the nucleotide sequence, we also confirmed no strain harboring identical sequence was isolated in our laboratory before and after the day of sample collection.

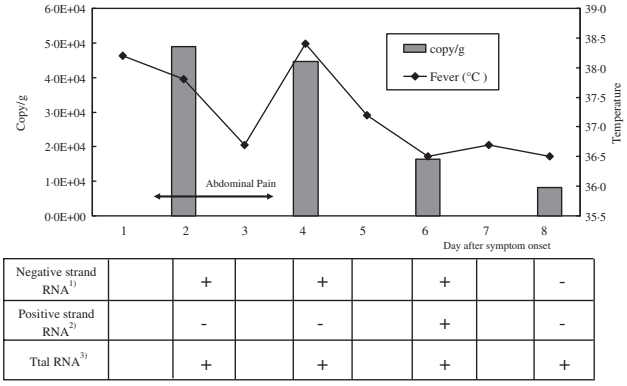
Results

AH1pdm viral RNA was detected in nine (25%) of the subjects from stool samples. Among nine subjects, one case (Case No. 1) was positive for viral isolation. Case1, a healthy 9-year-old girl, experienced fever and abdominal pain, and the others had gastrointestinal symptoms without upper respiratory symptoms. In case 1, influenza A virus was diagnosed by rapid antigen test on the day of symptom onset. Viable AH1pdm virus was isolated from the stool sample and nasal swab on the second day from onset using MDCK cells (Table 1). Viral load decreased gradually after

Table 1. Results of AH1pdm influenza virus detection and isolation in case 1

Days after symptom onset	1	2	3	4	5	6
Rapid antigen test result	+		+		–	
Collection of nasal swab						
Virus isolation (MDCK)		+				
Real-time RT-PCR Ct		33·42				
Collection of stool specimen						
Virus isolation (MDCK)		+		–		ND
Real-time RT-PCR Ct		33·92		37·99		39·22

MDCK, Madin–Darby canine kidney.



Negative strand RNA[1)]		+		+		+		-
Positive strand RNA[2)]		-		-		+		-
Ttal RNA[3)]		+		+		+		+

1) Negative stranded RNA ; genomic RNA 2) Positive stranded RNA ; replicating RNA, mRNA
3) Total RNA ; +/-

Figure 1. Persistent viral shedding of AH1pdm in stool specimens and results of strand-specific RT-nested PCR.

symptom onset. However, viral shedding was still present 8 days after symptom onset. Positive stranded RNA was detected 6 days after symptom onset from the stool specimen (Figure 1). Above two AH1pdm strains (isolated from nasal swab and stool specimen) bound exclusively to human type receptor, Neu5Acα2-6Gal. Sequence analysis demonstrated that isolated virus from stool samples was identical with that from nasal swabs in comparison of HA gene (990 bp).

Discussion

AH1pdm influenza virus was isolated from the stool and nasal swab samples in the same patient simultaneously by using MDCK cells. Our results suggests the detection of viral RNA and viable AH1pdm influenza virus from stool samples may serve as a potential mode of transmission and has important implications in understanding the context of AH1pdm influenza virus.

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Inactivation of influenza viruses by coated respirators: *in vitro* infectivity assays

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Keywords Acid polymers, Actiprotect, antiviral coating, antiviral masks, infectivity assay, respirators.

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Introduction

Strategies to prevent transmission of influenza include use of respirators. FFP2 and N95 respirators are certified to filter at least 95% of particles (0·3 μm in diameter), and many guidelines have recommended that healthcare workers wear respirators in certain healthcare settings to protect against infection from patients with pandemic influenza.[1–3]

We have developed a proprietary acid-polymer formulation to coat a standard FFP2 respirator with an antiviral layer. We aimed to test this coated respirator for antiviral efficacy against a range of influenza viruses. A series of tests compared the antiviral efficacy of coated and uncoated respirators in conditions designed to simulate real-life exposure to influenza by varying the route of inoculation, contact time, temperature, humidity, moisture, and contaminating substances. We also investigated whether infectious viruses could be transferred from contaminated respirator surfaces to gloves.

Materials and methods

We tested human, swine, and avian influenza viruses, including influenza A and B viruses. Influenza A subtypes were the A/H1N1 2009 pandemic strain, seasonal H1N1, H5N1, H3N2, H5N9, and H2N2. In each test, suspensions of influenza viruses were prepared to 4–8 $\log_{10}$ $TCID_{50}$/ml in MEM. In some tests, organic contaminants (yeast, BSA, and mucin) were added. One set of respirators was maintained at 40°C and 75% relative humidity for 24 hours before the viral challenge, and repeatedly sprayed with HEPES buffer to simulate respiratory secretions.

For each test, three coated (GlaxoSmithKline *Actiprotect*) and three uncoated (Sperian Willson *Easy Fit*) FFP2 respirator samples were inoculated with 0·2 ml of a viral suspension, which was applied with a pipette, sprayed, or aerosolised to create airborne droplets. After 1 minute at room temperature (on a shaker), the respirator samples were assayed for the presence of infectious viruses using standard methods.[4] In one test, after a 1 minute contact time of the respirator with the virus, nitrile gloves were applied with light pressure to the outer surface of inoculated respirator samples and then assayed after 1 minute.

Samples were put into test medium (MEM, supplemented with antibiotics [penicillin, gentamycin, or streptomycin] and amphotericin B or l-glutamine). The supernatants were vortexed, extracted, and used to prepare serial 10-fold dilutions in MEM. Each dilution was used to inoculate four wells of RMK cells in a multi-well plate, and these cultures were incubated and scored over 7 days for cytopathic effects, cytotoxicity, and viability. (Some tests substituted MDCK cells; others used inoculated embryonated chick eggs.) All tests included negative cell controls, cytotoxicity controls, and neutralisation controls. The Spearman–Karber formula was used to calculate viral loads as $TCID_{50}$ or EID_{50}.[4] Antiviral efficacy was calculated from the difference between the geometric mean loads of influenza virus on the coated and uncoated respirators after 1 minute of exposure.

Results

The viral loads applied to respirators in these experiments ranged from 5·5 to 8·1 $\log_{10}$ $TCID_{50}$, and were therefore high in comparison with respiratory secretions from infected patients at the peak of influenza symptoms (range 3–7 $\log_{10}$ $TCID_{50}$).[1] Tables 1–2 show that the average viral loads detected on uncoated FFP2 respirator samples remained high in all conditions tested, ranging from 3·2 to 6·9 $\log_{10}$ $TCID_{50}$ (or 4·5–5·0 $\log_{10}$ EID_{50}). In contrast, the average viral load on coated respirators after 1 minute of exposure ranged from below the limits of detection to ≤1·5 $\log_{10}$ $TCID_{50}$ (1·0 $\log_{10}$ EID_{50}). Therefore, the relative antiviral efficacy of the coating ranged from ≥2·7 to 6·4 $\log_{10}$. Table 1 shows that the relative antiviral efficacy of the coated mask remained high in simulated-use conditions such as organic contaminants and repeated saturation at high temperature and humidity.

In the experiment to test transfer of viruses from respirators, the gloves applied to regular uncoated inoculated respirators had a viral load of 3·5 $\log_{10}$ EID_{50} (Table 1). By contrast, no viruses were detected on either the coated respirators or the gloves applied to them. The relative reduction in contamination was therefore ≥2·5 $\log_{10}$.

Table 1. Viral loads on respirator surfaces

	Uncoated respirators	Coated respirators	Mean relative reduction in viral load
Viral load*	5·5	≤0·7	≥4·8 $\log_{10}$
Viral load with organic contaminants*	6·1	0·7	5·3 $\log_{10}$
Viral load after heat, moisture, and simulated secretions**	5·1	0·8	4·3 $\log_{10}$
Viral load transferred to glove**	3·5	≤1·0	≥2·5 $\log_{10}$ EID_{50}

*Influenza subtype was A/H5N1, and strain was VNH5N1-PR8/CDC-RG.
**Influenza subtype was A/H3N2, and the strain was Hong Kong/8/68. Results are mean $\log_{10}$ $TCID_{50}$, unless specified otherwise.

Table 2. Antiviral activity against different types and subtypes of influenza

Influenza subtype	Influenza strain	Uncoated respirators	Coated respirators	Mean relative reduction in viral load
A/H1N1	NYMC-X-179A*	5·2	≤1·5	≥3·7 $\log_{10}$
A/H1N1	Mexico/4108/2009*	6·7	≤0·9	≥5·8 $\log_{10}$
A/H1N1	Swine/Iowa/15/30	5·6	≤0·5	≥5·1 $\log_{10}$
A/H1N1	JPN/35/2007	6·5	≤1·4	≥5·1 $\log_{10}$
A/H1N1	JPN/36/2007	6·7	≤0·5	≥6·2 $\log_{10}$
A/H2N2	A2/JP/305/57	5·0**	≤1·0**	≥4·0 $\log_{10}$
A/H3N2	JPN/12/2007	6·9	≤0·5	≥6·4 $\log_{10}$
A/H3N2	JPN/31/2007	5·1	≤0·5	≥4·6 $\log_{10}$
A/H3N2	Hong Kong 8/68	5·0**	≤1·0**	≥4·0 $\log_{10}$
A/H5N1	VNH5N1-PR8/CDC-RG	4·8	≤0·5	≥4·3 $\log_{10}$
A/H5N9	TurkeyA/Wisc/68	5·0**	≤1·0**	≥4·0 $\log_{10}$
A/H5N9	MynaA/Mass/71	5·0**	≤1·0**	≥4·0 $\log_{10}$
B	JPN/128/2007	5·2	≤0·8	≥4·4 $\log_{10}$
B	JPN/143/2007	5·7	≤0·8	≥4·9 $\log_{10}$
B	JPN/85/2007	5·2	≤0·9	≥4·3 $\log_{10}$
B	JPN/115/2007	3·2	≤0·5	≥2·7 $\log_{10}$

Results are mean $\log_{10}$ $TCID_{50}$, unless specified otherwise, based on an infectivity assay in triplicate. Limits of detection varied.
*2009 pandemic strains.
**Results are mean $\log_{10}$ EID_{50}, based on a haemagglutinin assay in duplicate.

Discussion

The coated respirators inactivated a broad range of influenza strains within 1 minute, including the 2009 pandemic strain and human, swine, and avian influenza viruses. Antiviral effectiveness was not reduced by hot, humid conditions or repeated saturation, which might occur during prolonged use of respirators. In contrast, infectious virions were detected on the surfaces of all uncoated FFP2 respirators, and could be transferred to glove surfaces during handling of contaminated masks.

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Disclosure

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Filtration of aerosolised influenza viruses through respirators with antiviral coating: *in-vitro* infectivity assays

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Keywords Acid polymers, antiviral coating, antiviral masks, disposable respirators, FFP1, FFP2, FFP3, filtration.

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Introduction

Strategies to prevent transmission of influenza include use of respirators, and many guidelines have recommended that healthcare workers wear respirators in certain healthcare settings for protection against pandemic influenza.[1–3] FFP3 respirators are certified in Europe to filter at least 99% of NaCl particles (0·3 μm in diameter), and FFP2 and FFP1 respirators must filter at least 95% and 80% of these particles, respectively. Influenza A viruses are typically 0·1 μm, and can be carried in aerosolised droplets smaller than 1 μm in diameter, which can disperse widely, remain airborne for 8 hours, and be inhaled deeply into the respiratory tract.[4] We have developed an acid-polymer formulation to coat the outer layer of a standard FFP2 respirator, in order to provide antiviral activity on the outer surface. We compared this coated respirator against standard FFP1, FFP2, and FFP3 respirators for filtration of aerosolised influenza viruses. The aim was to simulate protection against infectious viruses in droplets released when infected people cough and sneeze, and during aerosol-generating procedures in healthcare settings.

Materials and methods

The first assay compared three samples of coated FFP2 respirators (GlaxoSmithKline *Actiprotect*) with three FFP2 controls (Sperian Willson *Easy Fit*). For each test, suspensions of influenza A (H3N2) at 7·1 $\log_{10}$ $TCID_{50}$/ml in 0·1× minimum essential medium (MEM) were aerosolised with a nebulizer. The airborne droplets were introduced into a sterile chamber upstream of a respirator sample for 2 minutes, at a flow rate of 28·3 l/minute. Constant airflow was maintained for another 2 minutes after exposure to the virus. Then the collection dish in the downstream sieve sampler (Anderson) was assayed for infectious viruses using standard techniques.[5] Briefly, serial dilutions of the collection medium (MEM with 1% FBS, 5% gelatine, and 2% HEPES, supplemented with antibiotics and amphotericin B) in MEM + trypsin were used to inoculate Madin–Darby canine kidney epithelial (MDCK) cells in quadruplicate in a multi-well plate. These cultures were then incubated and scored over 4–6 days for cytopathic effects, cytotoxicity, and viability. Negative cell controls and cytotoxicity and neutralisation controls were also performed. The Spearman–Karber formula was used to calculate $TCID_{50}$.

The second assay compared five samples of coated respirators with five FFP1 controls (3M *9310*) and five FFP3 controls (3M *1863*). A suspension of influenza A (H1N1), at 8·3 $TCID_{50}$/ml, was nebulized for 1 minute and 40 seconds into the aerosol chamber, at a flow rate of 28·3 l/minute, followed by constant airflow for 5 minutes after exposure to the virus. Then the collection medium in the downstream chamber (as before, with 1% $NaHCO_3$) was assayed as described above.

Results

Initial viral loads in the first and second assays were 8·1 and 7·9 $\log_{10}$ $TCID_{50}$, respectively, and were therefore high in comparison with respiratory secretions from infected patients at the peak of their influenza symptoms (range 3–7 $\log_{10}$ $TCID_{50}$). Table 1 shows that the average viral load that passed through the uncoated FFP2 respirators in the first assay was 3·6 $\log_{10}$ $TCID_{50}$. The average viral load that passed through the coated respirators was 1·4 $\log_{10}$ $TCID_{50}$. Therefore, for active filtration of viruses, the relative efficacy of the respirator with antiviral coating was 2·2 $\log_{10}$ greater than the uncoated respirator. For surface inactivation, the relative antiviral efficacy of the coated respirator was 3·9 $\log_{10}$.

In the second study, Table 2 shows that the average viral load that passed through the uncoated FFP1 respirators was 5·2 $\log_{10}$ $TCID_{50}$. In contrast, 3·1 $\log_{10}$ $TCID_{50}$ passed through the coated FFP2 respirators. By comparison with the

Table 1. Viral loads passing through coated and uncoated FFP2 respirators

	Uncoated FFP2 respirators	Coated FFP2 respirators	Mean relative reduction in viral load (coated versus uncoated FFP2
Viruses passing through respirators	3·6	1·4*	≥2·2 $\log_{10}$
Viruses remaining on respirator surfaces	5·2	≤1·3*,**	≥3·9 $\log_{10}$

Influenza subtype was A/H3N2, and strain was Hong Kong/8/68. Results are mean $\log_{10}$ $TCID_{50}$, unless specified otherwise.
*Limit of detection was 1·3 $\log_{10}$ $TCID_{50}$.
**No virus was detected on two of three masks.

Table 2. Relative reduction in viral load for uncoated FFP1 and coated FFP2 respirators

	Uncoated FFP1 respirators	Coated FFP2 respirators	Uncoated FFP3 respirators	Mean relative reduction in viral load
Viruses passing through respirators	5·2	3·1	5·2	2·1 $\log_{10}$ versus FFP1 2·1 $\log_{10}$ versus FFP3
Viruses remaining on respirator surfaces	7·3	3·6	7·1	3·8 $\log_{10}$ versus FFP1 3·6 $\log_{10}$ versus FFP3

Influenza subtype was A/H1N1, and strain was A/PR/8/34. Results are mean $\log_{10}$ $TCID_{50}$, unless specified otherwise. FFP1 sample was 3M9310. FFP3 sample was 3M1863. The total viral load detected with no respirator was 8·9 $\log_{10}$ $TCID_{50}$.

viral load when no respirator was present (8·9 $\log_{10}$ $TCID_{50}$), the FFP1 respirators reduced the viral load by 3·7 $\log_{10}$, and the coated FFP2 by 5·8 $\log_{10}$. Therefore, for active filtration of viruses, the respirators with antiviral coating reduced the viral load by 2·1 $\log_{10}$ more than the FFP1 respirators.

In this second study, the average viral load that passed through the uncoated FFP3 respirators was also 5·2 $\log_{10}$ $TCID_{50}$. By comparison with the viral load when no respirator was present (8·9 $\log_{10}$ $TCID_{50}$), the FFP3 respirators reduced the viral load by 3·7 $\log_{10}$. Therefore, for active filtration of viruses, the respirators with antiviral coating reduced the viral load passing through the mask by 2·1 $\log_{10}$ more than the FFP3 respirators.

Table 2 also shows that the coated respirators reduced the infectious viruses remaining on the mask surfaces by 3·8 $\log_{10}$ more than the FFP1 respirators, and 3·6 $\log_{10}$ more than the FFP3 respirators.

Conclusions

Even with a very high viral challenge, the coated respirators prevented passage of at least an additional 2·1 $\log_{10}$ infectious viruses, compared with uncoated respirators. Large numbers of infectious virions passed through all uncoated respirators tested. FFP3 respirators were no more effective than FFP1 respirators at blocking airborne influenza viruses. Based on these *in-vitro* results, respirators with the antiviral coating could be expected to provide more protection than standard respirators from the risk of inhaling influenza viruses.

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Disclosure

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Characterisation of stability, cytotoxicity, and skin sensitisation and irritation for respirators with antiviral coating

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Keywords Acid polymers, antiviral coatings, antiviral masks, cytotoxicity, disposable respirators, sensitization, skin irritation.

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Introduction

Strategies to prevent transmission of influenza include use of respiratory protection. FFP2 and N95 respirators are certified to filter at least 95% of NaCl particles (0·3 μm in diameter), and many guidelines have recommended that healthcare workers wear these respirators in certain healthcare settings to protect against infection from patients with pandemic influenza.[1,2] We have developed a proprietary acid-polymer formulation, designed to coat a standard respirator and inactivate influenza viruses on contact. We tested this coated respirator for cytotoxicity, skin irritation, and sensitisation potential. The antiviral coating was also tested for stability and leaching under extreme environmental conditions, such as physical abrasion and simulated breathing at different temperatures, levels of humidity and CO_2, and saturation with contaminants.

Materials and methods

Stability of coating under simulated conditions

Eight coated respirators were tested at standard relative humidity (60% RH) for 8 hours, and one at elevated humidity (80% RH) for 2 hours. Four coated masks were treated with synthetic blood or oral secretions, and then tested at 30% RH for 1 hour. The sample respirators were sealed onto a mannequin head inside an airtight chamber, and air at 30°C and 5000 ppm CO_2 was pumped through the masks by a cyclic breathing machine at 24 l/minute. A 37 mm glass-fibre filter was placed behind the respirator, over the mannequin's mouth opening. At the end of all tests, these filters were eluted and analysed using high-performance liquid chromatography (HPLC).

Cytotoxicity of mask materials

Standard *in vitro* methods were used to assess the cytotoxicity of the coated polyester and uncoated polypropylene layers of the respirator (GlaxoSmithKline *Actiprotect*).[3] Samples were extracted in minimum essential medium (MEM), supplemented with serum, penicillin, streptomycin, amphotericin B, and l-glutamine, at 37°C for 24 hours. Triplicate monolayers of mouse fibroblast cells (L-929) were dosed with each extract (including a reagent control and negative and positive controls), and incubated at 37°C in 5% CO_2 for 48 hours. After 48 hours of incubation with samples or controls, the monolayers of mouse fibroblast cells were examined microscopically for abnormal cell morphology or cellular degeneration.

Skin irritation or sensitisation by mask materials

Samples of the coated respirator (comprising four polypropylene layers bonded to the coated polyester outer layer) were applied under occlusive patch conditions to the skin of 51 adults. Controls, including individual layers, were applied in the same way. In a separate patch test, samples of the coated polyester outer layer and controls were

applied under the same conditions to 219 adults. After 24 hours, test patches and controls were removed. Sites were then scored for itching, erythema, oedema, epidermal damage, and papular response after 48 and 72 hours. The patches were applied three times a week for 3 weeks. To evaluate sensitisation, test patches were applied 10–15 days later for 24 hours at different sites to the original samples. After this challenge, skin was assessed and graded for sensitisation potential after 48 and 72 hours.

Results

Stability of coating under simulated conditions

Table 1 shows that no residues of the antiviral coating or degradation products were detected in the air that had passed through any of the eight respirators.

Table 1. High-performance liquid chromatography tests

	Relative humidity (%)	Time (hours)	Residues detected
Coated masks – Replicates A, B, C	60	8	None None None
Coated masks	80	2	None
Coated masks, simulated blood – Replicates A, B	30	1	None None
Coated masks, simulated oral secretions – Replicates A, B	30	1	None None

Cytotoxicity of mask materials

Cytotoxicity tests showed that the coated respirator material caused 10% cell lysis or toxicity, classified as slight reactivity (grade 1), and that uncoated material caused no cell lysis or toxicity (grade 0) (Table 2). Results for positive and negative controls were severe reactivity and no reaction, respectively.

Skin irritation or sensitisation by mask materials

From the results of the two human repeat-insult patch tests, neither the coated or uncoated layers nor the full-thickness respirator fabric caused irritation (including itching, erythema, edema, vesiculation, epidermal damage, papules, or reactions beyond the patch site) or sensitisation in any of the adult volunteers at any of the time points.

Conclusions

Based on these results, in conjunction with published data on acute and repeat-dose toxicity, mutagenicity, local irritation, dermal sensitisation, and inhalation safety for all components of the antiviral coating, the potential topical or inhalation exposure to the coated antiviral respirator does not pose a safety risk. The antiviral coating is durable and stable, and stays on the outer surface of the respirator, even in extreme environmental conditions. The coated respirator is non-irritating and non-sensitising. Therefore, this respirator is considered to be well-tolerated and safe for its intended use.

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Table 2. Cytotoxicity results for uncoated and coated mask material

	Rounded cells	Cells without intracytoplasmic granules	Cells with vacuolisation	Detached cells	Lysis	Grade*	Reactivity*
Uncoated material – Replicates A, B, C	0	0	0	0	0	0	None
	0	0	0	0	0	0	None
	0	0	0	0	0	0	None
Coated material – Replicates A, B, C	10%	0	0	10%	10%	1	Slight
	10%	0	0	10%	10%	1	Slight
	10%	0	0	0	10%	1	Slight

Scores are percentages of cells in each well.
*Grades: (0 = No reactivity = Discrete intracytoplasmic granules, with no cell lysis; 1 = Slight reactivity = No more than 20% of cells round, loosely attached, and without intracytoplasmic granules; 2 = Mild reactivity = No more than 50% of the cells round and devoid of intracytoplasmic granules; 3 = Moderate reactivity = No more than 70% of cell layers contain rounded cells or are lysed; and 4 = Severe reactivity = Near complete destruction of the cell layers).

ies were funded by GSK Consumer Healthcare, and GSK investigators were involved in all stages of the study conduct and analysis.

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Transmission of pandemic H1N1 2009 influenza in households and school settings: comparison with seasonal influenza

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Introduction

Knowing how influenza virus is transmitted at home and in school is the key to preventing its spread. At the previous two meetings of this Conference,[1,3] we introduced our study of household transmission of seasonal influenza and reported our conclusion that protracted survival of the virus even after treatment increases household transmission, and is a major factor in the transmission of the virus to infants. On the other hand, during the recent pandemic, many schoolchildren developed serious respiratory tract disorders, which again highlights the significance of school-based transmission of the disease. In this study, we compared transmission of a new influenza strain at home and in school with that of seasonal influenza and proposed countermeasures.

Materials and methods

The subjects used in the study of household transmission were 4844 individuals in 1271 households, including 1646 patients with seasonal influenza A (seasonal influenza), and 2404 individuals in 611 households, including 753 patients with the new influenza strain (pdmH1), during the past eight seasons (2001–2002 season to 2008–2009 season). Family members were classified as F (father), M (mother), or C (child), and children were further classified by age as C1 (0–6 years), C2 (7–12 years), C3 (13–18 years), or C4 (19 years or more), to give a total of seven groups in order to analyse the effect of family composition on household transmission.

For the analysis of school-based transmission, the epidemic status of seasonal influenza in 4237 children at six elementary schools over the past two seasons (2002–2003 and 2003–2004 seasons) was compared with that of pdmH1 in 1913 children at two primary schools.

Using observational data of school-based transmission, we also constructed a model for influenza transmission[3,4] and evaluated the effects of factors that could affect influenza transmission (e.g., antibody prevalence, transmission rate, non-infectious latent period, infectious latent period, school closure) through the use of 1000 simulations.

In this study, a diagnosis of influenza was confirmed by rapid influenza antigen detection kit. We previously reported the high sensitivity of the kits,[5–7] not only for seasonal influenza, but also for H1N1 pandemic 2009 compared to virus isolation and PCR. Serum antibody was not investigated. Most of the index patients were treated with oseltamivir or zanamivir, and 30 patients were treated with amatadine. No treatment was done for 16 patients. No NAI therapy was done as prophylaxis within the family.

Results

Incidence at home (household incidence) and household transmission rate

The incidence of households with an initial case patient who subsequently infected another member of the household was 21·6% (275 of 1271 households) for seasonal influenza or 18·3% (112 of 612 households) for pdmH1. Thus, the household incidence of pdmH1 was lower than that of seasonal influenza. In addition, the percentage of family members in households who were infected by initial case patients (household transmission rate) was 10·5% (375 of 3573 individuals) for seasonal influenza or 7·9% (141 of 1792 individuals) for pdmH1. Thus, the household transmission rate was also lower for pdmH1 than that for seasonal influenza.

Effect of family size on household incidence and household transmission rate

An analysis of the effect of family size on household incidence showed that, in families consisting of 2–7 individuals, the incidence of seasonal influenza in order of increasing family size was 3·5%, 17·4%, 24·5%, 24·2%, 32·4%, and 42·9%, respectively, and the incidence of pdmH1 was 0·0%, 13·5%, 17·8%, 24·7%, 42·9%, and 33·3%, respectively, indicating that household incidence tends to increase with increasing family size. In contrast, no definite relationship was noted between household transmission rate and family size. Transmission rates for seasonal influenza in order of increasing family size were 3·5%, 10·6%, 11·8%, 8·0%, 10·3%, and 7·1%, respectively, or 0·0%, 6·7%, 7·7%, 8·0%, 15·2%, and 5·6%, respectively, for pdmH1 (shown in Table 1).

Effect of age cohort of initial case patient in household on household incidence and household transmission rate

An analysis of the effect of the age cohort of the initial case patient in the household on household incidence and transmission rate showed that the household incidence of seasonal influenza in C1, C2, C3, and C4 was 26·7% (112 of 419 households), 21·4% (66 of 308 households), 16·3% (21 of 129 households), 9·9% (9 of 91 households), and for M and F was 19·6% (37 of 189 households) and 22·5% (29 of 129 households), respectively. Therefore, household incidence was the highest in C1, followed by the parents. When the initial case patient was a child, the household incidence increased with decreasing patient age. In contrast, the household incidence of pdmH1 in C1, C2, C3, and C4 was 23·1% (36 of 156 households), 17·3% (42 of 243 households), 8·1% (9 of 111 households), 10·0% (4 of 40 households), and for M and F was 30·8% (12 of 39 households) and 39·1% (9 of 23 households), respectively. Therefore,

Table 1. Incidence of influenza among family members living in the same household

	Seasonal Influenza A				pdmH1N1 2009			
	No. of families	No. of families with secondary infection (%)	No. of observed family members	No. of infected members (%) families	No. of infection (%)	No. of families with secondary members	No. of observed family members (%)	No. of infected
No. of family members								
Two	57	2 (3·5)	57	2 (3·5)	7	0 (0·0)	7	0 (0·0)
Three	407	71 (17·4)	814	86 (10·6)	171	23 (13·5)	342	23 (6·7)
Four	583	143 (24·5)	1749	207 (11·8)	326	58 (17·8)	978	75 (7·7)
Five	178	43 (24·2)	712	57 (8·0)	81	20 (24·7)	324	26 (8·0)
Six	37	12 (32·4)	185	19 (10·3)	21	9 (42·9)	105	16 (15·2)
Seven	7	3 (42·9)	42	3 (7·1)	6	2 (33·3)	36	2 (5·6)
Eight	2	1 (50·0)	14	1 (7·1)	0	–	–	–
Initial case age								
0–6 years old	419	112 (26·7)	1149	154 (13·4)	156	36 (23·1)	453	45 (9·9)
7–12 years old	308	66 (21·4)	937	95 (10·1)	243	42 (17·3)	692	48 (6·9)
13–18 years old	129	21 (16·3)	401	24 (6·0)	111	9 (8·1)	352	14 (4·0)
19 years old more	91	9 (9·9)	259	10 (3·9)	40	4 (10·0)	117	5 (4·3)
Mothers	189	37 (19·6)	468	48 (10·3)	39	12 (30·8)	110	14 (12·7)
Fathers	129	29 (22·5)	340	43 (12·6)	23	9 (39·1)	68	15 (22·1)
Grandparents	6	1 (16·7)	19	1 (5·3)	0	–	–	–
Total	1271	275 (21·6)	3573	375 (10·5)	612	112 (18·3)	1792	141 (7·9)

household incidence was higher when the initial case patient was a parent, rather than a child. The household transmission rates for seasonal influenza from C1 to F were 13·4%, 10·1%, 6·0%, 3·9%, 10·3%, and 12·6%, respectively. Therefore, as for household incidence, the highest rate (13·4%) was observed in C1. The corresponding household transmission rates for pdmH1 were 9·9%, 6·9%, 4·0%, 4·3%, 12·7%, and 22·1%, respectively, with the highest transmission rates observed for infections from parents (shown in Table 1).

Rate of individuals with a secondary infection in household transmission

If the rate of individuals with a secondary infection transmitted from the initial case patient in a household is presented as a percentage of the total number of affected individuals, the rates for seasonal influenza and pdmH1 were 22·8% (375 of 1646 individuals) and 18·9% (142 of 753 individuals), respectively. Therefore, the rate of individuals with a secondary infection was lower for pdmH1 than that for seasonal influenza. By age cohort, the corresponding rates of individuals for seasonal influenza in C1, C2, C3, and C4 were 22·3% (120 of 539 individuals), 11·5% (40 of 348 individuals), 12·2% (18 of 147 individuals), 7·1% (7 of 98 individuals), and for M and F was 42·7% (141 of 330 individuals) and 27·1% (48 of 177 individuals), respectively. For pdmH1, the corresponding rates in C1, C2, C3, and C4 were 24·3% (50 of 206 individuals), 9·3% (25 of 268 individuals), 9·0% (11 of 122 individuals), 4·8% (2 of 42 individuals), and for M and F was 51·9% (42 of 81 individuals) and 35·3% (12 of 34 individuals), respectively. These findings indicate that, especially in the case of pdmH1, most secondary infections in parents tend to be transmitted from another household member.

School-based transmission

The mean annual prevalence of seasonal influenza and the new influenza strain at the elementary schools for the two seasons was 19·6% and 10·3%, respectively, whereas the prevalence determined 7 days after appearance of the first case in school was 3·9% and 4·5%, respectively. In the recent season at the same elementary schools, however, the prevalence was a high 43·5%. Since the prevalence at 7 days after the appearance of the first case in school was already 17·5%, these data show that the influenza virus spread quickly throughout the schools. At the schools with high transmission rates in the early period of the pandemic, new infections were confirmed even 4 days after the school closure action was taken. These findings indicate that pdmH1, the current influenza virus, has a long latent period during which it becomes infectious and spreads from infected individuals to numerous others in their vicinity.

Model for influenza transmission

We constructed a model for influenza transmission in schools and estimated the time course of changes in the number of expected cases and the expected prevalence during the season. In this model, school children were divided into six groups depending on the stage of infection: uninfected period with no immunity, non-infectious latent period, infectious latent period, onset, post-onset infectious period, and immune period. It was assumed that school-based transmission occurred during the infectious latent period prior to onset and that no infections occurred during the post-onset infectious period because children were absent from school. Due to the long latent period of pdmH1, the distribution of the non-infectious latent period of pdmH1 was established as (Day 1, Day 2, Day 3, Day 4) = (10%, 40%, 40%, 10%) and the distribution of the infectious period as (Day 0, Day 1, Day 2) = (10%, 80%, 10%). When simulations were performed under these conditions using the model for school-based transmission of influenza in which children from classes with an outbreak were kept at home for 3 days, the time course of changes in the number of affected individuals actually observed and the time course of changes in the number of expected cases were determined. The expected prevalence under these conditions was 35%. To evaluate the effect of school closure, simulations were performed based on the assumption that children from affected classes were not kept at home for 3 days. It was shown that there was an increase in the expected number of cases during the 3 days corresponding to the period of actual school closure and that the expected prevalence increased to 52%. Based on these findings, it was concluded that keeping children home from classes with an outbreak is an effective means of controlling the transmission of influenza in schools (shown in Figure 1).

Discussion

If the transmissibility of pdmH1 virus at home is estimated based on the speed of transmission and the degree to which pdmH1 is prevalent in schools, it would be expected

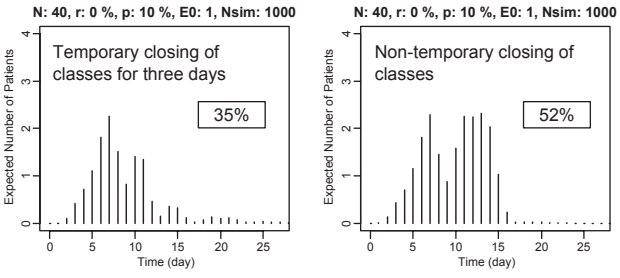

Figure 1. Simulation result in school setting with temporary closing of classes for 3 days or not by the proposed influenza transmission model.

that the household transmission of pdmH1 is also higher than that of seasonal influenza. In fact, the opposite is the case. This paradox can be explained in two ways.

1. The number of children aged 19 or more and parents with pdmH1 influenza as a percentage of the total number of affected individuals is lower than those with seasonal influenza (20·8% versus 37·2%). Further, although the number of parents with a secondary infection was high at home, the percentage of the total number of individuals with pdmH1 was a low 3·8% (29 of 754 individuals), compared to that for seasonal influenza (5·5% [91 of 1640 individuals]). In other words, adults are less susceptible to pdmH1 infections and there was a correspondingly small number of affected individuals. Therefore, it was considered that the transmission rate at home was lower than that at school for this reason.
2. The percentage of households with more than one affected individual within the same family was higher for pdmH1 at 28·5% (157 of 550 households) than for seasonal influenza at 24·4% (309 of 1265 households). In the patients secondarily infected with pdmH1, 26·8% of them showed symptoms of infection 10 days or more after the onset in the first patient, suggesting that they were not infected at home, and the actual household transmission was 20·9% (115 of 550 households). Therefore, although the prevalence was higher for pdmH1, it seems that household transmission was lower because households with an affected individual implemented satisfactory control measures against infection.

Conclusions

Seasonal influenza differs greatly from pdmH1 influenza in its transmissibility at home and in school. In the household transmission of pdmH1 influenza, both the household incidence and household transmission rate of pdmH1 were low compared to those for seasonal influenza. Although transmission of seasonal influenza from infants to parents was marked, in the case of pdmH1, the reverse was true with transmission from parents to children being predominant. It should be noted that household transmission in mothers was common in all eight seasons, suggesting the need to reconsider control measures against infection when nursing unwell family members.

In the case of school-based transmission, pdmH1 was more prevalent than seasonal influenza, indicating that the virus spread quickly throughout the schools. This difference was attributed to the long infectious latent period when pdmH1 rapidly became rampant in the schools. An analysis of school-based transmission using a model for influenza transmission showed that, when 10% of the student population is infected, schools should be closed for five consecutive days in order to minimize the spread of the disease.

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The effectiveness of seasonal influenza vaccine in preventing pandemic and seasonal influenza infection: a randomized controlled trial

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Introduction

Household transmission has been estimated to account for one-third of all influenza transmission,[1,2] and children are at high risk of spreading the disease. With reference to previous evidence,[3–15] some vaccine deployment strategies target children to prevent them from infection and transmitting influenza.[16] Nevertheless, few studies evaluated the effectiveness of vaccinating children in reducing household transmission.[10,11] During 2008–09, a pilot randomized controlled trial was conducted to investigate such effect by studying households with school age children randomized to receive trivalent inactivated seasonal influenza vaccine (TIV).[17]

The monovalent vaccine against pandemic influenza A (H1N1) (pH1N1) had yet been available until the end of the first wave. Various conclusions have been made as to whether seasonal influenza vaccine might possibly protect against pH1N1.[18–25] We report findings on the effectiveness of TIV against pH1N1 observed in our cohort.

Materials and methods

Households were screened if they expressed interest after receiving invitation letters distributed via their children's school or an existing pediatric cohort study.[26] To be eligible, the household had to include at least one child aged 6–15 years who was not allergic or hypersensitive to any of the TIV components. Children known to have immunosuppressive conditions or other contraindications against TIV were also excluded. Written consent and assent were obtained from participants aged above 18 years and those aged 8–17 years, respectively. Proxy written consent was obtained from legal guardians or parents for participants younger than 18 years. Ethical approval was obtained from the Institutional Review Board of the University of Hong Kong.

Consented households were allocated to the TIV and placebo group (in ratio 3:2) according a code generated by block randomization with random block sizes of 5, 10, and 15. An independent nurse prepared 0.5 ml of TIV (VAXIGRIP, Sanofi Pasteur; A/Brisbane/59/2007(H1N1)-like, A/Brisbane/10/2007(H3N2)-like, and B/Florida/4/2006 strains for 2008–09) and 0.5 ml of sterile saline solution into identical syringes labeled with the randomization code. One child (study subjects) from each household in the TIV group received a single dose of TIV with one child from each household in the placebo group receiving a single dose of saline placebo. Parents and legal guardians were asked to report any adverse reactions 4 days following vaccination. All participants, study nurses, and other research staff were blinded to the allocation and administration of vaccine or placebo. The vaccine allocation sequence was only disclosed to the investigators at completion of the study.

Serum specimens were collected from subjects shortly before (November–December 2008), one month after vaccination (December 2008–January 2009), and after the winter (April 2009) and summer influenza seasons (August–October 2009). Serum specimens were obtained from household contacts at baseline and after the winter and summer influenza seasons.

All household members recorded any fever ≥37.8°C, chills, headache, sore throat, cough, presence of phlegm, coryza, or myalgia daily on a symptom diary. They were

also invited to report to the study hotline immediately if they experienced at least 2 of the above signs or symptoms. As a response, the study nurse would visit the households with any sick members and collect nose and throat swab from all household members. The households were also telephoned monthly or increased to fortnightly during influenza seasons to monitor for signs and symptoms and remind them to report to the hotline. Supermarket or book vouchers (for children) were given to the households including US$13 for each serum specimen collected, US$6.5 for each home visit, and US$65 for completion of the study.

Serologically-indicated influenza infection was the primary outcome of this study. It was define as a ≥4 fold rise in antibody titer within each influenza season. Other study outcomes included RT-PCR confirmed influenza virus infection, acute respiratory illness (ARI) (two of any of the above listed signs or symptoms), and influenza-like illness (ILI) (fever ≥37.8°C with cough or sore throat).

Antibody titers against the vaccine strains were obtained by testing each serum specimens by haemagluttination inhibition (HAI). Viral microneutralization (VN) using standard methods was found to be more sensitive than HAI in detecting antibody response against A/California/04/2009(H1N1) in another study conducted by our group [27] and was, therefore, used in this study. The sera was initially diluted at 1/10 and further tested in serial doubling dilutions. Nose and throat swabs were tested by reverse transcription polymerase chain reaction (RT-PCR) for influenza A and B viruses. Technical details of the laboratory methods have been reported elsewhere.[27,28]

Fisher's exact test and chi-squared tests were used to compare count data including occurrence of side effects, laboratory confirmed, and clinically defined influenza infections. Wilcoxon signed-rank test were used to compare the serum antibody titers between groups. Exact binomial method or the Wald approximation was used to estimate 95% confidence intervals where appropriate. All analyses were carried out in R version 2.8.1 (R Development Core Team, Vienna, Austria).

Results

Twenty-five primary and secondary schools in the district of the study clinic were invited to participate. To parents of three schools that agreed to take part and another study cohort, 3690 invitation letters were sent and 105 households were enrolled. Personal referrals were made from these parents to enroll 14 additional households.

Among 119 enrolled households, 1 subject with history of epileptic seizure was assessed to be contra-indicated against receiving the vaccine. Blood taking failed in another subject, and both of them withdrew from the study. Eleven households did not complete the study. Table 1 shows subject and household contacts of the TIV and placebo group were similar in demographics and prior influenza vaccination history.

Table 1. Baseline characteristics of children who received TIV or placebo and their household contacts

Characteristics	TIV	Placebo
Study subjects	(*n* = 71)	(*n* = 48)
Male	41 (58%)	23 (48%)
Age group		
6–8 years	20 (28%)	16 (33%)
9–11 years	42 (59%)	28 (58%)
12–15 years	9 (13%)	4 (8%)
Received influenza vaccination for 2007–08 season	8 (11%)	7 (15%)
Household contacts	(*n* = 189)	(*n* = 123)
Male	86 (46%)	52 (42%)
Age group		
<15 years	47 (25%)	26 (21%)
16–45 years	94 (50%)	69 (56%)
>45 years	48 (25%)	28 (23%)
Received influenza vaccination for 2007–08 season	24 (13%)	12 (10%)
Received influenza vaccination for 2008–09 season	9 (5%)	5 (4%)
Households	(*n* = 71)	(*n* = 48)
Mean number of members (SD)	3.9 (0.9)	3.9 (1.2)
Mean flat size (square meters) (SD)	48 (22.8)	49 (23.6)

SD = standard deviation

Antibody titers before vaccination were comparable between groups (data not shown). Most study subjects who received TIV showed antibody titer ≥40 against the vaccine strains 1 month after receiving TIV, and the proportion was significantly higher than those who received placebo (A/H1N1 93% in TIV versus 68% in placebo group, $P < 0.01$; A/H3N2 97% versus 61%, $P < 0.01$; B 99% versus 91%, $P = 0.15$). None of the study subjects had antibody titer ≥40 against pH1N1 following receipt of seasonal TIV. No serious adverse reactions were reported, and only pain at injection sites was slightly higher in TIV group (data not shown).

Subjects who received TIV had lower rates of serologically confirmed seasonal influenza A(H1N1) (8% versus 21%, $P = 0.10$), A(H3N2) (7% versus 12%, $P = 0.49$) and B infection (3% versus 8%, $P = 0.36$, although the differences were not statistically significant (Table 2). Study subjects had higher rate of serologically confirmed pH1N1 infection (32% versus 17%, $P = 0.09$), yet it was not statistically significant. After adjusting for potential cross reactive antibody response, 31% of subjects in TIV versus 12%

Table 2. Attack rates of laboratory-confirmed influenza infections and acute respiratory illnesses in children who received trivalent inactivated vaccine (TIV) or placebo and their household contacts

	TIV		Placebo		
Outcome	**%**	**(95% CI)**	**%**	**(95% CI)**	***P*-value**
Study subjects	(*n* = 71)		(*n* = 48)		
Serologically-confirmed					
Seasonal A/H1N1	0.08	(0.02, 0.15)	0.21	(0.09, 0.32)	0.10
Seasonal A/H3N2	0.07	(0.01, 0.13)	0.12	(0.03, 0.22)	0.49
Pandemic A/H1N1	0.32	(0.22, 0.43)	0.17	(0.06, 0.27)	0.09
Seasonal B	0.03	(0.00, 0.07)	0.08	(0.01, 0.16)	0.36
RT-PCR-confirmed					
Seasonal A/H1N1	0.03	(0.00, 0.07)	0.04	(0.00, 0.10)	0.91
Seasonal A/H3N2	0.01	(0.00, 0.04)	0.02	(0.00, 0.06)	0.66
Pandemic A/H1N1	0.03	(0.00, 0.07)	0.00	(0.00, 0.07)	0.66
Seasonal B	0.00	(0.00, 0.05)	0.02	(0.00, 0.06)	0.84
ILI[†]	0.35	(0.24, 0.46)	0.38	(0.24, 0.51)	0.95
ARI[†]	0.66	(0.55, 0.77)	0.67	(0.53, 0.80)	0.89
Household contacts	(*n* = 189)		(*n* = 123)		
Serologically-confirmed					
Seasonal A/H1N1	0.13	(0.08, 0.17)	0.14	(0.08, 0.20)	0.91
Seasonal A/H3N2	0.21	(0.15, 0.26)	0.16	(0.10, 0.23)	0.41
Pandemic A/H1N1	0.17	(0.12, 0.23)	0.14	(0.08, 0.20)	0.48
Seasonal B	0.06	(0.02, 0.09)	0.10	(0.05, 0.15)	0.28
RT-PCR-confirmed influenza					
Seasonal A/H1N1	0.01	(0.00, 0.03)	0.01	(0.00, 0.02)	0.71
Seasonal A/H3N2	0.02	(0.00, 0.04)	0.01	(0.00, 0.02)	0.66
Pandemic A/H1N1	0.03	(0.00, 0.05)	0.01	(0.00, 0.02)	0.47
Seasonal B	0.00	(0.00, 0.02)	0.00	(0.00, 0.03)	1.00
ILI[†]	0.16	(0.11, 0.22)	0.11	(0.06, 0.17)	0.29
ARI[†]	0.42	(0.35, 0.49)	0.39	(0.30, 0.48)	0.64

*Winter infection confirmed by four-fold rise in antibody titers from post-vaccination to mid-season; summer infection confirmed by four-fold rise in antibody titers from mid-season to post-season. Results displayed reflect either winter or summer infection in the aggregate (see table S1 for winter and summer results separately).

[†]Influenza-like illness (ILI) defined as temperature ≥37.8°C plus cough or sore throat; acute respiratory illness (ARI) defined at least any two of fever ≥37.8°C, chills, headache, sore throat, cough, presence of phlegm, nasal congestion, runny nose, muscle or joint pain.

in placebo groups showed pH1N1 infection confirmed by either serology or RT-PCR ($P = 0.04$).

Little differences were observed for RT-PCR confirmed infection, ARI, and ILI in results combining the winter and summer influenza seasons. During winter season when seasonal influenza predominated, study subjects who had received TIV showed a lower tendency to develop ILI (15% versus 23%, $P = 0.43$) or ARI (46% versus 54%, $P = 0.52$). An opposite tendency was seen (ILI 27% versus 19%, $P = 0.43$; ARI 49% versus 44%, $P = 0.68$) during summer when pH1N1 predominated. However, these differences were not statistically significant. Rates of ILI in subjects infected with pH1N1 did not differ statistical significantly between subject who received TIV and placebo (40% versus 25%, $P = 0.30$). The study was not powered to detect indirect benefits to household contacts of vaccines resulting from reduced household transmission. Attack rates were found to be similar between household contacts of subjects received TIV and placebo (data not shown).

To examine potential factors that might affect risk of laboratory confirmed pH1N1 infection, a multivariable logistic regression model was fitted to study all subjects and their household contacts. Younger participants aged below 16 years were found to have a higher risk (<16 years OR = 6.60, 95% CI 2.17, 20.13; 16–45 years OR = 2.53, 95% CI 0.80, 7.99, >45 OR = 1.00). After adjusting for age, sex, and date of study completion, receipt of TIV for the 2008–9 influenza season was not found to affect risk of pH1N1 infection. However, participants who had laboratory confirmed seasonal influenza infection during the study period had 65% lower risk of pH1N1 infection (infected with seasonal influenza OR = 0.35, 95% CI 0.14, 0.87; not infected with seasonal influenza OR = 1.00). As

limited by the sample size, we were not able to differentiate between the protective effect of seasonal A(H1N1) and A(H3N2) infection against pH1N1. Other details of the results from the study were published elsewhere.[17]

Discussion

A non-significantly higher rate of pH1N1 infection was observed in study subjects who received TIV compared to placebo. Results from a multivariable logistic regression suggested that such a pattern might be explained by more common seasonal influenza infection in placebo group prior to the pandemic, protecting the placebo group against pH1N1. Seasonal influenza infection within 3–6 months observed in our study might have conferred better cross protection than TIV against pH1N1. This resembles similar previous findings on cross protection between influenza infections in human and animal studies.[29–36]

However, the same phenomenon has not been observed in some studies on seasonal influenza vaccine against pH1N1.[18,21,23–25] Apart from differences in study design and vaccine used, we speculate that a short time interval between pH1N1 and most recent seasonal influenza peak activities might be crucial for the phenomenon. Hong Kong is a subtropical area where the 2009 pandemic was preceded immediately by summer seasonal influenza circulation and a few months apart from the winter 2008–9 influenza peak. If cross protection from seasonal influenza lasts for only a short period, it might have waned below partial cross protection from TIV over time from last seasonal influenza infection. The current study is limited by a small sample size, and further studies are required to confirm our hypothesis.

While TIV is only effective against matching strains, a universal influenza vaccine could provide better protection against the ever evolving influenza viruses.

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Relationships between reactogenicity and age, dose number and immunogenicity in 937 children receiving two doses of $ASO3_B$-adjuvanted split virion or whole virus H1N1 influenza A 2009 pandemic vaccines

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Keywords AS03-adjuvanted pandemic influenza vaccine, immunogenicity, non-adjuvanted pandemic influenza vaccine, pandemic influenza vaccine, reactogenicity.

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Introduction

Immunisation of healthy, as well as high risk, children has been the focus of much recent attention both in prevention of seasonal influenza and during the 2009 H1N1 pandemic. Detailed information on reactogenicity, particularly for newer vaccine formulations that include adjuvants, is limited. We recently reported results of a head-to-head comparison of two 2009 H1N1 pandemic influenza vaccines in children in the UK.[1] Here we present new, detailed analyses of reactogenicity data from that study, which has important potential implications for future paediatric influenza vaccine development and use.

Materials and methods

We compared the safety, reactogenicity, and immunogenicity of two H1N1 influenza vaccines, one $AS03_B$ (tocopherol based oil in water emulsion) adjuvanted egg culture derived split virion, the other non-adjuvanted cell culture derived

whole virion, given as two dose schedules 21 days apart, in a randomised, open label trial as previously reported. The study was age stratified (6 months to under 3 years & 3–12 years) to ensure adequate data in young children. Age appropriate safety data (simplified for under 5 year olds) were collected for 7 days after each vaccine dose and serum was collected at enrolment & 21 days after the second dose. Nine hundred-thirty seven children received vaccines as per-protocol.

Results

When comparing the two vaccines, grade 3 (≥50 mm) local reactions were seen more frequently following the adjuvanted than the non-adjuvanted vaccine in both age groups, after both vaccine doses. In children over 5 years old, 7·2% versus 1·1%, $P < 0·001$, after dose one; 8·5% versus 1·1%, $P = 0·002$, after dose two, in children under 5 years old, 1·5% versus 0·0%, $P = 0·06$, after dose one (non significant, NS); 5·9% versus 0·0%, $P < 0·001$ after dose two. Fever ≥38°C (axillary measurement) was seen more frequently following the second dose of the adjuvanted vaccine compared to the non-adjuvanted vaccine in <5 year olds (22·4% versus 12·5%; $P < 0·05$).

Looking specifically at the adjuvanted vaccine in under 5 year olds, comparing the second dose with the first, there were significantly higher rates of fever ≥38°C (axillary measurement) (22·4% versus 8·9%, $P < 0·001$), local grade 3 (≥50 mm) reactions (5·9% versus 1·5%, $P = 0·02$), pain (39·4% versus 31·5, $P = 0·02$), use of analgesia or antipyretic medication (43·7% versus 31·5%, $P < 0·001$), and decreased activity (31·9% versus 20·4%, $P < 0·001$).

The adjuvanted vaccine was significantly more immunogenic, most notably in the younger children. In <3 year olds, haemagglutination inhibition (HI) seroconversion rates were 98·2% versus 80·1%, $P < 0·001$.

Among all general and local reactions measured, only the maximum temperature measured during the 7 days after the second dose of the adjuvanted vaccine showed a significant (positive) association with post vaccination HI titres. For each 1°C rise in temperature there was a 27% increase in titre ($P < 0·001$).

Discussion

These reactogenicity data demonstrate a step towards the future possibility of one-dose influenza immunisation programmes for young children associated with low rates of fever and other reactions. The occurrence of fever following adjuvanted vaccine, seen particularly after a second dose in younger children, was quantitatively associated with enhanced antibody titres. This association was not seen with unadjuvanted vaccine. This apparent difference between the relatedness of the pyrogenic and immunogenic effects of the two vaccines merits further investigation. Novel adjuvants appear to have the potential to overcome the relatively poor immunogenicity previously experienced with inactivated influenza vaccines in infants and young children. However, careful adjustment may be needed to optimise the balance between high protection and acceptable reaction rates.

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Development of pandemic live attenuated influenza vaccine (LAIV) in Russia

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The 2009 influenza pandemic was caused by a new strain of H1N1 influenza virus. At the same time, highly pathogenic avian influenza H5N1 virus with potential to cause a pandemic continues to circulate in poultry, in some coun-

tries causing sporadic human infections. Vaccination has been used as an effective public health tool for influenza prophylaxis. The goal of this study was to evaluate live attenuated influenza vaccine (LAIV) vaccine candidates for subtypes H1 and H5. The attenuated phenotype of H1 and H5 LAIV candidates has been proven in experiments *in ovo* and *in vivo*. In randomized clinical trials among adult volunteers, no significant adverse reactions attributable to the live vaccine occurred. Our results indicate that pandemic LAIV candidates were well tolerated and elicited serum, local, and cellular immune responses.

Introduction

The emergence and spread of highly pathogenic avian influenza H5N1 viruses in avian populations and concurrent infections in humans since 1997 has prompted efforts to develop vaccines for use in the event of an influenza pandemic. In 2009, the world faced a new H1N1 pandemic. Immunization with inactivated or live vaccines is the primary measure for preventing influenza. LAIVs appear to be safe and efficacious, and might possibly provide broader immune responses than inactivated vaccines. Our study evaluated LAIV pandemic candidates as part of the global influenza pandemic preparation project outlined by the WHO.

Materials and methods

Viruses

A/California/7/2009 (H1N1) pandemic virus, A/duck/Potsdam/1406-86 (H5N2) non-pathogenic avian virus, A/Leningrad/134/17/57 (H2N2), master donor virus (MDV) for LAIV, A/17/duck/Potsdam/86/92 (H5N2), and A/17/California/2009/38 (H1N1) pandemic LAIV candidates were used. The wild type (*wt*) viruses were obtained from US CDC (Atlanta, GA). Pandemic LAIV candidates were generated by classical reassortment of *wt* viruses with MDV in embryonated hen eggs.

Phenotype analysis

Capacity of the viruses to grow at optimum, low, and elevated temperatures (*ca/ts* phenotype) was evaluated by routine technique in embryonated hen eggs.

Study vaccine, study population, study design

LAIV and placebo were supplied by MICROGEN (Irkutsk, Russia). The monovalent LAIV was produced from the pandemic vaccine candidates and formulated to contain 10^7 and $10^{6.9}$ EID_{50} per dose (0·5 ml) of A/17/California/2009/38 and A/17/duck/Potsdam/86/92, respectively. The vaccine or placebo was administered intranasally with a single-use dosing nasal sprayer. Two doses were given at an interval of 21 days.

One hundred-ninety healthy adults aged 18–60 years were randomly divided into groups to receive either pandemic vaccine candidates (204) or placebo (28). Subjects were informed about purposes and methods of the study and potential risks associated with participation. All participants had an HAI antibody titer of ≤1:10 to A/California/7/2009 (H1N1) pandemic virus. In all there were 47 and 42 vaccines and 19 and 8 participants who received placebo, and were further tested for immune responses to H1N1 or H5N2 pandemic vaccine, respectively. Another 29 participants vaccinated with H1N1 LAIV were children between 12 to18 years old. Before the children were vaccinated, their parents were advised about study and their consent was required before any child was enrolled. On the advice of the National Ethics Committee, we did not include a placebo group in this study. Individuals were not enrolled if they had an acute illness or fever at the beginning of the study or a history of egg allergy.

Immune responses

Immune responses of subjects were assessed by routine HAI test (evaluation of serum IgG antibodies), ELISA (evaluation of IgA antibodies eluted from the nasal swabs into steril PBS), and cytokine flow cytometry assay (evaluation of virus–specific $CD3^+$ $CD4^+$ $IFN\gamma^+$ and $CD3^+$ $CD8^+$ $IFN\gamma^+$ peripheral blood mononuclear cells).

Results

Preclinical attenuation analysis

The results of phenotypic analysis *in ovo* showed that pandemic vaccine candidates retained the cold adapted-temperature sensitive (*ca/ts*) phenotype, typical of the cold-adapted parental MDV. In contrast and as expected, A/California/07/2009 and A/duck/Potsdam/1406-86 parental strains had the *non-ts/non-ca* phenotype typical of *wt* viruses. The H5N2 pandemic vaccine candidate demonstrated an attenuated phenotype in mice and in Java macaques and did not infect chickens. The vaccine attenuation study confirmed the attenuated phenotype of a A/17/California/2009/38 pandemic LAIV candidate in mouse, ferret, and guinea pig models.

The phase I/II randomized, controlled, double-blind clinical study

Safety evaluation of pandemic vaccine candidates in adults

Clinical examination of subjects who received two doses of pandemic vaccine candidates indicated that both vaccines were well tolerated. No fever reactions were observed after

the first or second vaccination. After the first vaccination, 33·0% and 40·0% of reactogenicity events consisting of catarrhal symptoms, such as pharyngeal irritation or hyperemia, were observed for H1N1 and H5N2 vaccine candidates, respectively. After revaccination, subjects did not report local or systemic reactions.

Immunogenicity of pandemic vaccine candidates in adults

To determine whether a serological response occurred in the cohort of immunologically naïve subjects vaccinated with pandemic vaccine candidates, HAI and ELISA tests were used (Table 1).

Post-vaccination geometrical mean titers (GMT) among 20 subjects who received two doses of H5N2 vaccine were significantly higher than pre-vaccination titers. The frequency of ≥4 fold antibody rises was significantly higher (47·1%) after revaccination than after one dose (5·9%). The percentage of subjects with post-vaccination serum HAI titers to H5N2 ≥ 1:20 was 47·1% and for titers ≥1:40, it was 29·4%. No seroconversions in the placebo group were detected. The virus-specific nasal IgA antibody response to vaccination after two doses of the H5N2 vaccine candidate demonstrated significant increases of ≥4 fold rise IgA antibodies (65%) compared to one dose.

Cumulative data of H5N2 vaccination (all applied tests) showed 35% and 80% of conversions after the first and the second vaccination, respectively.

Increasing H5N2 vaccine virus infectivity from $10^{6·9}$ to $10^{8·3}$ EID_{50}/dose lead to an enhancement of post-vaccination HAI titers in vaccinees after the first vaccination to homologous H5N2 antigen from 5·9% to 31·0% of ≥4 fold antibody rises.

Values of post-vaccination serum HAI antibody titers in subjects vaccinated with another pandemic vaccine candidate, A/17/California/2009/38, also proved to be rather low. After the primary vaccination, the percentage of subjects with HAI protective antibody titers ≥1:40 were 2·1%. After revaccination, this parameter increased to 17·0%. Four-fold increases in serum HAI antibody titres were four-fold conversions after the first and the second vaccination was 23·4% and 34·4%, respectively. ELISA antibodies in nasal swabs showed had an advantage in detecting induction of local IgA as compared to serum HAI antibodies. After revaccination four-fold serum HAI antibody conversions were 34·4% vs. 63·8% of IgA conversions in nasal swabs, respectively.

Taking into account cumulative data of H1N1 vaccination (HAI and ELISA data), the obtained results were

Table 1. Antibody responses in seronegative volunteers vaccinated with A/17/California/2009/38 (H1N1) and A/17/duck/Potsdam/86/92 (H5N2) pandemic live attenuated influenza vaccine (LAIV) candidates

				HAI test (blood serum)			Number (%) of persons with reliable Ab conversions			
Treatment group		***n***	**Sample was taken at day**	**GMT***	**Ab titers ≥1:20, *n* (%)**	**Ab titers ≥1:40, *n* (%)**	**HAI test in serum**	**ELISA IgG in serum**	**ELISA IgA in nasal swabs**	**Total data (HAI test + ELISA)¶**
H1N1	Vaccine	47	0†	5·4	0	0	11 (23·4%)	12 (25·5%)	18 (38·3%)	20 (42·5%)
			1‡	8·8	12 (25·5%)	1 (2·1%)				
			2§	11·0	16 (34·1%)	8 (17·0%)	16 (34·4%)	21 (44·7%)	30 (63·8%)	33 (70·2%)
	Placebo	19	0	6·5	0	0				
			1	8·4	2 (22·2%)	0	0	0	0	0
			2	9·0	2 (22·2%)	0	0	0	0	0
H5N2	Vaccine	20	0	4·0	0	0				
			1	7·5	3 (17·7%)	2 (11·8%)	1 (5·9%)	nd**	6 (30·0%)	7 (35·0%)
			2	15·7	8 (47·1%)	5 (29·4%)	8 (47·1%)	nd	13 (65·0%)	16 (80·0%)
	Placebo	8	0	3·9	0	0				
			1	4·6	0	0	0	nd	0	0
			2	4·6	0	0	0	nd	0	0

Here and in the Table 2:
*Geometric mean titer.
†Day before vaccination.
‡Day 21 after the first vaccination.
§Day 21 after revaccination.
¶Antibody conversions were detected in one or several tests.
**Not determined.

42·5% and 70·2% of conversions after the first and the second vaccination, respectively.

Fourty-seven subjects were vaccinated with H1N1 LAIV, and 19 who received a placebo were chosen for evaluation of cellular immune response by cytokine assays. After revaccination, the mean increases of both $CD4^+$ and $CD8^+$ memory cells were significantly higher in vaccinated subjects compared to the placebo group. Interestingly, the same effect of vaccination was observed in vaccinees without detectable conversions of HAI antibody titers. Even after a single vaccination, the rate of subjects with significant increases of these cells in the blood was 37·5% ($CD8^+$) and 75% ($CD4^+$). After the revaccination, the percentage of subjects with significant increases in $CD8^+$ and in $CD4^+$ cells was 68·8%.

Immunogenicity of H1N1 pandemic vaccine candidate in children

HAI antibody results among children aged 12 to 18 years proved to be significantly higher when compared to adult subjects: after the first vaccination, 41·4% of the children seroconverted; after revaccination, seroconversions reached 83·3% (Table 2). The GMT rise to H1N1 vaccine with primary vaccination was 3:1; after revaccination it increased to 6:7.

Discussion

Benefits of vaccination with LAIV to aid in the control of influenza outbreaks are acknowledged by the WHO.[1] Many years of LAIV seasonal trials have shown excellent tolerability and low reactogenicity.[2–4] Indeed, data showed that live influenza vaccines cause minimal systemic, local, and thermal reactions, generally from 0 to 3%. A different situation was observed in the cohort of immunologically naïve volunteers vaccinated with pandemic vaccines. The rate of local reactions to A/17/California/2009/38 and A/17/duck/Potsdam/86/9 vaccine candidates increased to 33·0% and 40·0%, respectively. After revaccination no significant local and systemic reactions were observed. This confirms, indirectly, the development of a sufficiently high level of protection after the first vaccination with pandemic LAIV.

The most important criterion for assessing the quality of vaccines is their estimated safety, epidemic effectiveness, and immunogenicity. However, current regulatory documentation [5] mandates that induction of serum antibodies, measured by HAI, as the only criterion for a LAIV immunogenicity evaluation.

In addition to the standard HAI assay, we determined serum (IgG) and local (IgA) antibodies in adult subjects vaccinated with an H1N1 pandemic vaccine candidate. Evaluation of overall results obtained in these additional serological tests, as well as those from the HAI assay, showed an immune response to the vaccine in the majority of subjects (42·5% of Ab seroconversions after the single vaccination and 70·2% after revaccination, respectively).

These data show that methods used to routinely measure LAIV immunogenicity should be revised to include a number of additional immunological methods such as IgG and IgA ELISA, and cytokine assays consistent with the recently updated WHO recommendations on LAIV monitoring.

These clinical studies clearly demonstrated that pandemic LAIV candidates are effective at generating pandemic specific influenza immunity. A key finding from this study is that it may be practical to give the vaccine as a single dose to both children and adults.

Evaluation of our LAIV pandemic vaccine candidates was performed as part of the global influenza pandemic preparation project outlined by the WHO.[6] It was considered that LAIV could be produced in greater quantities and more rapidly than inactivated vaccines. Together with the generation of herd immunity by LAIV, this suggests that LAIV implementation during the first wave of a pandemic may provide significant social, economic, and health benefits to the community.

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Table 2. Immunogenicity of A/17/California/2009/38 (H1N1) live attenuated influenza vaccine (LAIV) for children 12–18 years of age

n	Sample was taken at day	GMT	GMT rise	Seroconversions, *n* (%)	Ab titers ≥1:40, *n* (%)
29	0	6·7			
29	1	20·5	3·1	12 (41·4%)	13 (44·8%)
18	2	44·9	6·7	15 (83·3%)	15 (83·3%)

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Cross-protection elicited by MF59-adjuvanted vaccine during seasons with good or partial matching between vaccine strain and clinical isolates

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Addition of MF59 to subunit influenza vaccine can enhance the breadth of antibody response against mismatched influenza seasonal and avian strains. However, little is known on the effect of MF59 on immunogenicity of influenza vaccines when ''apparent'' good matching between circulating and vaccine strains exists. In this study, a comparison of the immune response elicited by MF59-adjuvanted or non-adjuvanted subunit vaccine, containing A/California/7/04(H3N2) strain, against circulating viruses isolated between 2004/2005 and 2006/2007 seasons, belonging to different clades, is shown. MF59 offers advantages in terms of higher immunogenicity, expressed as higher post-vaccination HI titres, also against viruses that show antigenic and molecular pattern undistinguishable from vaccine strain; the advantages became even more evident as the antigenic and molecular distance between vaccine and circulating strains grew. The availability of a vaccine able to confer an improved cross-protection versus viruses showing good, partial, or poor matching with the vaccine strains, such as MF59 adjuvanted vaccine, represents a precious tool to better prevent influenza and influenza-correlated complications.

Introduction

The main evolutionary mechanism of influenza viruses during inter-pandemic period is the antigenic drift, but the epidemiological picture of circulating viruses is complicated by a high level of heterogeneity of strains, even though drift does not occur, due to co-circulation of drifted and old strains or to co-circulation of viruses belonging to the same type/subtype but with different antigenic patterns.[1–6] Lack of data exists on the impact of the wide heterogeneity of circulating strains on the seroprotection and on-field effectiveness of influenza vaccine: in particular, little is known about the ability of influenza vaccine to elicit an effective immune response against isolates with few amino acid mutations with respect to vaccine strains that represent the majority of circulating viruses. MF59-adjuvanted vaccines, which are currently used for the prevention of seasonal

influenza epidemics in elderly, are showed to confer higher seroprotection against homologous and drifted A(H3N2) strains than non-adjuvanted vaccines.[7–9] The broader immune response showed by MF59-adjuvanted vaccine was measured using HI and NT assays against egg-grown drifted strains representing vaccine composition changes during the following seasons, but its ability to elicit a broader immune response against circulating viruses belonging to vaccine cluster and presenting amino acid mutations onto antigenic sites or against on-field isolates not-antigenically distant from vaccine strains has not yet been investigated. The main aim of the present study is to compare the immune response elicited by MF59-adjuvanted or non-adjuvanted subunit influenza vaccine, containing A/California/7/04(H3N2) strain, against circulating viruses isolated between 2004/2005 and 2006/2007 seasons, belonging to different clades and, in particular, to A/California/7/04 cluster, presenting amino acid mutations onto antigenic sites with respect to the vaccine virus in ''apparent'' good antigenic matching condition.

Materials and methods

Prior to the 2005–2006 influenza season, 50 healthy, elderly subjects aged more than 65 were randomly assigned (1:1) to receive either a single dose of MF59-adjuvanted subunit influenza vaccine (FLUAD®; Novartis Vaccines, Siena, Italy) or a non-adjuvanted subunit influenza vaccine (Agrippal®; Novartis Vaccines, Siena, Italy). The two groups are comparable in terms of age (mean ± SD 71·68 ± 4·76 and 72·08 ± 4·43 years in subunit and MF59 group, respectively). They received influenza vaccines containing the A/California/7/04 influenza subtype A(H3N2) virus, as recommended by WHO for the 2005–2006 season. Strains used for the analisys included vaccine egg-grown viruses A/Wyoming/3/03, A/California/7/04, A/Wisconsin/67/05, and A/Brisbane/10/07, and cell-grown isolates circulating concomitantly with vaccine strain A/California/7/04 during the 2004/2005 and 2005/2006 seasons and drifted viruses isolated during the following winter.

A/Genoa/13/2004, A/Genoa/27/04, and A/Genoa/47/04 isolated during the 2004/05 season, appear more philogenetically close to 2004/05 vaccine strain A/Wyoming/3/03 showing amino acid changes onto antigenic sites in position 145 (N145K), 189 (N189K), and 227 (P227S) with respect to A/California/7/04. In particular, A/Genoa/13/04 and A/Genoa/27/04 presents N126D amino acid mutation detected in clade 2 A/Wyoming/3/03-like viruses.[1] The HA sequences of A/Genoa/59/04, A/Genoa/2/05, Genoa/11/05, A/Genoa/47/05, and A/Genoa/62/05 fell within the clade represented by the HA of A/California/7/04; among these isolates, A/Genoa/2/05 and A/Genoa/11/05 showed antigenic site sequences very close to that of the 2005/06 vaccine strain, whereas HA sequences of A/Genoa/62/05, A/Genoa//47/05 and A/Genoa/59/05 posses amino changes onto antigenic site A(R142K), C(G50E) and D(R208K), respectively.

The HA sequences of more recent isolates fell within the clade represented by the HA of A/Brisbane/10/07 and characterized by the amino acid changes, relative to the HA of A A/California/7/04, G50E and K140I, with the exception of A/Genoa/3/07, showing R142G and L157S amino acid changes present in viruses belonging to A/Nepal/921/06 clade.

Measure of genetic distance between vaccine and circulating strains was calculated as previously described by Gupta.[10] Two blood samples were collected from each subject, just before and 22 ± 2 day post-vaccination. All sera were stored at −20°C. All samples were tested at the Laboratory of Health Sciences Department, University of Genoa, by Haemagglutination-Inhibition (HI) and Neutralization (NT) assays, performed following the WHO criteria and standardised method in our laboratory, respectively.[11–13] Guinea pig red blood cells were used for HI assay. All samples were assayed twice for HI and for NT. The obtained antibody titre was expressed as the reciprocal of the last sera haemagglutinating or inhibiting virus dilution. Immunogenicity was determined by: geometric mean titre (GMT); mean-fold increase (MFI; ratio of post- to pre-vaccination titre); seroprotection rate (the percentage of subjects achieving an HI and NT titre ≥40 IU); and seroconversion rate (percentage of subjects with a fourfold increase in HI or NT antibody titers, providing a minimal post vaccination titer of 1:40). Post-vaccination GMT was reported as ratio, with the corresponding 95% confidence interval, of GMTs after vaccination with MF59-adjuvanted vaccine and with non-adjuvanted subunit vaccine. Seroprotection and seroconversion rate 95% confidence interval was calculated using modified Wald method. Comparisons of seroconversion and seroprotection rates between subunit and MF59-adjuvanted vaccine groups have been analyzed by Fischer's exact test. The results were evaluated against the committee for medicinal products for human use (CHMP) criteria for approval of influenza vaccines in the elderly, which require that at least one of the following criteria be met: MFI >2; seroprotection rate >60%, or seroconversion rate >30%. Furthermore, HI titres were also transformed into binary logarithms, corrected for pre-vaccination status, as described by Beyer *et al.* [14] and were expressed as median titres, with the corresponding 25°–75° inter-quantile range. Comparisons of corrected post-vaccination titers between subunit and MF59-adjuvanted vaccine groups were analyzed by Wilcoxon test. Difference in

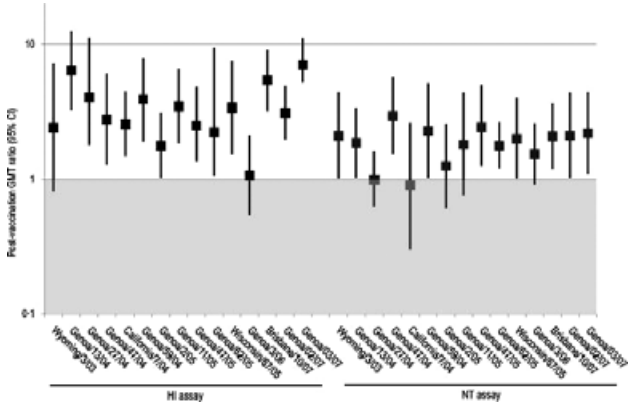

Figure 1. Post-vaccination geometric mean titer (GMT) ratio (95% C.I.) between MF59-adjuvanted and non-adjuvanted vaccine groups determined using HI and NT assays, according to viral strain.

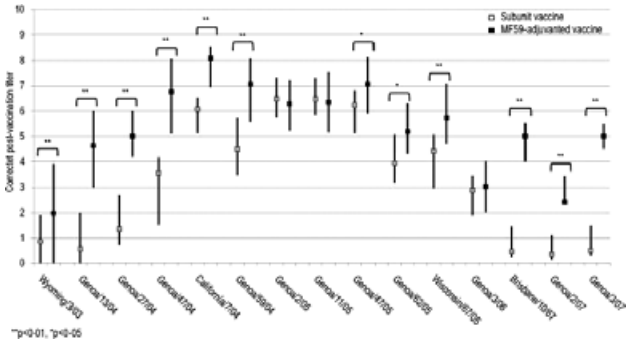

Figure 2. Post-vaccination HI titres, corrected for pre-vaccination status, in subjects vaccinated with an MF59-adjuvanted vaccine or a non-adjuvanted vaccine, each containing A/California/7/2004 antigen, according to viral strain. Data expressed as median. Error bars denote 25°–75° inter-quantile range.

immunogenicity profile between vaccine groups, expressed by ratio of different parameters, was correlated with genetic and antigenic distance between vaccine and viruses used in the study using Spearman test.

Results

Pre-vaccination titres were not significantly different between vaccine groups, for all 15 strains (data not shown). Post-vaccination GMT ratios between MF59-adjuvanted and non-adjuvanted vaccine groups determined using HI and NT assays, with the corresponding 95% confidence interval, according to viral strain are shown in Figure 1.

Both vaccines met CHMP requirements for MFI (>2), seroconversion (>30%), and seroprotection rate (>60%) against A/Wyoming/3/03-like, with the exception of A/Genoa/13/04 and A/California/7/04-like circulating viruses and against egg-grown A/Wyoming/3/03, A/California/7/04, and A/Wisconsin/67/05 strains; the immune response against A/Genoa/13/04 met the requirements for MFI and seroprotection rate only in MF59-adjuvanted vaccine group. Requirements for MFI, seroconversion, and seroprotection rate against the A/Brisbane/10/07-like virus A/Genoa/2/07 and the A/Nepal/921/06-like Genoa/3/07 viruses and against egg-grown A/Brisbane/10/07 strain were reached only in subjects vaccinated with the MF59-adjuvanted vaccine. A similar pattern emerged from the analysis of MFI, seroconversion and seroprotection rates using NT assays. Subjects vaccinated with the MF59-adjuvanted vaccine showed significantly higher post-vaccination HI GMTs against A/Wyoming/3/03-like, A/California/7/04-like, A/Nepal/921/06-like and A/Brisbane/10/07-like viruses, with the exception of A/Genoa/3/06, and against egg-grown A/California/7/04, A/Wisconsin/67/05, and A/Brisbane/10/07 strains, compared with individuals immunized with the non-adjuvanted vaccine (Figure 1). The MF59-adjuvanted vaccine also induced significantly higher seroconversion and seroprotection rates against A/Genoa/13/04, A/Genoa/27/04, A/Brisbane/10/07, A/Genoa/2/07, A/Genoa/3/07 (data not shown).

Following correction for pre-vaccination status, HI titres were significantly higher for the MF59-adjuvanted vaccine group when evaluated against A/Wyoming/3/03-like viruses, A/Brisbane/10/07-like A/Genoa/2/07, and A/Nepal/921/06-like A/Genoa/3/07 strain (Figure 2).

Pre-vaccination titre corrected response was higher in subjects vaccinated with MF59 adjuvanted vaccine also against egg-grown A/Wyoming/3/03, A/California//7/04, A/Wisconsin/67/05, and A/Brisbane/10/07. Among viruses more closely related to A/California/7/04, subjects immunized with MF59-adjuvanted vaccine showed a significantly higher corrected titres against A/Genoa/59/04, A/Genoa/47/05, and A/Genoa/62/05 strains compared with the non-adjuvanted vaccine (Figure 2). Spearman test showed a clear correlation between the distances and the advantage offered by MF59 expressed by ratio between MFI, post-vaccination GMTs, corrected post-vaccination median, seroconversion, and seroprotection rates calculated using HI test in the two vaccine groups. Similarly, ratio between MFI, seroconversion, and seroprotection rates calculated with NT test correlated with the genetic and antigenic distance between vaccine and viruses used for the study.

Discussion

The ability of MF59 to enhance the immunogenicity and to elicit a broader immune response against drifted strains than non-adjuvanted vaccine is consistent with other findings reported during the last decade.[7–9,15] In subjects vaccinated with the MF59-adjuvanted vaccine containing A/California/7/04, the immune response, expressed by a

number of parameters, such as crude and corrected post-vaccination titers, seroconversion, and seroprotection rates calculated using HI and NT assays, is higher than that observed in individuals immunized with subunit vaccine when it is evaluated against a drifted strains, such as A/Brisbane/10/07-like and A/Nepal/921/06-like strains, and against egg-grown A/Brisbane/10/07 virus. For the first time in this study, the impact of heterogeneity of circulating strains antigenically close to the vaccine on the antibody response elicited by MF59- and non-adiuvanted vaccines is evaluated. Immune response against viruses isolated during the 2004/05 season, that appear more phylogenetically close to 2004/05 vaccine strain A/Wyoming/3/03, was higher in subjects vaccinated with MF59-adiuvanted vaccine as demonstrated by higher crude and corrected post-vaccination HI titres and higher post-vaccination NT titres, with the exception of A/Genoa/27/04, against whom the NT post-vaccination GMT is identical in MF59 and subunit vaccine groups. Furthermore, HI seroconversion and seroprotection rates were higher in MF59 vaccine group when evaluated against A/Genoa/13/04 and A/Genoa/27/04. As far as the immune response against A/California/7/04-like viruses, the small number of enrolled subjects did not allow appreciating differences using qualitative response indicators, but crude post-vaccination HI titres were higher in MF59 vaccine group for all the strains. Interestingly, A/California/7/04-like viruses with at least one amino acid change onto antigenic sites, i.e. A/Genoa/59/04, A/Genoa/47/05, and A/Genoa/62/05, showed a more marked difference in terms of response between the two vaccine groups. Individuals immunized with MF59-adiuvanted vaccine showed higher corrected post-vaccination HI titres and post-vaccination NT titres in comparison with subjects vaccinated with plain vaccine. These response indicators were similar in the two vaccine groups when the response was evaluated against A/Genoa/2/05 and A/Genoa/11/04, which present no amino acid changes onto antigenic sites and identical HI titers respect with A/California/7/04 at molecular and antigenic characterization, respectively.

Thus, the advantage offered by MF59 in terms of higher immunogenicity expressed by higher post-vaccination HI titres is observable also against viruses showing antigenic and molecular pattern undistinguishable from vaccine strain, but it became even more evident as the antigenic and molecular distance between vaccine and circulating strains grew. As emerged for A/Genoa/59/04, A/Genoa/47/05, and A/Genoa/62/05, one amino acid was a sufficient change in antigenic sites for 2-fold decrease of HI titre against homologous vaccine strain to observe 2-fold higher post-vaccination NT titers (MF59/subunit postvaccination GMT ratio range between 1·79 and 2·45, Figure 1) and one-dilution higher corrected post-vaccination HI titers in MF59 vaccine group (Figure 2). Finally, the correlation between the distance and the improvement offered by MF59 in terms of higher immunogenicity clearly emerged by Spearman correlation analysis: it remains well-founded both using a number of different response parameters obtained from HI and NT assays and calculating the distance by serological and genetic methods.

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Safety and efficacy of a novel live attenuated influenza vaccine against pandemic H1N1 in swine

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The pandemic H1N1 (H1N1pdm) has crossed the species barriers and caused outbreaks in pigs in several countries. Thus, the development of a vaccine that is effective in this livestock species is urgently needed. We have previously demonstrated that introduction of temperature-sensitive mutations in the PB2 and PB1 genes of an avian H9N2 combined with the insertion of an HA tag in PB1 resulted in an attenuated (att) vaccine backbone for both chickens and mice. Because the new pandemic strain is a triple reassortant (TR) virus, we chose a swine-like TR virus isolate, A/turkey/OH/313053/04 (H3N2) (ty/04), to introduce the double attenuating modifications with the goal of producing live attenuated influenza vaccines (LAIV). The ty/04 att-based vaccines were attenuated in pigs and conferred complete protection against the 2009 H1N1 pandemic virus. Our studies highlight the safety of the ty/04 att vaccine platform and its potential as a master donor strain for the generation of live attenuated vaccines for swine.

Introduction

Outbreaks of H1N1pdm in pigs in commercial swine operations have been reported in several countries. In all incidents, epidemiological investigations have linked humans as the possible source of the infection to pigs. Experimentally, it was established that the virus is pathogenic and transmits readily in pigs.[1] The natural outbreaks of H1N1pdm and laboratory studies underscore the threat that the virus poses to the swine industry and highlight the need for developing effective control strategies. In the United States, a trivalent live attenuated influenza vaccine (FluMist®) has been licensed for use in humans since 2003.[2] In swine medicine, however, temperature-sensitive LAIVs are not available. Currently, only inactivated vaccines are available for pigs, but they provide limited protection against antigenically diverse influenza viruses. Additionally, the use of inactivated vaccines has been associated with enhanced pneumonia when immunized pigs were challenged with divergent viruses.[3] Thus, the development of LAIVs has the potential to circumvent the drawbacks associated with commercial vaccines.

With the aim of developing LAIV temperature-sensitive influenza vaccines against the H1N1pdm virus, we have used reverse genetics to introduce attenuation markers in the polymerase genes of a swine-like TR H3N2 influenza virus, A/turkey/Ohio/313053/04 (H3N2) (ty/04).[4] We chose this isolate because it grows well in both eggs and cell culture-based substrates, displays a broad host range, and has internal genes similar to the H1N1pdm virus. Safety and efficacy studies of the ty/04 *att* vaccine candidates in pigs demonstrated that this vaccine backbone is attenuated in swine and conferred sterilizing immunity upon an aggressive intratracheal challenge of pigs with the 2009 H1N1 pandemic virus. Thus, introduction of genetic signatures for *att* in the backbone of a swine-like TR influenza virus resulted in highly attenuated and efficacious live influenza vaccines with promising applications veterinary medicine.

Material and methods

Cell lines and virus strains

293-T cells and MDCK cells were maintained as previously described.[5] A/turkey/Ohio/313053/04 (H3N2) (ty/04) has

been previously described and it was kindly provided by Yehia Saif, Ohio State University.[4] A/California/04/09 (H1N1) (Ca/04) was kindly provided by the Centers for Disease Control and Prevention (CDC). Generation of recombinant viruses by reverse genetics (RG) was done using a previously described method.[6] The genetic signatures for attenuation[5] were introduced into the PB2 and PB1 genes of ty/04. NY 2:6 ty/04 *att* is a 2:6 reassortant with the surface genes from the A/New York/18/2009 (H1N1) virus and the ty/04 *att* internal genes. All viruses were amplified in MDCK cells to produce viral stocks.

Safety of ty/04 att-based vaccines in pigs

Twenty-five pigs were divided into five groups ($n = 5$) and intranasally inoculated with 10^5 $TCID_{50}$/animal of either H3N2:6ty/04 *att* or with NY(H1N1)2:6ty/04 *att* vaccines diluted in 2 ml of MEM. Two other groups were similarly inoculated with H3N2:6ty/04 WT and H3N2:6ty/04 RG and served as controls, whereas a fifth group was mock-vaccinated with PBS alone. Clinical observations were performed as previously described.[1,7]

Efficacy of H1N1 ty/04 att vaccine in pigs

Fourty pigs were divided in four groups ($n = 10$)(Table 1). Group 1 was vaccinated with 10^5 $TCID_{50}$/animal of NY(H1N1)2:6ty/04 *att* through intranasal route, whereas group 2 was vaccinated intramuscularly with 2 ml of an adjuvanted UV-inactivated Ca/04 vaccine (UVadj-Ca/04).[7] Group 3, non-vaccinated and challenged (NV+Ca/04), and group 4, non-vaccinated, mock-challenged (NV+Mock), were also included. Pigs were boosted two weeks later. Fourteen days post boost (dpb), pigs from groups 1–3 were challenged intratracheally with 2 ml of 1×10^5 $TCID_{50}$ of Ca/04. Following challenge, pigs were monitored using methods as previously described.[7]

Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5·00 (GraphPad Software Inc., San Diego, CA). The differences were considered statistically significant at $P < 0{\cdot}05$.

Results

The ty/04 att-based vaccines are attenuated in swine

Pigs inoculated with WT ty/04 viruses developed fever (>40°C) that peaked 24 hpi (Figure 1A) and shed large amounts of in nasal secretions (Figure 1B). Similarly, viral titers in bronchoalveolar lavage fluid (BALF) collected at 3 dpi ranged from 10^5 to 10^6 $TCID_{50}$/ml (Figure 1C). At necropsy, the lungs from animals inoculated with these viruses had severe pneumonia (Figure 1D). In contrast, none of the animals inoculated with H3N2 or H1N1 ty/04 *att* viruses developed clinical signs following vaccination, indicating that the ty/04 *att* viruses were safe for administration to pigs (Figure 1A). Correspondingly, there was 100–1000 fold less virus shedding from the nose of pigs vaccinated with ty/04 *att* viruses as compared to unmodified ty/04 viruses. In general, NY(H1N1)2:6ty/04 *att* –vaccinated pigs shed less virus than H3N2:6 ty/04 *att* inoculated pigs (Figure 1B). In addition, viral titers in BALF were significantly reduced ($P < 0{\cdot}01$) in ty/04 *att*-vaccinated pigs as compared to ty/04 WT-infected pigs (Figure 1C). Although both vaccines caused mild gross and microscopic lesions in the lungs, the percentage of lung

Table 1. Clinical performance of H1N1 ty/04 att-based vaccine in swine after challenge with pandemic H1N1

Group	HI titer[†]	Macroscopic pneumonia[‡]	Nasal swabs[§]		BALF 5 dpc[¶]
			3 dpc	5 dpc	
NY(H1N1)2:6ty/04 *att*	16·2	0·3 ± 0·1*	0 ± 0 *	0 ± 0*	0 ± 0*
UVAdj-Ca/04	20·0	1·2 ± 0·6*	0 ± 0 *	0·4 ± 0*	0·3 ± 0·3*
NV+Ca/04	<10	5·5 ± 1·3	2·2 ± 0·4	2·8 ± 0·2	3·9 ± 0·2
NV+Mock	<10	0·2 ± 0·1*	0 ± 0*	0 ± 0*	0 ± 0*

BALF, bronchoalveolar lavage fluid, UVAdj-Ca/04, UV-inactivated Ca/04 vaccine; NV+Ca/04, non-vaccinated, challenged positive control group; NV+Mock, non-vaccinated, non-challenged negative control group.
*Significantly different from NV+Ca/04 control group at $P < 0{\cdot}05$.
[†]Geometric mean HI titer against Ca/04 at the day of challenge.
[‡]Percentage of macroscopic lung lesions given as mean score ± SEM.
[§]Average viral titer (log_{10}) measure as $TCID_{50}$ per ml.
[¶]Average viral titer (log_{10}) in BALF at 5 dpc.

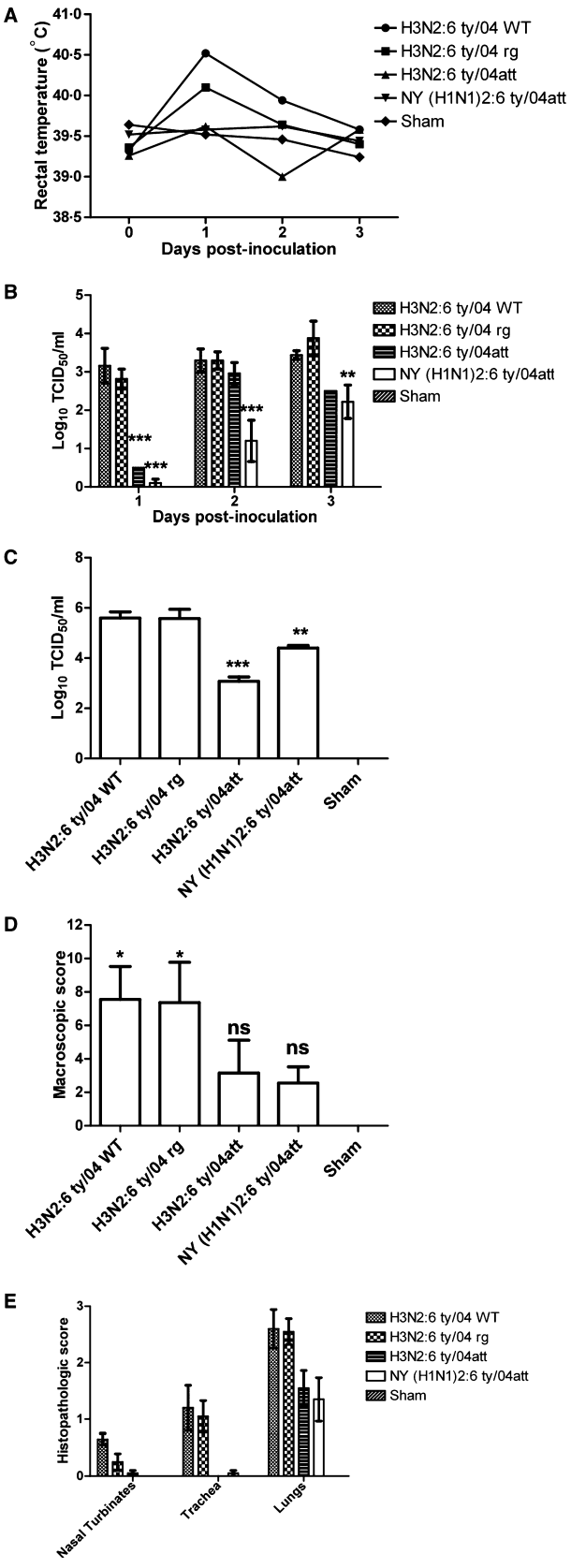

Figure 1. Attenuation phenotype of H1N1 and H3N2 ty/04 *att* viruses in swine. (A) Daily mean rectal temperatures (B) Viral shedding in nasal secretions of pigs. (C) Viral titers in bronchoalveolar lavage fluid (BALF) collected at necropsy. (D) Percentage of macroscopic lung lesions and (E) Histopathologic scores of nasal turbinates, trachea and lungs at 3 dpi. NY(H1N1)2:6ty/04 att (a virus that carries the surface genes of A/New York/18/09 (H1N1) and ty/04 att internal genes). All H3N2 viruses have their surface genes derived from ty/04. Values are shown as the mean ± SEM. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

involvement was not significantly different from mock-vaccinated pigs, corroborating the clinical findings that these vaccines are sufficiently attenuated in pigs (Figure 1D, E). Histopathologically, nasal turbinates and trachea obtained from pigs immunized with either vaccine were similar to control animals, as opposed to the WT-inoculated pigs (Figure 1E).

Vaccination with H1N1 ty/04 att-based vaccines provides sterilizing immunity against H1N1pdm in pigs

The clinical performance in pigs of the H1N1 vaccines is summarized in Table 1. NV+Ca/04 animals had macroscopic pneumonia, viral replication in BALF and shedding in the nose. UVAdj-Ca/04 vaccine provided satisfactory protection, but this protection was not sterilizing. Remarkably, animals vaccinated with NY(H1N1)2:6ty/04 *att* had sterilizing immunity. In both vaccine groups there was a significant reduction ($P < 0{\cdot}001$) in the percentage of macroscopic lung pathology compared to the NV+Ca/04 group. Control pigs had neither significant macroscopic nor microscopic lesions in the lungs. HI antibody titers measured at the day of challenge in both vaccine groups were approximately the same (Table 1).

Discussion

In the present study, we developed for the first time, temperature-sensitive LAIV for use in pigs. Data from our safety studies showed that both the H3N2 and H1N1 ty/04 *att* vaccines were attenuated in pigs. Although the ty/04 *att* vaccines were detected in BALF samples, the level of viral replication was significantly reduced in comparison to unmodified virus and, more importantly, caused no overt clinical signs. A minimal amount of replication is likely beneficial for eliciting T-cell responses to internal genes that may provide heterologous cross-protection.

One of the most challenging tasks in producing effective live attenuated vaccines is to achieve an adequate balance between safety and efficacy. By introducing the *att* modifications into the polymerase genes of a swine-like TR strain, this desirable balance was achieved. The vaccines were

attenuated in pigs and, more importantly, provided sterilizing immunity upon an aggressive challenge with pandemic H1N1 as opposed to an experimental Ca/04 inactivated vaccine, which elicited protective but not sterilizing immunity in all animals.

In the face of influenza pandemics that have the ability to overcome the species barriers such as the 2009 H1N1, the supply of vaccines for use in agriculture could be jeopardized. Our cell culture-based live *att* H1N1 vaccines could be an attractive alternative for this possible pandemic vaccine shortage. Because the ty/04 *att* live vaccines developed here are efficacious in swine, are easier to manufacture than inactivated vaccines, and do not require adjuvants, our study represents a major advance in vaccine development for the 2009 H1N1 pandemic.

In conclusion, our second generation of live *att* influenza vaccines based on modifications of the PB2 and PB1 genes of ty/04 retains its safety properties *in vivo* and can induce excellent protection against aggressive H1N1 challenges in the swine host.

Disclosure

Data presented in this manuscript were previously published in Pena L, Vincent AL, Ye J, Ciacci-Zanella JR, Angel M, Lorusso A, Gauger PC, Janke BH, Loving CL, Perez DR. Modifications in the polymerase genes of a swine-like triple reassortant influenza virus to generate live attenuated vaccines against 2009 pandemic H1N1 viruses. J Virol. 2010 Oct 20. [Epub ahead of print].

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Protection of mice against influenza A and B virus infection by mucosal immunization with *Bacillus firmus* as an adjuvant

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Keywords adjuvat, Bacillus firmus, influenza viruses A and B, intrasubtyp and intersubtyp cross protection, mucosal immunization.

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Introduction

Influenza virus is one of the most important respiratory pathogens worldwide.[1,2]

Type A influenza causes an acute disease of the upper airways, and affects 20–40 million persons yearly. Moreover, the threat of human influenza epidemic and pandemic has dramatically increased in recent years.[3] Vaccination is one of the crucial interventions for reducing the spread and impact of influenza. The generally used parenteral inactivated influenza vaccines induce mainly systemic antibody responses and only weak cell-mediated immunity and low levels if any mucosal immunity. On the other hand, intranasal immunization with live virus can induces a broad spectrum of both systemic and mucosal antibodies, and the immune response localized in the

mucosa blocks the virus even during the first phase of infection. Unfortunately, the use of live vaccines is always associated with a certain risk. The development of a cross-protective vaccine against potentially pandemic strains is an essential part of the strategy to control and prevent a pandemic outbreak. We induced intrasubtypic and intersubtypic cross-protection in BALB/c mice by intratracheal (IT) immunization with inactivated influenza viruses together with dead delipidated *Bacillus firmus* (DBF) as an adjuvant.

Materials and methods

Influenza viruses

B/Lee/40, B/Yamanashi 166/98, A/PR 8/34 (H_1N_1), and A/California 7/04 (H_3N_2) were propagated by standard methods in the allantoic sac of chicken embryos. All viruses were inactivated by formaldehyde at a final concentration of 0·025% for 72 hour at +4°C.

Animals

Adult 9-14-week-old BALB/c female mice.

Adjuvant

Inactivated and methanol-chloroform DBF (strain CCM 2212).

Immunization

Mice were immunized intratracheally under halothane anesthesia with 25 μl of immunization mixture administered into each nostril (total volume of 50 μl comprised 25 μl of virus [haemagglutination titre (HT) 1:256] and 25 μl of DBF (500 μg). Two equal doses were given at an interval of 4 weeks.

Protective experiment

Ten days after the 2nd immunization dose, the mice were infected with live influenza virus B/Lee/40 lethal for mice (total infection dose corresponded to $5 \times LD_{50}$) or A/PR 8/34 (total infection dose corresponded to 0·5–5 LD_{50}).

Results

DBF adjuvant markedly increased both systemic and mucosal anti-viral antibody formation when applied together with inactivated influenza A or B viruses.[4] Protective significance was tested *in vivo*. Mice were preimmunized with 1) PBS (controls), 2) DBF alone, 3) virus alone, and 4) virus+DBF. Influenza B virus strains B/Lee and B/Yamanashi 166/98 (58 years phylogenetically distant and antigenically substantially different, especially in terms of the main protective antigen – surface haemagglutinin) or two different influenza A subtypes - A/PR 8/34 (H_1N_1) and A/California 7/04 (H_3N_2) - were used (Figures 1 and 2). The mice were challenged with $5 \times LD_{50}$ of either B/Lee/40 or A/PR 8/34 as appropriate. All controls died. The mice treated with DBF alone died with a delay or survived, which could be explained by stimulation of innate immunity. The animals immunized with virus alone were protected against homologous strains. Adjuvant immunization was cross-protective: the mice immunized with a heterologous B strain (Figure 2) fell ill (pronounced body mass loss), but almost all survived and recovered.[5] The mice immunized with a heterologous A subtype were excellently protected (negligible weight loss and zero mortality).[6] Intratracheal DBF (500 μg per mouse) given to non-immunized mice 24 hour before influenza infection eliminated the lethal effect in 40–100% of infected animals depending on infection dose (0·5–5 LD50); in mice infected with lower than lethal doses (0·5 LD50), weight loss was minimized or did not occur.

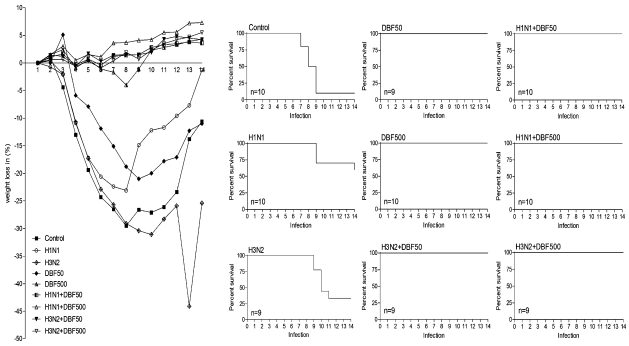

Figure 1. Protection experiment – weight loss and mortality after immunization of mice with influenza virus A(H_1N_1) and A(H_3N_2) and subsequent infection of all mice with live influenza virus A/PR 8/34.

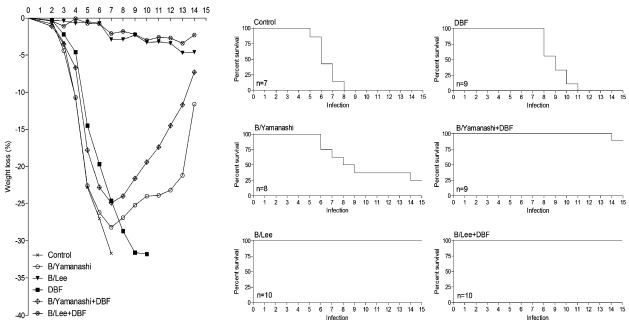

Figure 2. Protective experiment – weight loss and mortality after immunization of mice with influenza virus B/Lee and B/Yamanashi 166/98 and subsequent infection of all mice with live influenza virus B/Lee.

Discussion and conclusions

The current mode of vaccination-induced immunity is mostly effective against a homologous strain of the virus

used for vaccination. The attention is therefore focused on vaccines that are able to induce cross-protection and could be effective also in case of sudden appearance of a new virus variant. Inactivated influenza viruses are known to be often insufficiently effective when used for mucosal immunization and for induction of cross-protection against drifted influenza viruses or novel subtypes. The drawback of vaccination with dead virus can be overcome by using a suitable adjuvant. Mouse models were successfully immunized with vaccine containing inactivated virus in combination with cholera toxin or the *Escheria coli* heat-labile toxin (LT).[7–9] The use of cholera toxin in humans is precluded because of its high toxicity; a number of LT mutants that retain their adjuvant activity have been prepared; these mutants were likewise tested on the mouse model and should not cause any serious side effect in humans. For this reason, current studies aim at finding a suitable and safe mucosal and systemic immune response. DBF has been shown to be a very efficient adjuvant for mucosal immunization stimulating both innate and adaptive immunity. Intratracheal immunization with inactivated influenza viruses and DBF as adjuvant induced efficient and even heterosubtypic cross-protection. DBF given 24 hour before infection provided partial protection probably because of its strong stimulatory effect on the innate immunity.

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Peculiarities of reassortment of a cold-adapted influenza A master donor virus with influenza A viruses containing hemagglutinin and neuraminidase of avian H5N1 origin

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Keywords influenza virus, live attenuated influenza vaccine, reassortant.

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Temperature-sensitive and cold-adapted candidates for live attenuated influenza vaccine with genomic composition of 7:1 based on highly pathogenic influenza A/H5N1 viruses with pandemic potential were generated by the replacement of six internal genes from the influenza A/Puerto Rico/8/34 (PR8) virus from PR8-based RG-candidates for inactivated vaccine with appropriate internal genes of influenza A/Leningrad/134/17/57 (H2N2) master donor virus

(MDV) for Russian LAIV by methods of classical reassortment. All attempts to capture avian N1 neuraminidase into the genome of the MDV LAIV production were ineffective. 6:2 reassortants were not generated. Step by step co-infection of triple reassortants (H5N1-H1N1-H2N2) with H2N2 MDV in some cases was the only possibility to generate influenza A/H5N2 cold-adapted vaccine reassortants. Difficulties in generating 6:2 reassortants could be explained by a substantial gene constellation in the genome of PR8-based H5N1 reassortant viruses. Strong coupling of PB2/PR8 and avian N1 genes in A/H5N1-PR8-RG reassortants was revealed.

Introduction

Annually updated LAIV strains are generated by classical reassortment of circulating influenza viruses with well characterized, attenuated, *ts/ca* MDVs. Resulting attenuated reassortants inherit the relevant HA and NA of wild type parental virus and six internal genes of the MDV.[1] Candidates for inactivated influenza vaccines based upon avian influenza viruses with pandemic potential are generally generated by reverse genetics methods.[2] In these cases, like with LAIV, vaccine strains are 6:2 reassortants which possess the modified HA and NA from potentially pandemic virus and six internal genes from the PR8 virus. The PR8 virus is considered to be of low virulence, i.e. attenuated, for humans, yet offers properties of high seed virus growth for influenza vaccine production. The HA of avian H5 influenza viruses with pandemic potential is engineered to remove four basic amino acid codons from the cleavage site of HA, resulting in a virus that is considered attenuated for natural hosts and safe for people.

The objective of this study was to safely generate vaccine candidates for a LAIV using highly pathogenic avian influenza viruses by the replacement of six internal PR8 genes in the genome of candidates for inactivated vaccine subtype H5N1 (A/H5N1-PR8-RG) with internal genes of the LAIV MDV by methods of classical reassortment.

Materials and methods

Viruses

A/Leningrad/134/17/57 (H2N2), *ca/ts* MDV for Russian LAIV. Reassortants for inactivated vaccine subtype H5N1, A/H5N1-PR8-RG (NIBRG-23, VN-PR, Indo-PR) prepared from A/turkey/Turkey/1/05, A/Vietnam/1203/2004, and A/Indonesia/05/2005 avian influenza viruses with PR8 strain as a donor of internal genes. PR8-based reassortant viruses were obtained from the United States Centers for Disease Control and propagated in 10 to 11 day old embryonated chicken eggs.

Genetic reassortment procedure

Len17-MDV and A/H5N1-PR8-RG virus were co-infected in embryonated chicken eggs. Five rounds of selective propagation were performed, three of which were at low temperature (25°C). The production and selection of reassortants were carried out in the presence of rabbit antiserum to Len17-MDV. Cloning by endpoint dilution was performed in each of the last three passages.

Partial inactivation of parental virus by UV light

A virus sample in an open Petri dish was rocked gently for 20 sec while being irradiated with a GE 15 watt germicidal lamp at a distance of 20 cm from the dish. The residual infection titer was measured by titration in embryonated chicken eggs.

Genome composition and attenuated phenotype analysis

Genome composition of reassortant viruses was monitored by RFLP analysis.[3] In addition, capacity of reassortant viruses to grow at optimum, low, and elevated temperatures (*ca/ts* phenotype) for influenza viruses was determined by virus titration in chicken eggs.

Results

Reassortment of the MDV with the VN-PR or INDO-PR viruses either resulted in reassortants that contained six internal genes from Len17-MDV. However, all generated clones contained the NA from the MDV. Of ten such 7:1 reassortants based on VN-PR three reassortants had the PA gene from PR8 and one had NS gene from PR8. 6:2 reassortants from the targeted H5N1 composition were not generated. After repeated attempts, 7:1 temperature sensitive and cold adapted reassortants based on VN-PR and INDO-PR viruses were obtained, but again, none had inherited the avian N1 neuraminidase (Table 1).

In contrast, NIBRG-23 didn't reassort with the MDV at all. Twelve unsucsessful attempts to develop 6:2 or 7:1 reassortants of NIBRG-23 with MDV showed that the classical reassortment procedure (cloning by limited dilutions in the presence of anti-MDV serum, followed by co-infection of equal doses of two parental viruses in eggs and two selective passages at 25°C) did not work for this virus pair. To disharmonize the incredibly strong gene constellation of NIBRG-23, various modifications of the co-infection step were studied, such as: altering the NIBRG-23 to MDV ratio (from 1:1 to $1:10^6$); altering the sequence of infection in chicken eggs with parental viruses (simultaneous co-infection of both parental viruses, H5N1 virus 30 min prior MDV, MDV 30 min prior H5N1); and altering concentra-

Table 1. Generation of reassortants of VN-PR or Indo-PR with Len17 MDV. (Classical reassortment procedure)

Virus/Clone	PB2	PB1	PA	HA	NP	NA	M	NS
Len17	Len	Len	Len	Len	Len	Len	Len	Len
VN-PR	PR8	PR8	PR8	VN	PR8	VN	PR8	PR8
Indo-PR	PR8	PR8	PR8	INDO	PR8	INDO	PR8	PR8
8 clones	Len	Len	Len	INDO	Len	Len	Len	Len
10 clones	Len	Len	Len	VN	Len	Len	Len	Len
3 clones	Len	Len	PR8	VN	Len	Len	Len	Len
1 clone	Len	Len	Len	VN	Len	Len	Len	PR8

MDV, master donor virus.

tions of anti-MDV serum alone or together with anti-PR8 serum. It was noted that even if the H5N1 to MDV ratio was $1{:}10^6$, the clones obtained were presumably parental H5N1 viruses without the transfer of any MDV-genes into genome of NIBRG-23. In all, 234 clones were isolated, and 209 of them were identical to NIBRG-23 parental virus. In nine clones, only the PA gene from MDV was included, whereas in three clones only the 'cold' NS gene was included (data not shown).

Using UV inactivation of NIBRG-23 prior co-infection was more encouraging. After the first round of co-infection of partially UV-inactivated NIBRG-23 with MDV (at ratio $1{:}10^2$), reassortants that inherited several internal genes of MDV were obtained in the context of the NIBRG-23 background (B3, C2, C4, D1) (Table 2). Some of them (C2, C4, D1) were chosen for the next round of co-infection. After the second round of co-infection, C2, C4, and D1 'intermediate' reassortants with MDV (at ratio 1:1 or 1:10) 7:1 vaccine reassortants finally were obtained.

Discussion

Live attenuated influenza vaccine is considered as one of the most promising pandemic vaccines. According to the WHO there is evidence that LAIV might be more effective than inactivated vaccines.[4] This study attempted the safe development of LAIV for potential pandemic highly pathogenic avian A/H5N1 viruses on the base of RG-reassortants for inactivated vaccine with modified H5 hemagglutinin and MDV for LAIV. Replacement of PR8-based internal genes into genome of VN-PR and INDO-PR reassortants with appropriate genes of MDV was realized by the classical reassortment procedure. Difficulties were encountered in obtaining 6:2 reassortants that contained both the HA and NA from the wild type avian H5N1 parental virus. In attempts to reassort the NIBRG-23 with MDV, the classical reassortment procedure was unsuccessful. The challenge faced was to break an incredibly strong gene constellation of the NIBRG-23 virus. Partial UV-inactivation of NIBRG-23 was encouraged in replacement of some PR8 internal genes with MDV genes

Table 2. Generation of reassortants of NIBRG-23 with Len17 MDV. (Reactivation in eggs. Two rounds of reassortment)

Virus/Clone	PB2	PB1	PA	HA	NP	NA	M	NS
Len17	Len	Len	Len	Len	Len	Len	Len	Len
NIBRG-23	PR8	PR8	PR8	Tur	PR8	Tur	PR8	PR8
Isolates (first round of reassortment of UV-inactivated NIBRG-23 with Len17)								
Clone B3	PR8	PR8	Len	Tur	PR8	Tur	PR8	PR8
Clone C2	PR8	Len	Len	Tur	PR8	Tur	PR8	PR8
Clone C4	Len	Len	Len	Tur	PR8	Len	PR8	Len
Clone D1	PR8	Len	Len	Tur	PR8	Tur	Len	PR8
Isolates (second round of reassortment)								
Clone C235	Len	Len	Len	Tur	Len	Len	Len	Len
Clone C488	Len	Len	Len	Tur	Len	Len	Len	Len
Clone D132	Len	Len	Len	Tur	Len	Len	Len	Len
Clone D133	Len	Len	Len	Tur	Len	Len	Len	Len

MDV, master donor virus.

in reassortment event. After the second round of co-infection of 'intermediate' reassortants with MDV, *ts/ca* vaccine candidates with 7:1 genome formula were obtained. Regardless of the strategy used, all generated reassortants from three different H5N1 viruses contained NA of MDV.

This challenge of obtaining the desired genome composition may be a result of crossing human viruses with avian H5N1 strains. In our repeated attempts of numerous strategies, 6:2 avian-MDV reassortant viruses that had the genotype of six MDV genes with avian influenza A H5 and N1 genes were not isolated. Difficulties in obtaining 6:2 vaccine reassortants of avian H5 and human H2N2 influenza viruses have been observed previously with Len-17 MDV based A/17/duck/Potsdam/86/92 (H5N2) LAIV strain inherited from A/duck/Potsdam/1406/86 (H5N2) wild type virus HA gene only and 7 other genes including NA - from MDV.[5] In some cases avian-human reassortant viruses with gull H13N6 and human influenza H1N1 genes were difficult to generate, and reassortants with the desired genotype of six gull virus genes with human influenza A H1 and N1 genes were not isolated despite repeated attempts. The gull PB2, NP, and NS genes were not present in any of the gull-human H1N1 reassortants generated.[6]

It is difficult to fully understand potential reasons for observed difficulties to reassort some avian viruses with human strains. Unsuccessful attempts to develop 6:2 vaccine reassortants may be caused by an observed strong connection of PB2 and NA genes in the genome of A/H5N1-PR8-RG viruses. In our attempts, each reassortant that possessed avian N1 neuraminidase inheritied PB2 gene of PR8 as well. And vice versa, the 'cold' PB2 gene always appeared to be coupled with the N2 neuraminidase of the MDV. In some cases, step by step co-infection of triple reassortants (H5N1-H1N1-H2N2) with H2N2 MDV may be the only possibility to generate a cold-adapted vaccine reassortant.

Our studies demonstrate unique and significant challenges that are faced in the development of influenza vaccines for avian influenza viruses with pandemic potential. Such challenges must be further studied to identify methodologies to allow for rapid development and response to emerging viruses in a crisis. It is imperative that these studies be continued and expanded to identify either mechanisms of such tight gene constellations in influenza viruses produced by RG-derived vaccine strains or inability some genes of human H2N2 and avian H5N1 viruses to cross. In addition, further studies to improve the efficiency of classical reassortment processes will be conducted.

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Basic results of development of a production technology and control of a pandemic influenza A/H5N1 vaccine

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Introduction

During the period from 1997 to 2009, avian influenza outbreaks among humans have been registered in 15 countries of Asia, Europe, and Africa. Morbidity and mortality of humans followed the global spread of avian influenza H5N1 among wild and domestic birds, which caused great economic loss to the poultry industry in many regions including some highly developed countries.

The global threat from avian influenza forced scientists to develop technologies for the production of A/H5N1 human vaccine. The development of AI A/H5N1 vaccines using strains isolated in Kazakhstan and the organization of local production and creation of strategic stockpiles of effective vaccines is the an important issue for public health protection in the Republic of Kazakhstan.

To address this, a scientific program 'Influenza A/H5N1 vaccine development for public health protection in Kazakhstan' was approved and financed from 2008 to 2010. In this article we give basic results of the development of a recombinant AI A/H5N1 inactivated whole virion vaccine with aluminium hydroxide as adjuvant for public health protection in Kazakhstan.[1–3]

Materials and methods

The development of vaccine technology was conducted with the use of A/AstanaRG/6:2/2009(A/H5N1) recombinant strain made of A/chicken/Astana/6/05(H5N1) and A/PR/8/34(H1N1) strains by the reverse genetics. Inactivation of virus containing allantoic fluid was carried out with the use of formalin in different concentrations. Completeness of the virus inactivation was tested by 3-fold virus passaging in embryos.[4,5]

Purification and concentration of the inactivated virus-containing allantoic fluid was conducted with the use of ultra filtration in tangential flow, which was followed by gel filtration. Then we evaluated the content of total protein, hemagglutinin, and ovalbumin in purified and concentrated material.[6] Vaccine was composed of clarified and inactivated virus concentrate with the known HA dose containment, and 0·4% aluminum hydroxide was added in 1:1 proportions.

Composition components and quality control of finished vaccine was determined in the stages of semi-finished product and finished biopreparation. Determination of quantitative ovalbumin content was conducted by ELISA applying a strip test-system Chicken Egg Ovalbumin ELISA Kit Cat. N 6050 (Alpha Diagnostic International, USA). Vaccine immunogenicity was evaluated by HAI micro test in U-bottom 96-well plates produced by 'Costar' (USA).[7] Vaccine apyrogenicity was evaluated after intravenous injection of the studied preparation to rabbits.[8,9] For confirmation of the results vaccine series were tested for bacterial endotoxins with the use of Limulus Amebocyte Lysate produced by Charles River Laboratories, Inc. USA.[8]

The vaccine toxicity was evaluated in white mice with body weight 18–20 gm and in rats with body weight 180–210 g both males and females according to GLP principles.[9]

Allergenic characteristics of the inactivated vaccine was determined in white outbred mice and guinea-pigs both males and females according to 'Methodic Guideline for evaluation of allergenic characteristics of pharmacological substances'.[10]

Results

In the first series of experiments, we conducted work for obtaining influenza A(H5N1) recombinant strain. Bidirectional expression plasmid pHW_B754 with full-length sequences of HA and NA gene segments of the strain A/chicken/Astana/6/05 (H5N1) isolated in Kazakhstan were synthesized in Geneart AG, (Regensburg, Germany). HA gene was modified by deleting the region encoding multiple basic amino acid RRRK motif in HA cleavage site. Moreover, to prevent recovery of repeating basic amino acids motif due to polymerase slide, we inserted replacements G → T and K → T. Thus the HA cleavage site consists of the following sequence NTPQGERRRKKRGLFGAI NTPQTETRGLFGAI.

The basic amino acid motif of highly pathogenic strain A/chicken/Astana/6/05 (H5N1) was replaced by the sequence TETR/GLF, which is characteristic of low pathogenic strains of influenza H5N1. Sequence of gene coding NA in the strain A/chicken/Astana/6/05 (H5N1) was cloned without modifications. The other segments PB1, PB2, PA, NP, M and NS were obtained from influenza virus IVR-116 and synthesized and cloned in two-forked expression plasmid pHW_B754 in Geneart AG company, Germany. The origin of genetic segments of vaccine strain A/AstanaRG/6:2/2009 (H5N1) is presented in Table 1.

Vero cell culture (134 passage) (WHO) was received from European Cell Culture Collection (Salisbury, Wiltshire SP4 0JG, Great Britain). The cell culture was grown in DMEM/F12 medium with the addition of 10% of fetal bovine serum and 2 mm l-glutamine. To obtain reassortant virus A/Astana/6/05R-6:2, Vero cells were infected with correlative plasmids by way of electroporation using Nucleofector II (Amaxa) equipment. Infected cells were placed in 6-well plates. After 6 hour, DMEM/F12 medium was changed into 4 ml of Opti-Pro SFM (Gibco) medium adding 2 mm L-glutamine and 1μg/ml trypsin. Two days after cytopathic effect appearance supernatant was collected and used for infection of SPF-eggs.

The virus A/AstanaRG/6/05-6:2 was grown in chicken embryos, and then virus titer was determined in chicken embryos and Madine-Darby canine kidney (MDCK) cell culture. The titer of two final A/AstanaRG6/05-6:2 virus stocks was 9·1 $\log_{10}$ EID_{50}/ml (Chicken embryos); 1·8 $\log_{10}$ $TCID_{50}$/ml (MDCK cells); HA titer 1:512. A/chicken/Astana/6/05 (H5N1) virus contains motif of repeating basic amino acids in HA cleavage site. It is known that this sequence is the main determinant of AI virus pathogenicity. That is why this site was deleted in vaccine candidate strain. Sequence results confirmed that influenza virus A/AstanaRG/6/05R-6:2 strain HA gene sequence contains modified HA cleavage site and keeps mutations inserted for prevention of return to virus wild type. To confirm stability of modified HA gene sequence, five additional passages of recombinant strain A/Astana RG/6/05-6:2 were conducted in chicken embryos. Sequencing and following phylogenetic analysis of the recombinant strain A/Astana RG/6/05-6:2 HA gene sequence proved the presence of modification in HA cleavage site. Deletion of pathogenicity site of the obtained virus was confirmed by lethality test for chicken embryos, intravenous pathogenicity test in chicken, and in plaque-forming test with trypsin. Pathogenicity test in chicken embryos showed that recombinant strain A/AstanaRG6/05-6:2 is capable of growing up to high titers without causing embryos' death.

A/AstanaRG6/05-6:2 strain pathogenicity evaluation was conducted in 5–6 week-age white leghorns chicken, and this study proved that the strain A/AstanaRG6/05-6:2 (H5N1) is not virus pathogenicity inductor in chickens, which got intravenous injections of this virus (pathogenicity index is equal to 0). H5N1 strain HA cleavage site modification provides its cleavage capability only with tripsin-like proteases, which shows low level of pathogenicity. Aiming at confirmation of HA cleavage site modification, we experimentally studied virus replication ability both with trypsin and without this enzyme. And we got the following results. In the plaque-forming test, A/AstanaRG6/05-6:2 strain produced plaques in MDCK cells only with trypsin, proving the trypsin-dependent phenotype characteristic of low pathogenic avian influenza viruses.

To prove the HA subtype antigenic analyses of A/Astana/6/05R-6:2 strain was conducted by means of serological methods in hemagglutinin inghibition test with the use of postinfection antisera of rabbits and rats (Influenza Research Institute SWD RAMS), standard serum received from CDC, Atlanta, USA. HAI test proved that A/Astana/6/05R-6:2 strain belongs to H5 subtype. Furthermore, toxicity of vaccine candidate strain was evaluated by way of subcutaneous injection of viral material to BALB mice. The strain appeared to be non-toxic for white mice getting subcutaneous injection of 0·5 ml of the preparation. The conducted research showed that according to all tested characteristics, A/Astana/6/05R-6:2 strain can be used for

Table 1. Segments of A/AstanaRG/6:2/2009 (H5N1) strain genome and their origin

Virus genome segments	A/AstanaRG/6:2/2009 (H5N1) (gene ratio 2:6)
HA	A/chicken/Astana/6/05(H5N1) with the modification of cleavage site
NA	A/chicken/Astana/6/05
PB1, PB2, PA, NP, M, NS	IVR-116

influenza A/H5N1 inactivated vaccine production. According to its genetic characteristics, this strain belongs to the group of vaccine strains recommended by WHO for the development of influenza pre-pandemic inactivated vaccines. We determined basic cultivation parameters of the recombinant strain A/AstanaRG6/05-6:2 in 10–11 day chicken embryos. The determined parameters are the following: infection dose, 1000–10 000 EID_{50}; cultivation period, 72 hour; incubation temperature, 33°C. These cultivation parameters allow obtaining virus containing material with biological EID and hemagglutinating activity of 8·5–9·0 $\log_{10}$ EID_{50}/cm^3 and 1:512 HA titre and even higher.

In the next series of experiments, we conducted research on the determination of optimal sequence of technological stages of virus clarification, concentration, and inactivation in the order of vaccine production. Samples of viral material were subjected to inactivation before and after clarification and concentration. The regimen of virus inactivation by formaldehyde with final concentration of 0·05%, period of inactivation of 3 days, temperature of inactivation medium of 4–6°C, pH of inactivation medium of 7–7·5.

On the basis of the conducted experiments we determined that the selected regimen of inactivation provides complete and irreversible inactivation of viral suspensions of the HPAI strain irrespective of the kind of inactivated material. We did not observe reduction of HA activity in non-clarified viral suspensions. However, when we inactivated clarified and concentrated material, HA activity reduced by an order of magnitude. Comparison of forms and sizes of virion structural elements in native (non-clarified) and formalin inactivated preparations did not reveal any significant differences. Concentration of virus particles in the studied preparations was similar. The selected inactivation regimen provides obtaining completely avirulent viral suspension of the strain A/AstanaRG6/05-6:2, and it does not influence the structure of the virus. On the basis of the experiments results, we selected method of viral allantoic fluid inactivation without preliminary clarification.

During further research, we tried to get highly clarified viral concentrate. This study resulted in the combined scheme, which includes clarification of inactivated viral allantoic fluid by low speed centrifugation at 4000 circulations per min for 30 minutes, filtration through membrane filters with pore diameter of 0·45 μm, ultrafilatration/diafiltration, gel filtration in 6B sepharose, and sterilization of viral suspension through membrane filters with pore diameter of 0·22 μm.

The experiments resulted in the development of production technology of embryonic inactivated vaccine based on recombinant strain A/AstanaRG/6:2/2009 (H5N1) containing aluminium hydroxide as adjuvant. The developed influenza A/H5N1 human vaccine has the trade name Kazfluvac®. Its composition components are presented in Table 2.

Table 2. Composition components of Kazfluvac® vaccine preparation

Components	Function/purpose	Concentration for 0.5 ml dose
A/AstanaRG/6:2/2009 (H5N1)	Active component	HA equivalent 15 μg
Aluminium hydroxide	Adjuvant	0.5 mg
Thyomersal	Preservative	50 μg
KH2PO4	Buffer component	10 mm
K2HPO4	Buffer component	10 mm
NaCl	Buffer component	150 mm

Preclinical testing of the vaccine Kazfluvac® was conducted according to the following parameters: general health condition of animals, change of body weight and temperature of immunised animals (for ferrets), presence of post vaccination antibodies response in sera, forming protective immune response against reassortant viruses of H5 subtype, study of acute and chronic toxicity of three experimental vaccine series in different doses and semi-finished vaccine product applying different ways of injection, study of allergic and immunotoxic characteristics of the vaccine, as well as study of pyrogenic reaction and analysis for bacterial endotoxins presence.[11–14]

Preclinical tests of Kazfluvac® vaccine safety showed that this vaccine does not have toxic effect on organisms of warm-blooded laboratory animals.

Double intramuscular injection of Kazfluvac® vaccine in inoculative dose does not effect appearance, general health condition, behaviour of animals, their muscular strength and physical activity, does not have negative effect on biochemical parameters of blood and basic physical functions of animals organism, and does not cause pathomorphological changes. This shows the safety of the vaccine. Local irritation action was not observed. The results of the vaccine allergic action study showed that the vaccine does not have allergic effect at the intravenous injection. The research also showed that the vaccine does not have negative effect on immune system of laboratory animals.

Research conducted on mice and ferrets showed high immunogenic activity of the vaccine at one- and two-dose regimen of injection. The research showed 100% of protective effect of Kazfluvac® vaccine at two-dose injection regimen in ferrets infected by homological strain of influenza virus.

Discussion

The implemented studies resulted in development of the technology for production of the whole-virion allantoic influenza vaccine with aluminium hydroxide as an adjuvant under the brand name Kazfluvac®. The preparation is based on the vaccinal strain A/AstanaRG/6:2/2009 that belongs to the endemic for Kazakhstan and Russia clade 2, subclade 2 of influenza A/H5N1 viruses. The strain was constructed by the method of reverse genetics and contains HA (with modified pathogenicity site) and NA genes of influenza virus A/Astana/6/2005 (H5N1) as well as PA, PB1, PB2, NP, M, and NS genes of the high yielding influenza virus strain A/PR/8/34.

The devised inactivated influenza A/H5N1 vaccine Kazfluvac® is a safe and immunogenic biopreparation that is not worse than the overseas analogues in its immunobiological characteristics.[15–18]

To date the whole-virion inactivated influenza A/H5N1 vaccines of the producers such as Omnivest (Hungary), Biken, Denka Seiken, Kitasato Institute, Kaketsuken (Japan), GSK Biologicals (Belgium), Sinovac Biotech (China) are registered. All of them are produced on the basis of chicken embryos and aluminum is used as an adjuvant. Kazfluvac® differs from its analogues in the flowchart of the virus purification and concentration that makes possible to produce a safer preparation.[19,20]

The results of the conducted research and preclinical testing allow starting work towards implementation of Phase I preclinical tests on volunteers. It is planned to conduct a randomized blind placebo-controlled Phase I study on double application of Kazfluvac® vaccine in increasing doses. The preparation will be administered to volunteers aged 18–60 years for assessment of its safety and immunogenicity in doses of 7·5 and 15·0 μg of HA.

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Development of production technology and pre-clinical testing of a pandemic influenza A/H1N1 vaccine

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Introduction

When the World Health Organization (WHO) announced the sixth phase of A/H1N1v influenza pandemic, scientists all over the world started investigation to develop technology for production of prophylactic means against the disease. Having taken into consideration the threat of a pandemic for Kazakhstan, the Ministry of Education and Science of the Republic of Kazakhstan launched the program ''Monitoring, Study, and Development of Diagnostic, Prophylactic, and Therapeutic Means for Influenza A/H1N1.'' This paper presents the experimental data obtained at the RIBSP in the course of the studies towards the development of technology for production of an inactivated A/H1N1 influenza vaccine, as well as the results of pre-clinical testing of the developed vaccine.

Materials and methods

The development of vaccine production technology was conducted with the use of WHO recommended vaccine strain NIBRG-121xp constructed by the method of reverse genetics in the National Institute for Biological Standards and Control (NIBSC, Great Britain). The virus was inactivated with formalin at different final concentrations, and the extent of inactivation was evaluated via threefold virus passages in developing chicken embryos.[1] The inactivated virus was purified and concentrated by the method of ultrafiltration in tangential flow followed by gel filtration. The purified and concentrated material was evaluated judging on the total protein, hemagglutinin (HA), and ovalbumin.

The vaccine was prepared by pooling the purified and concentrated virus material with the certain weight content of HA and the work solution of aluminum hydroxide (0·4%) in the ratio 1:1. The ovalbumin content was quantified in ELISA with the use of the strip test system Chicken Egg Ovalbumin ELISA kit (Cat. No. 6050 Alpha Diagnostic International, San Antonio, Texas, USA). Weight content of the virus HA was determined according to Sominina, Burtseva.[2] The content of the residual formaldehyde, aluminum (Al^{+3}) ions, and thiomersal in the vaccine was measured according to the operating instructions.[3]

The vaccine immunogenicity was assessed in the hemagglutination inhibition test, which was carried out as a microassay in 96-welled U-bottomed plates (''Costar'', New York, USA).[3,4] Apyrogenicity of the vaccine was assessed post intravenous administration of the tested preparation to rabbits.[5,6] To confirm the obtained results the vaccine batches were tested for bacterial endotoxins with use of the Limulus Amebocyte Lysate (Charles River Laboratories, Inc., Wilmington, MA, USA).[7]

The toxicity of the vaccine was assayed in white mice weighing 18–20 g and in rats weighing 180–210 g (male and female) in compliance with the principles of good laboratory practice.[8] Allergenic properties of the inactivated vaccine were determined according to the ''Operating instructions on assessment of allergenic properties of pharmaceutical substances''[9] in white outbred laboratory mice and guinea-pigs of both sexes.

Results

The first step in the course of developing technology for vaccine production was to determine the major conditions for influenza virus cultivation: usage of 10-days embryonated chicken eggs at the infectious dose within 1000–10 000 EID_{50}, incubation temperature (34 ± 0·5)°C, and duration of the incubation period 72 hours. The established parameters for virus cultivation made it possible to produce virus-containing materials of infectious activity within 8·5–9·0 log EID_{50}/cm^3 and hemagglutinating activity 1:256 and higher.

In the subsequent experiments, an optimal method for virus inactivation was selected. On the basis of the experimental findings, the following conditions for inactivation of the native virus-containing material were elected: formalin of 0·05% final concentration as an inactivating agent; inactivation period of 72 hours at temperature (4 ± 2)°C. These conditions provide the complete inactivation of the virus (NIBRG-121xp strain) material, did not impact distinctly the structural organization of the virus, and did not reduce the antigenic activity. As it is well known, virus purification and concentration means very much in the development of technology for production of an inactivated whole-virion influenza vaccine. The investigation into optimization of the technological step of purification and concentration of the recombinant influenza virus NIBRG-121xp strain resulted in selection of an optimal pattern including such steps as clarification of the virus suspension by filtration through membranes with pore size 0·45 μm, virus concentration by ultrafiltration in a tangential flow, dialysis filtration in a tangential flow, gel filtration on Sepharose 6B, and sterilization of the viral suspension through membrane filters with pore size 0·22 μm.

The studies conducted by the RIBSP specialists resulted in the development of technology for production of the first domestic whole-virion inactivated A/H1N1 influenza vaccine with aluminum hydroxide as adjuvant and with the brand name Refluvac®. The key processing characteristics of the whole-virion inactivated A/H1N1 influenza vaccine vaccine Refluvac® are shown in Table 1.

Simultaneous with the performance of all process operations, the parameters such as sterility, inactivation extent, pH, vaccine specificity, total protein content, weight content of HAs, aluminum and formalin contents, content of thiomersal, and ovalbumin, pyrogenicity of the vaccine and its immunogenicity for mice, were optimized. The key qualitative characteristics of the designed influenza A/H1N1 vaccine Refluvac® are shown in Table 2.

Before implementation of Phase I clinical trials on volunteers, preclinical testing of three experimental batches of Refluvac for immunogenic activity and safety was carried out. It was conducted in three laboratory bases of research institutions: the Toxicology Institute/Federal Medicobiological Agency, Russia (St Petersburg), the Research Institute for Biological Safety Problems (Republic of Kazakhstan), and the Influenza Research Institute/North-Western Branch of the Russian Academy of Medical Sciences (St Petersburg), with use of different animal models (mice, rats, chinchilla rabbits, guinea-pigs, ferrets).

The results of the preclinical testing are as follows:

- Electron microscopy of the preparation has shown that the viral particles are well dispersed and do not aggregate. The portion of whole (intact) particles is over 95%, which is evidence of virion integrity;
- Assessment of polypeptide composition of the vaccine Refluvac by electrophoresis in 10% polyacrylamide gel with sodium dodecyl sulfate has shown the vaccine to contain both surface antigens (HA, NA) and highly purified inner virion proteins (NP, M1) that are type-specific antigens, so the vaccine is a preparation of full immunological value;
- Judging on the parameters of acute and chronic toxicity for white mice and rats of both sexes, the vaccine is a non-toxic and safe preparation;

Table 1. Process-dependent parameters of the vaccine Refluvac®

Parameters	Method applied	Results/comments
Recombinant influenza virus NIBGR-121xp strain	Virus passaging in chicken embryos	Virus stocks have been produced in embryonated chicken eggs and is stored freeze-dried at minus 70°C
Production of the virus-containing suspension	Virus passaging in chicken embryos	Virus-containing suspension was produced in 11-days chicken embryos
Inactivation of the virus containing suspension	Inactivation of the recombinant avian influenza virus strain with formalin	The virus was inactivated with formalin at the final concentration 0·05% for 72 hours
Purification concentration of the inactivated virus containing suspension	Virus purification by the method of ion-exchange chromatography on DEAE-cellulose Virus concentration by ultrafiltration Gel filtration on Sepharose 6B Microfiltration through membrane filters	Clarification, ultrafiltration, diafiltration, gel filtration, microfiltration
Adjuvant	Preparation of the aluminum hydroxide work solution	Alhydrogel-85
Preservative	Preparation of the thiomersal work solution	Thiomersal at 0·1 ml/ml

Table 2. Major qualitative characteristics of the influenza A/H1N1 Refluvac®

Parameters	Obtained result
Description	Transparent liquid with loose deposit. Its shaking for 1–2 minutes results in formation of a homogenous whitish-gray suspension
Originality (at the stage of an intermediate product)	Interacts with the type-specific serum and does not interact with the sera of other types and subtypes of the influenza virus
Dispersibility	The suspension readily enters a syringe through a needle No. 0840.
Mechanical inclusions	Meets the requirements of RD 42-501-98.
pH	7·1
Total protein (at the stage of an intermediate product)	32·4 μg/0·5 ml
Sterility	Sterile.
Pyrogenicity	Apyrogenic
Bacterial endotoxins (at the stage of an intermediate product)	26 IU/0·5 ml
Toxicity	Non-toxic
Specific safety (at the stage of an intermediate product)	Live virus is absent
Weight content of hemagglutinin (at the stage of an intermediate product)	7·5 μg/0·5 ml
Immunogenicity	Geometric mean titer of antibodies to the homologous virus in blood sera of mice in HAI test in 14 days post administration is 1:147.
Ovalbumin	0·2 μg/0·5 ml
Thiomersal	103 μg/ml
Aluminum ions (Al^{+3})	0·5 mg/ml
Formaldehyde	0·002%

HAI, hemagglutination inhibition.

- Under conditions of a chronic experiment on white mice and rats, it was found that Refluvac does not produce changes in behavior, somatic, or vegetative responses;
- Assay of hematological and biochemical blood characteristics of white mice and rats following vaccine administration did not reveal any significant differences as compared to the animals of the control group;
- Refluvac does not cause allergenic and immunotoxic impact;
- The vaccine Refluvac does not cause local irritative effect;
- Refluvac is apyrogenic for laboratory animals;
- The pathomorphological and hystopathological analysis did not reveal any changes due to immunization in animal organs;
- Testing of immunogenic characteristics of the vaccine on mice and ferrets has shown formation of hemagglutinating antibodies in animals after single administration;
- Refluvac induces 100% protection in immunized ferrets at their challenge with the wild-type influenza virus A/California/07/2009 (H1N1v).

The results of the performed preclinical testing have allowed concluding that Refluvac, an inactivated whole-virion vaccine with aluminum hydroxide as adjuvant, is a safe and highly effective preparation against influenza A/H1N1v.

Discussion

The implemented study resulted in development of technology for production of the first domestic inactivated allantoic whole-virion influenza A/H1N1 vaccine with aluminum hydroxide as an adjuvant under the brand name Refluvac® based on the recombinant strain NIBRG-121xp. The devised pandemic vaccine meets WHO requirements as well as requirements concerning safety and immunogenicity of the National Pharmacopeias of the Republic of Kazakhstan and Russian Federation.[9–12]

The devised technology for vaccine production differs from the previous technologies for production of allantoic whole-virion influenza A/H1N1 vaccines in its process-dependent parameters. Presence of an adjuvant (aluminum hydroxide) increases significantly the vaccine immunogenicity and allows maximal reduction of the dose of the administered antigen that, in turn, results in diminished reactogenicity of the vaccine. Aluminum hydroxide is an adjuvant that is most frequently used in clinical practice.[13]

To date the results of the double-centered randomized study of the Europe-licensed vaccine Fluval P [monovalent inactivated whole-virion influenza vaccine with aluminum phosphate based on strain A/California/07/2009 (H1N1) NYMC X-179A (Omninvest, Pilisborosjeno, Hungary)] that is similar to the Refluvac preparation are published. The data of this research are an evidence of safety and high

immunological effectiveness of the vaccine in dose 6 μg HA at single administration both in adults and elderly persons.[14]

The results of the pre-clinical tests allow recommending carrying out Phase 1 clinical testing of the Refluvac® vaccine for safety and immunogenicity. Single immunization of volunteers with Refluvac® in doses 3·75, 7·50, and 15·00 μg of HA are planned.

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Preclinical study of live cold-adapted reassortant influenza H1N1 pandemic and H7N3 vaccine candidates

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Keywords Immunogenicity, live attenuated influenza vaccine, preclinical studies.

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Abstract

The following paper describes the preclinical study of new H1N1 and H7N3 live attenuated influenza A vaccine (LAIV) candidates in mice. The study demonstrated that A/17/mallard/Netherlands/00/95 (H7N3) and A/17/California/2009/38 (H1N1) vaccine candidates were indistinguishable from parental A/Leningrad/134/17/57 (H2N2) master donor strain in terms of replication in the lungs and noses of mice at 3 and 6 days post-infection. It was found that both H7N3 and H1N1 LAIV and A/Leningrad/134/17/57 (H2N2) show no neuroivasive capacity and fail to replicate in the brains of mice. Immunization of mice with either 1000 MID_{50} (50% mouse infectious dose) or 100 MID_{50} of H7N3 LAIV protected mice from infection following a homologous challenge with wild-type H7N3 virus. Both H1N1 and H7N3 LAIV candidates were demonstrated to be immunogenic. After one dose of 100 MID_{50} of H1N1 LAIV, the geometric mean titer (GMT) of hemagglutination inhibition (HI) antibodies was 17·6. One dose of 1000 MID_{50} or 100 MID_{50} H7N3 LAIV elicited an HI antibody with a GMT of 8·7 and 6·6, respectively. The second dose of H7N3 LAIV further increased of serum HI antibodies to a GMT 40·0 and 23·3 for 1000 MID_{50} or 100

$MID_{50,}$ respectively. The study results confirm that new H1N1 LAIV and H7N3 LAIV candidates are safe and immunogenic and confer protection from homologues influenza virus infection in mice.

Introduction

The recent emergence of a new pandemic H1N1 virus and the threat of transmission of avian viruses to humans had stimulated research and development of live attenuated cold-adapted influenza vaccines against newly appeared influenza viruses. Formulations of live attenuated influenza A vaccine (LAIV) against pandemic influenza strains, including H1N1, H5N1, H9N2, and H7N3 are currently being tested in preclinical and Phase I clinical studies.[1] The following paper describes the preclinical study of new H1N1 and H7N3 LAIV candidates in mice.

The study addressed the following three objectives: (i) to demonstrate that cold-adapted (ca) reassortant influenza A(H1N1) and A(H7N3) vaccine candidates are indistinguishable from the parental A/Leningrad/134/17/57 (H2N2) Master Donor Strain (MDS) virus with regard to replication efficiency in upper and lower respiratory tract of mice; (ii) to demonstrate the immunogenicity of different doses of cold-adapted (ca) reassortant influenza A(H1N1) and A(H7N3) vaccine candidates in mice; and (iii) to demonstrate the protective efficacy of cold-adapted (ca) reassortant influenza A(H5N1) and A(H7N3) vaccine candidates in mice against a homologous wild-type virus challenge.

Materials and methods

Viruses

The A/17/mallard/Netherlands/00/95 (H7N3) reassortant containing the HA and NA genes from A/mallard/Netherlands/00 (H7N3) and six other genes from MDS, the A/17/California/2009/38 (H1N1) reassortant containing the HA and NA genes from A/California/7/2009 (H1N1) and six other genes from A/Leningrad/134/17/57 (H2N2) were generated by classical genetic reassortment in embryonated chicken eggs (EC). Viruses were propagated in 10-days old eggs (34°C, 48 hours). Fifty percent egg infectious dose (EID_{50}) titers were determined by serial titration of viruses in eggs. Titers were calculated by the method of Reed and Muench.[2]

Mice

Female Balb/c mice, 6–8 weeks of age were used in all experiments. Mice were lightly anesthetized with ether and then inoculated intranasally (i.n.) with 50 μl of infectious virus diluted in phosphate-buffered saline (PBS).

Viral replication kinetics and neuroinvasion

Mice were inoculated with 100 MID_{50} (50% mouse infectious dose) of A/17/California/2009/38 (H1N1), A/17/mallard/Netherlands/00/95 (H7N3), and A/Leningrad/134/17/57 (H2N2) MDS. Viral loads were measured in respiratory and brain tissues collected at 3 and 6 days post-infection (dpi). Tissue homogenates prepared using a disruptor and clarified supernatants were titrated on eggs at permissive temperature to determine infectious concentrations.

Immunogenicity and protection studies

Groups of animals were inoculated with 1000 MID_{50} or 100 MID_{50} of either H1N1 LAIV or H7N3 LAIV intranasally after collecting a pre-immunization blood sample. A second blood sample was collected at 28 dpi. On the same day, the animals received a second intranasal inoculation with the same virus that was used for priming at 0 dpi. To assess protection, all animals were infected 42 dpi with either 100 MID_{50} of A/California/7/2009 (H1N1) or 100 MID_{50} A/mallard/Netherlands/00 (H7N3) virus by the intranasal route. Four animals from each group were euthanized at 45 dpi, and the respiratory and systemic organs were harvested for virus titration. A forth blood sample was collected at 56 dpi from the remaining animals. HI antibody titers were determined for individual serum samples collected on days 0, 28, 42, and 56. Body weights were taken daily following challenge through day 14 post-challenge. Sera were tested for HI against homologous H1N1 and H7N3 viruses.

Results and discussion

The H1N1 LAIV, H7N3 LAIV and H2N2 MDS influenza viruses replicate in mice lungs at level 2·1–2·3 $lgEID_{50}$/ml at 3 dpi (Figure 1). At 6 dpi, replication of the viruses in the lungs decreased to 1·6–2·0 $lgEID_{50}$/ml (data not shown). In contrast, the wild-type virus A/mallard/Netherlands/00 (H7N3) demonstrated high level replication in lungs – 6·4 $lgEID_{50}$/ml. The levels of replication of studied viruses in nasal turbinates were 2·5–3·7 lg EID_{50}/ml at 3 dpi (Figure 1), and 2·0–2·2 $lgEID_{50}$/ml at 6 dpi (data not shown). There were no significant differences between the viruses in regard to replication in upper respiratory tract of mice. Thus, it was shown that A/17/mallard/Netherlands/00/95 (H7N3) and A/17/California/2009/38 (H1N1) vaccine candidates was indistinguishable from parental A/Leningrad/134/17/57 (H2N2) in terms of replication in the lungs and noses of mice at 3 and 6 dpi. No virus was found in the brain tissue of immunized mice at 3 and 6 dpi (in undiluted samples tested). Thus, it was shown that A/17/mallard/Netherlands/00/95 (H7N3), A/17/Cali-

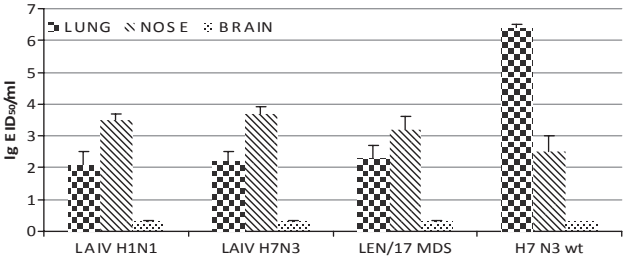

Figure 1. Replication of influenza viruses in upper and lower respiratory tract and neuroivasions in mice.

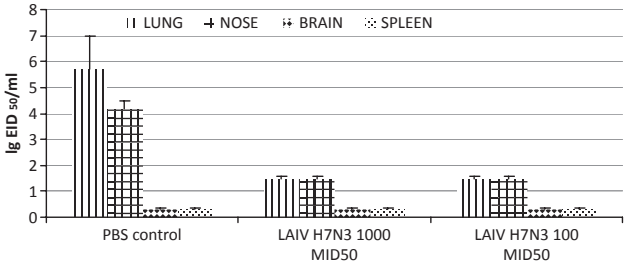

Figure 2. Replication of challenge virus A/mallard/Netherlands/00 (H7N3) in respiratory and systemic mouse organs.

fornia/2009/38 (H1N1) vaccine candidates are identical to A/Leningrad/134/17/57 (H2N2) in lacking neuroivasive capacity, and all three viruses similarly fail to replicate in the brain.

It was shown that all immunized animals survived after challenge with wild-type A/mallard/Netherlands/12/00 (H7N3) virus. The mice in vaccine groups showed no signs of morbidity. Average weight changes were tracked from day 2 to day 6 in all study groups, but the changes did not exceed 5%. As shown in Figure 2, the challenge virus actively replicated in respiratory tissue taken from mock immunized animals (5·7 $lgEID_{50}$ in the lung and 4·2 $lgEID_{50}$ in the nose), but failed to infect the brain and spleen. On the other hand, in both H7N3 LAIV vaccinated groups, all tested organs were free from presence of challenge virus. Thus, immunization of mice with either 1000 MID_{50} or 100 MID_{50} H7N3 LAIV protected the animals from the subsequent challenge infection with a homologous with wild-type H7N3 virus.

Both H1N1 and H7N3 LAIV candidates were found to be immunogenic. After one dose of 100 MID_{50} of H1N1 LAIV, GMT of HI antibodies were 17·6. One dose of 1000 MID_{50} or 100 MID_{50} H7N3 LAIV elicited HI antibody level with GMT of 8·7 and 6·6, respectively. The second dose of H7N3 LAIV further stimulated serum HI antibody levels to GMT 40·0 and 23·3, for 1000 MID_{50} or 100 $MID_{50,}$ respectively (data not shown).

The mouse model is widely used to better understand the pathogenicity of avian influenza viruses for mammalian species, to be able to predict the pandemic potential of such viruses, and to develop improved methods for the prevention and control of the virus in a potential pandemic.[3] A subset of the H7 viruses was evaluated for the ability to replicate and cause disease in BALB/c mice following intranasal administration. H7 subtype viruses were able to infect mice without adaptation and manifested different levels of lethality and kinetics of replication.[4] There is limited preclinical information available for LAIV. Thus, live monovalent vaccine against pandemic influenza virus H1N1 (Influvir) was tested for acute toxicity and its effect on the systems and organs of laboratory animals. According to toxicology and necroscopy results, the live monovalent influenza vaccine Influvir, when applied intranasally, was safe and was well tolerated.[5] In our current study we demonstrate that A(H1N1) and A(H7N3) LAIV are indistinguishable from the parental MDS virus with regards to replication kinetics in the upper and lower respiratory tract of mice. Both H1N1 and H7N3 LAIV candidates were immunogenic and protect mice against subsequent a challenge with the wild-type virus.

Conclusions

Live attenuated cold-adapted (ca) influenza vaccines are an effective means for the control of influenza, most likely due to their ability to induce both humoral and cellular immune responses. In our study we confirm that new H1N1 LAIV and H7N3 LAIV candidates are safe, immunogenic, and confer protection from influenza infection in mice.

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Low doses of AF03-adjuvanted pandemic influenza A/H5N1 and A/H1N1 vaccines protect ferrets against infection after homologous and heterologous viral challenge

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Keywords Adjuvant, ferrets, H1N1 2009, H5N1, influenza, vaccine.

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Introduction

The global response to an influenza pandemic requires the availability of a maximum number of vaccine doses in a very short time frame. Faced with limited manufacturing capacity, the development of efficacious low-dose pandemic influenza vaccines using adjuvants is considered to be a key component in antigen-sparing strategies. In 2009, the novel swine-origin 2009 A (H1N1) influenza viruses were responsible for influenza-like illness in Mexico and the United States in April 2009,[1] and on June 11, 2009 the World Health Organization (WHO) declared a pandemic by raising the worldwide pandemic alert level to phase 6. Therefore, H1N1 inactivated monovalent vaccine formulated with our proprietary oil-in-water emulsion based adjuvant was evaluated in ferrets for its potential to induce with low antigen dose efficient, robust, and rapid protective immunity against a wild type challenge virus (A/Netherlands/602/2009). This adjuvant was also tested in ferrets in a H5N1 avian influenza model for its ability to induce a cross-clade immunity and cross-protection.

Materials and methods

Two independent studies (A&B) were carried out with male and female outbred ferrets (*Musleta putorius furo*) in compliance with "Guide for the care and use of laboratory animals," ILAR recommendations and AAALAC standards. Ferrets used in both studies were influenza seronegative by anti-nucleoprotein ELISA and by HI assay against the pandemic and seasonal strains. In study A, four groups of seven ferrets aged approximately of 6 months received one or two IM vaccinations 3 weeks apart of either AF03-adjuvanted (3·8 μg of HA with AF03) or unadjuvanted (15 μg of HA) A/H1N1/California/07/2009 monovalent vaccine. Seven control animals received adjuvant alone. Four weeks after the last vaccine administration, ferrets were inoculated intratracheally with 10^6 $TCID_{50}$ of wild-type H1N1 homologous challenge virus A/Netherlands/602/2009. In study B, groups of 6 ferrets aged of approximately 12 months received two doses 3 weeks apart of AF03-adjuvanted A/H5N1/Indonesia/5/05/ CDCRG-2 (3·8 μg of HA). Control animals received AF03 adjuvant alone. Three months later, all ferrets were challenged intra-tracheally with 10^4 $TCID_{50}$ of either the homologous A/Indonesia/5/05 (clade 2.1 A/H5N1) virus or with one of the heterologous viruses A/turkey/Turkey/1/05 (clade 2.2 A/H5N1), A/Vietnam/1194/04 (clade 1 A/H5N1), or A/Hong Kong/156/97 (clade 0 A/H5N1). In both studies, ferrets were monitored after challenge for clinical signs, temperature, and weight change. To assess viral shedding in the upper respiratory tract, nasal and pharyngeal swabs were collected on days −2, 2, 4, and 5. Animals were necropsied 4 days after the A/H1N1 challenge and 5 days after the A/H5N1 challenges. Macroscopic examination was conducted to assess lung pathology and lung tissue was collected for measurement of viral loads and microscopic examination. Viral titers were determined as previously described[2] on Madin-Darby canine kidney cells. Data are expressed as $\log_{10}$ $TCID_{50}$ per gram of lung tissue or per mL of swabs. The HI assay was used following a standard protocol with either 1% chicken erythrocytes, for H1N1 pandemic[3,4] or with 1% horse erythrocytes for H5N1. HI titrations were performed on individual RDE (Receptor Destroying Enzyme, Sigma-Aldrich, France) treated serum samples collected at each time point. For HI geometric mean titre calculation, non-responders were assigned a titre of 1:5. Serum samples were also tested using a seroneutralization (SN) assay[4] and using an HRP-labeled mouse monoclonal antibody (Serotec, Oxford, UK) to detect viral protein.

Results

In study A, HI and MN antibody responses were detected in all animals after vaccination with A/California/07/2009

(H1N1) in the presence or absence of adjuvant (Table 1), whereas no antibody responses were observed in control animals. A single administration of a human dose of unadjuvanted A/California/07/2009 monovalent vaccine elicited HI antibody titers specific to A/California/07/2009 strain (mean HI titer = 26) with 100% of animals with HI titers ≥10 and 50% of animals with HI titers ≥40, a titer associated with protection of humans against seasonal influenza.[5] HI and MN antibody titers were markedly increased (≥3·4-fold) after the second vaccine dose of unadjuvanted vaccine with 75% of ferrets showing HI titers ≥40. Vaccination with 3·8 μg of AF03-adjuvanted A/California/07/2009 monovalent vaccine, induced higher HI and MN titers (>7-fold) in ferrets that received one or two vaccine doses as compared to those observed in animals vaccinated without adjuvant. Strikingly, HI titers ≥40 were elicited in 100% of ferrets after a single administration of the AF03-adjuvanted vaccine. Moreover, functional antibody titers induced by a single dose of 3·8 μg of adjuvanted vaccine were as high as those induced by two doses of 15 μg of unadjuvanted vaccine, showing a marked antigen dose-sparing effect of the AF03 adjuvant.

Four weeks after the last vaccination, ferrets were challenged intratracheally with wild type A/Netherlands/602/2009 (H1N1) virus. Parameters used for the assessment of protective efficacy the adjuvanted and unadjuvanted vaccines are shown in Table 1. All ferrets, including unvaccinated controls survived the H1N1 2009 challenge. Body weight loss was monitored as an indicator of disease and a mean body weight loss of 20% was recorded in the control group at day of necropsy. Body weight loss was reduced to ≤10% and ≤8% in animals that had received 1 and 2 doses of either unadjuvanted or AF03-adjuvanted vaccine, respectively.

Viral lung titration showed high levels of virus replication (≥4·7 TCID50/g tissue) in the lungs of all control ferrets 4 days after challenge. One or two administrations of unadjuvanted vaccine reduced lung viral load by 2 and 3 $\log_{10}$, respectively. Interestingly, ferrets that received either one or two doses of AF03-adjuvanted H1N1 2009 vaccine, showed significantly greater reduction of lung viral loads (>4 $\log_{10}$). No virus was detected in the lungs of 6/7 (86%) animals immunized with a single injection of the AF03-adjuvanted vaccine and in 100% of ferrets vaccinated twice.

Assessment of viral shedding from the upper respiratory tract showed that the AF03-adjuvanted A/H1N1 monovalent vaccine was able to reduce the viral load in the nose and in the throat by 3·8 and 3·1 $\log_{10}$, respectively, as compared to the control group. Conversely, viral loads were only slightly reduced in the nose and mostly unchanged in the throat in ferrets immunized with either one or two doses of unadjuvanted A/H1N1 monovalent vaccine.

Gross pathology and histology examinations revealed lung lesions consistent with influenza A/H1N1 virus infec-

Table 1. Immunogenicity and protection of ferrets challenged with A/H1N1/Netherlands/602/2009 4-weeks after the last immunization with one or two doses of either 15 μg of unadjuvanted or 3·8 μg of AF03-adjuvanted A/H1N1/California/07/2009 monovalent vaccine

	Immunogenicity	Clinical signs	Viral detection		Gross pathology and histology	
Vaccine and vaccine regimen (μg of HA)	**HI titers (geometric mean titre)***	**Body weight loss*** (%)**	**Lung tissue (Log_{10} $TCID_{50}$/g)**	**Upper respiratory tract and shedding (nasal and pharyngeal swabs)**	**Affected lung parenchyma (%)**	**Alveolar damages****
Unadjuvanted A/H1N1 (15 μg)						
Single (D21)	36	10	3·4	≈1 Log_{10} reduction	7	+
D0, D21	95	9	2·5	of viral shedding	3	+
AF03-adjuvanted A/H1N1 (3·8 μg)						
Single (D21)	190	8	1·3	>3 Log_{10} reduction	4	+/−
D0, D21	987	8	≤1·03 (detection threshold)	of viral shedding	1	+/−
AF03 alone (control)						
D0, D21	5	20	5·6	++++	34	++++

*HI titers measured 3 weeks after the last vaccine administration.
**Severity of alveolitis, alveolar oedema and hemorrhage.
***[(Weight at D-1 pre-challenge)/(weight at D+4 post-challenge)] × 100.

tion in control animals. Based on the percentage of affected lung parenchyma, lung lesions were reduced in all vaccinated animals as compared to controls, irrespective of vaccine formulation or regimen. Histopathology studies showed that formulation of vaccine with AF03-adjuvant reduced alveolitis, alveolar damage, alveolar oedema and hemorrhage, and type II hyperplasia.

In study B, vaccination of ferrets with H5N1 clade 2.1 vaccine strain induced HI and MN antibodies responses against the clade 2.1 vaccine strain. Detectable HI responses were induced by a single dose of vaccine (data not shown). However, a second dose of AF03-adjuvanted vaccine strongly increased HI and MN titers, which persisted for 3 months (Table 2).

Antibody responses cross-reactive to heterologous clade 2.2 strain were elicited ferrets vaccinated with the AF03-adjuvanted clade 2.1 vaccine. HI antibody titers ≥40 cross-reactive to clade 2.2 and persistent up to D110 were observed in vaccinated animals. An inter-clade low cross-reactive HI response to a clade 1 strain was only detected in a few ferrets that had been vaccinated with the AF03-adjuvanted clade 2.1.

All AF03-adjuvanted clade 2.1 antigen vaccinated animals survived challenge either with the homologous or heterologous virus until euthanized day 5. After challenge, mean body temperature and mean body weights were monitored as indicators of disease. In the control ferrets, mean body temperature increased by 2–3°C (depending on the challenge virus strain) 24 h post challenge, with an accompanying mean body weight loss ranging from 15·4% to 20·7%. Ferrets vaccinated with the AF03-adjuvanted clade 2.1 vaccine showed a lower and delayed fever compared to control ferrets that received the same viral challenge, whereas no significant differences were observed between vaccinated animals and their respective controls upon challenge with clade 2.2 or clade 0 viruses. Body weight loss was reduced in all vaccinated animals when compared to controls after challenge with either the homologous clade 2.1 strain or with one of the heterologous strains.

Lung virus titration showed high levels of virus replication in all control animals 5 days after homologous challenge with the clade 2.1 virus. Lung viral loads of all ferrets immunized with the AF03-adjuvanted clade 2.1 vaccine were reduced more than 4 $\log_{10}$. Vaccination resulted in complete viral clearance from the lungs of 80% of animals assessed 5 days after challenge. As compared to controls, a reduction of the mean viral load of about 2 $\log_{10}$ was observed in ferrets vaccinated with the AF03-adjuvanted clade 2.1 vaccine after heterologous challenge with either the clade 0 or clade 1 virus. Conversely, vaccination with AF03-adjuvanted clade 2.1 vaccine did not result in reduction of lung viral loads after challenge with the clade 2.2 heterologous virus strain.

Titration of pharyngeal swabs showed high levels of viral shedding in all control ferrets after challenge with clade 2.1 strain, whereas virus was not detected in any vaccinated animal. Similarly, 5 $\log_{10}$ reduction of viral shedding was seen in vaccinated versus control ferrets following clade 1 heterologous challenge. Lower reductions in viral shedding were observed after clade 2.2 challenge (2·6 $\log_{10}$) and clade 0 challenge (1·4 $\log_{10}$).

Table 2. Immunogenicity and protection of ferrets challenged with homologous and heterologous A/H5N1 virus strains 3 months after the last immunization with 3·8 μg of AF03-adjuvanted A/H5N1 clade 2.1 monovalent vaccine

	Protection parameter							
			Clinical signs reduction		Viral detection		Reduction of macro- and microscopic lung damages	
Challenge strain	H I titers (geometric mean titre)*	% Survival [% control]	Body weight loss	Fever	Lung tissue ($\log_{10}$ $TCID_{50}$/g)	Upper respiratory tract and shedding (pharyngeal swabs)	Affected lung parenchyma	Alveolar damages**
Homologous								
Clade 2.1	72	100 [80]	++	++	Undetectable	Undetectable	++++	+++
Heterologous								
Clade 0	Not done	100 [83]	++	=	2 $\log_{10}$ reduction	1·4 $\log_{10}$ reduction	++	++
Clade 1	12	100 [100]	+++	+	2 $\log_{10}$ reduction	Undetectable	N/A***	N/A***
Clade 2.2	52	100 [50]	+/−	=	=	2·6 $\log_{10}$ reduction	=	+/−

*Pre-challenge (day 110) HI titers specific to the clade 2.1 vaccinal strain or cross-reactive to the heterologous challenge strain.
**Severity of alveolitis, alveolar oedema and hemorrhage.
***N/A, not applicable as few or only mild macro- and microscopic damages were observed with clade 1 challenge in control group.

Gross pathology and histology revealed lung lesions consistent with influenza A/H5N1 virus infection all control animals challenged with the clade 2.1, clade 2.2 or clade 0 strains. Mild to moderate lung lesions were observed in control animals following challenge with clade 1 virus. Macroscopic evaluation (percentage of affected lung parenchyma) and histopathological analysis (extent and severity of alveolitis, alveolar oedema and hemorrhage) showed that lung lesions were significantly reduced in AF03-adjuvanted clade 2.1 vaccinated animals after challenge with the homologous clade 2.1 virus strain as compared to controls. Similarly, a reduction of the macroscopic and microscopic lung lesions was observed in vaccinated animals upon heterologous challenge with clade 2.2 and clade 0 virus strains, whereas no differences were observed between control and vaccinated animals after challenge with clade 1 virus.

Discussion

The results of these ferret challenge studies demonstrated that low doses of pandemic influenza vaccines formulated with an oil-in-water emulsion adjuvant, AF03, elicited strong antibody responses specific to the immunizing strain. Importantly, these vaccines provided protection after homologous challenge with complete virus clearance in ferret lungs and reduced viral shedding from the upper respiratory tract suggesting an ability to reduce virus transmission. Moreover, AF03-adjuvanted H5N1 vaccine can provide cross-protection upon challenge with different H5N1clades by preventing mortality and reducing the viral burden in the lower and the upper respiratory tract. In conclusion, the results of these studies highlighted the ability of AF03-adjuvanted influenza vaccines to induce potent immune responses and full protection in ferrets against homologous challenge and suggested that protection may be mediated, at least in part, by antigen-specific humoral immunity.

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H9N2 influenza virus vaccine prepared from a non-pathogenic isolate from a natural reservoir conferred protective immunity against the challenge with a human H9N2 virus in mice

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Introduction

Since 1993, outbreaks of H9N2 influenza virus infection in poultry have occurred in Eurasian countries. Phylogenetic and antigenic analysis of H9N2 isolates revealed that there are three sublineages, consisting of G1, G9, and Korean, among HA genes of the Eurasian H9N2 viruses. H9N2 viruses do not cause severe disease in poultry, but co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, or attenuated coronavirus vaccine may exacerbate the disease.[1,2] H9N2 viruses were isolated from domestic pigs in China and Korea and from humans with febrile respiratory illness in Hong Kong in 1998, 1999, and 2003.[3–5] It is, thus, postulated that

H9N2 virus may cause pandemic influenza in humans. We, therefore, prepared test vaccine from H9N2 influenza viruses isolated from feral duck.

In the present study, H9 virus strains were analyzed antigenically and phylogenetically to select a proper H9N2 vaccine strain. Inactivated whole virus particle vaccine was prepared, and its potency against H9 virus challenge was assessed in mice.

Materials and methods

Viruses

A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2), A/quail/Hong Kong/G1/1997 (H9N2), A/chicken/Hong Kong/FY20/1999 (H9N2), A/silkie chicken/Hong Kong/SF43/1999 (H9N2), and A/quail/-Hong Kong/A17/1999 (H9N2) were provided by Dr K. F. Shortridge, the University of Hong Kong, China. A/ostrich/South Africa/9508103/1995 (H9N2) and A/chicken/Pakistan/2/1999 (H9N2) were provided by Dr I. H. Brown, Veterinary Laboratories Agency, Weybridge, UK. A/Hong Kong/1073/1999 (H9N2) (HK/1073/99) was provided by Dr A. J. Hay, MRC National Institute for Medical Research, UK. These viruses were grown in 10-day-old embryonated chicken eggs, and infectious allantoic fluids were stored at −80°C until use.

Phylogenetic analysis

Viral RNAs were extracted from the allantoic fluid of chicken embryos infected with viruses by using a commercial kit (TRIzol LS Reagent; Invitrogen, California, USA) and reverse-transcribed with the Uni12 primer[6] and M-MLV reverse transcriptase (Invitrogen). The primers used for the HA gene amplification were H9-101F[7] and H9-1341R. For phylogenetic analysis, sequence data of the genes together with those from public database were analyzed by the neighbor-joining method.[8]

Antigenic analysis

H9 influenza viruses were analyzed by hemagglutination-inhibition (HI) test.[9] Chicken hyperimmunized antisera against seven H9 viruses were prepared according to previous report.[10]

Virus replication and pathogenicity against embryonated chicken eggs

Viruses were inoculated into 10-day-old embryonated chicken eggs and incubated for 48 hours at 35°C. HA titers and 50% egg infectious dose (EID_{50}) were measured every 12 hours post-inoculation. Pathogenicity of Dk/Hok/49/98 against embryonated chicken eggs was evaluated by mean death time (MDT) as described previously.[11]

Vaccine preparation

Dk/Hok/49/98 was injected into the allantoic cavities of 10-day-old embryonated chicken eggs and propagated at 35°C for 48 hours. The virus in the allantoic fluids (512HA) was purified by differential centrifugation and sedimentation through a sucrose gradient according to previous report.[12] The concentration of protein was measured by OD using Ultrospec 3100 *pro* (Amersham Biosciences, Tokyo, Japan). The purified virus was inactivated with 0·1% formalin at 4°C for 7 days.

Immunization of mice and challenge of immunized mice with HK/1073/99

Four-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were injected subcutaneously with 10, 2, 0·4, or 0·08 μg proteins of inactivated Dk/Hok/49/98 whole virus vaccine. Two weeks later, the mice were boosted by subcutaneous injection with the same dose of the vaccine. Control mice were injected with PBS. Serum samples were tested by enzyme-linked immunosorbent assay (ELISA) according to previous report.[10] One week after the second vaccination, 10 mice in each group were challenged intranasally with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under anesthesia. On 3 days post-infection, five mice in each group were sacrificed, and the lungs were separately homogenized to make a 10% (w/v) suspension with minimal essential medium (Nissui, Tokyo, Japan). The virus titers of the supernatants of lung tissue homogenates were calculated in 10-day-old embryonated chicken eggs and expressed as the EID_{50}/gram of tissue. The other five mice in each group were monitored for body weight for 14 days after challenge.

Results

Phylogenetic analysis of the HA gene of H9 influenza viruses

The HA genes of 22 H9 viruses were sequenced and analyzed by the neighbor-joining method. All of the 22 H9 viruses were classified into the Eurasian lineage (Figure 1). Eleven, seven, and four strains were classified in the Korean, G9, and G1 sublineages, respectively. The H9 viruses of the Korean and G9 sublineages were isolated from waterfowl, poultry, pigs, and humans in the east Asian countries, and those of the G1 sublineage were isolated from poultry in the west Asian countries.

Antigenic analysis of the H9 influenza viruses

The cross-reactivity between these antisera and H9N2 viruses were analyzed by HI test. The antisera against H9 viruses belonging to the Korean sublineage were broadly cross-reacted to H9 viruses belonging to the G9 and G1

sublineages. H9 viruses belonging to the Korean lineage were reacted to the antisera against H9 viruses belonging to the G9 and G1 sublineage compared with H9 viruses belonging to the other sublineage (data not shown). Thus, it was suggested that H9 vaccine strain should be selected from the viruses of Korean sublineage to prepare for the vaccine strain of H9 viruses.

Selection of H9 vaccine strain for mice

Dk/Hok/49/98 replicated efficiently in 10-day-old embryonated chicken eggs (data not shown). Pathogenicity of Dk/Hok/49/98 against embryonated chicken eggs was determined by MDT. Dk/Hok/49/98 was low pathogenic against embryonated chicken eggs (data not shown) and was selected as an H9 vaccine strain.

Protective efficacy of the test vaccine in mice against H9 virus challenge

To assess the potency of the vaccine against H9 virus infection, mice vaccinated subcutaneously with inactivated Dk/Hok/49/98 were challenged intra-nasally with HK/1073/99. Immunogenicity of the inactivated vaccine was assessed by measuring the IgG antibodies in mouse sera by ELISA. Antibody was detected in the group of mice injected 10 μg protein after the first immunization and detected in the group of mice injected 2 μg protein after the second immunization. Thus, potency of the present inactivated whole virus vaccine was demonstrated in mice. Next, to assess the protective immunity of the inactivated vaccine in mice, viral titers in the lungs was determined. The virus titers in the lungs were $10^{1.5}$–$10^{3.7}$ EID_{50}/g in the groups of mice injected 10, and 2 μg protein, and $10^{5.3}$–$10^{6.0}$ EID_{50}/g in the other vaccinated groups. Body weight reduction of mice were observed in the group of mice injected 0·4, 0·08 μg protein, and control groups from 3 dpi, and reached to 10% body weight loss from 4- to 6-day post-infection (Figure 2). This result correlates with antibody titer in mouse sera and viral titers in the lungs. These results suggest that the test H9 inactivated whole vaccine confers prevent of weight loss and reduction of virus replication against H9 influenza virus infection in mice.

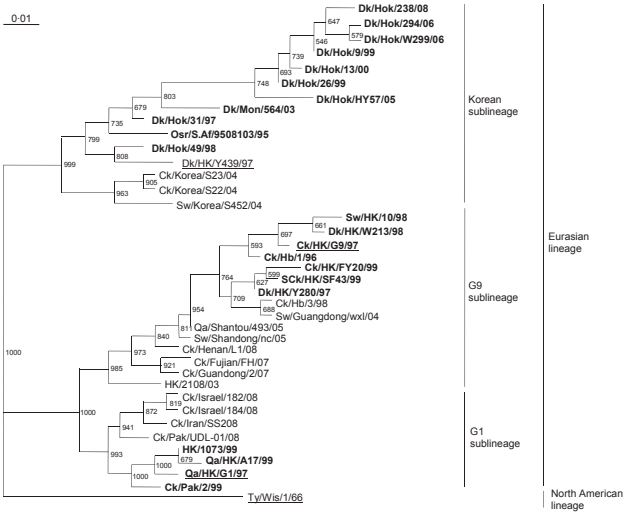

Figure 1. Phylogenetic tree of the HA genes of H9 influenza viruses. Nucleotides 163–1048 (886 bases) of the H9 HA genes were used for the analysis. Viruses stocked in our laboratory are bolded. Representative viruses in each sublinage are underlined. Sw, swine; Ck, chicken; SCk, silky chicken; Dk, duck; Osr, ostrich; Qa, quail; Ty, turkey; HK, Hong Kong; Hb, Hebei; S.Af, South Africa; Hok, Hokkaido; Pak, Pakistan; and Wis, Wisconsin.

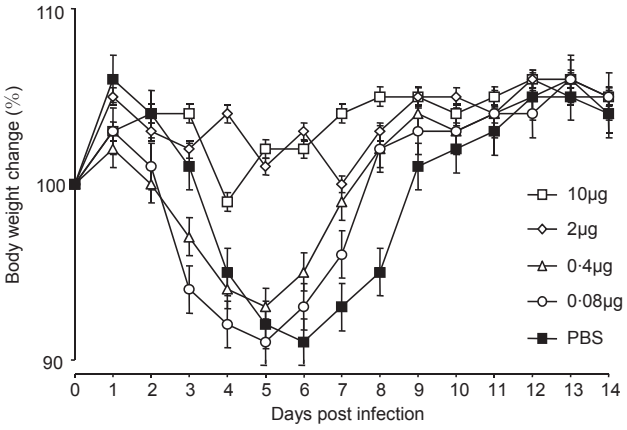

Figure 2. Changes in body weight of challenged mice. Data presented correspond to average body weight changes in each group with corresponding SD.

Disucussion

Recently, H9N2 viruses of all of three sublineage have been isolated from wild birds and poultry in worldwide. H9N2 viruses were isolated from pigs and humans in China[3] and Korea, suggesting that H9N2 virus would be a potential for a pandemic influenza virus in human population. H9N2 viruses were isolated from pigs in China and Korea and were classified into the G9 and Korean sublineage. In human cases, all H9N2 virus isolated from humans in China was classified into the G1 sublineage. It was suggested that H9N2 viruses isolated from pigs and humans vary in antigenicity of isolates between the Korean, G9, and G1 sublineages. Therefore, it is important for the preparedness of influenza pandemic to develop H9 influenza virus vaccine, which could broadly cross-react to antisera of all sublineage viruses. So, we selected the vaccine candidate strain, Dk/Hok/49/98, which could broadly cross-react to antisera of all sublineage viruses, and which could replicate

efficiently in embryonated chicken eggs. Thus, an inactivated whole particle virus vaccine of Dk/Hok/49/98 was prepared for animal experiment in mice.

In this study, it was suggested that the test vaccine has potency to protect against challenge with H9 virus using mice for mammalian model. The challenge virus, HK/1073/99, was isolated from human, replicates efficiently in mice, and shows pathogenicity in mice. The test vaccine inhibited viral replication and body weight loss in mice. Whole inactivated vaccine produced protective immunity, supporting our approach of using whole virus particles for vaccine development. Furthermore, whole particle virus vaccine could induce IgG and mucosal IgA levels after intranasal vaccination with whole particle vaccine.[13] The present results may facilitate the studies of the vaccine for future pandemic caused by H9 influenza virus in humans.

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Evaluation of growth, genetic, and antigenic characteristics of pandemic H1N1 viruses isolated and passaged in qualified MDCK suspension cells (MDCK33016PF) and embryonated hen eggs

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Keywords Cell culture, pandemic H1N1, vaccine development.

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Abstract

Shortly after the new swine-origin pandemic H1N1 influenza virus emerged, virus isolates were distributed by the WHO for the urgent development of suitable vaccine strains. Unfortunately, these wildtype H1N1pdm viruses grew poorly in cell lines and eggs, requiring the generation of a series of three conventional and eight reverse genetics http://www.who.int/csr/resources/publications/swineflu/summary_candidate_vaccine.pdf derived reassor-

tants to attempt to improve growth. To determine whether wild type H1N1pdm grew better in the Novartis MDCK suspension cell line (MDCK33016PF) than in eggs, isolations from H1N1pdm positive clinical samples were attempted in both substrates. The isolation rate of H1N1pdm viruses was higher in MDCK33016PF cells (89%) (31/35) compared to allantoically inoculated eggs (66%) (23/35). However the yields were lower than observed with seasonal viruses. Little improvement in virus yield was seen with extra passaging or dilutions of H1N1pdm viruses isolated in MDCK33016PF cells.

Introduction

With the emergence of the swine-origin pandemic H1N1 (H1N1pdm) influenza in April 2009,[1] the need for efficient production of a suitable vaccine was a high priority.[2] Virus isolates were distributed by the WHO for the urgent development of suitable vaccine strains early in the pandemic. Vaccine viruses can be grown in embryonated chicken eggs [2] or in certified mammalian cells.[3,4] Unfortunately wildtype H1N1pdm virus strains distributed by the WHO grew poorly in cell lines and eggs, requiring the generation of a series of 3 conventional and 8 reverse genetics [5] derived reassortants to attempt to improve growth. From these reassortants, only the conventional egg derived reassortants NYMC-X-179A and NYMC-X-181 (both based on one of the earliest known viruses A/California/7/2009) showed high enough growth and yield in eggs and cell culture to make them suitable for vaccine manufacture. These reassortants, while acceptable, still only gave haemagglutinin (HA) yields of approximately 60% that of seasonal H1N1 reassortants. To determine if more recent wild type H1N1pdm viruses grew better in the Novartis MDCK suspension cell line (MDCK33016PF), H1N1pdm positive clinical samples were cultured in MDCK33016PF cells and also in embryonated hen's eggs. In addition, to improve virus yields from MDCK33016PF isolates, extended passaging of three wild type H1N1pdm influenza viruses was performed using various virus dilutions at each passage level. The results were assessed using various serological and molecular biology techniques and compared to viruses isolated in eggs and conventional MDCK cells.

Materials and methods

Viruses

H1N1pdm viruses were received at the centre from WHO National Influenza Centres, WHO Influenza Collaborating Centres and other regional laboratories and hospitals in Australia, New Zealand, and the Asia/Pacific region. Viruses were received as original clinical specimens consisting of nasal swabs, throat swabs, nasopharyngeal aspirates, or nasal washes that had previously been shown to be H1N1pdm positive by real time RT-PCR.

Isolation

These specimens were then cultured in MDCK33016PF cells with serum free medium containing trypzean (Optaflu) [3] and also independently inoculated into the allantoic cavity of 11 day-old embryonated hen's eggs. Virus cultures in MDCK33016PF cells were sampled at 48 and 72 hour and evaluated by various means including HA titres. At 72 hour, virus cultures were further passaged at varying dilutions ranging from 10^{-5} to 10^{-8} up to a total of 10 passages. Embryonated hen's eggs were incubated at 35°C for 3 days and allantoic fluid was harvested and HA titres performed to determine whether a further passage was required in order to improve growth.

Reassortants

The conventional reassortants were produced by a mixed infection of eggs or MDCK 33016PF cells with the wild type virus and a donor virus carrying the internal genes of the A/Puerto Rico/8/34 virus. The reassortants were obtained by sequential passages using immuno-selective antisera against the surface antigen of the donor virus to remove virus populations carrying the HA and NA protein of the donor strain.[6] The reverse genetics viruses were rescued in Vero cells using the 12 plasmid system.[7] Both types of reassortants were generated and supplied by WHO Collaborating Centres and essential regulatory laboratories except the NVD-c-07 strain, which was produced by Novartis.

Evaluation

Growth of the viruses was monitored by HA titer using turkey red blood cells, by quantitative real time RT-PCR (qRT-PCR) to detect the influenza A matrix gene, and also by flow cytometry to detect virus positive cells using monoclonal antibodies (IMAGEN Influenza virus A and B).[8,9] Matrix gene copy number was determined using qRT-PCR and analysed using the sequence detection software on a 7500 Fast System SDS (Applied Biosystems, California, USA). Further characterisation was performed through sequence analysis and the HA inhibition (HAI) assay.[8] Sequence analysis was performed using DNAStar 8 and all sequences obtained were compared with the sequence of either the original clinical specimen if available or the conventional ATCC derived MDCK cell isolate. The HAI assay was used to characterize the viruses against a panel of known standard reference viruses and their homologous ferret antiserum.

Results

In this small study with recent H1N1pdm viruses, the isolation rate was higher in MDCK33016PF cells (89%) (31/35) compared to allantoically inoculated eggs (66%) (23/35). Assessment of HA titres, however, showed higher HA titres in egg-isolated viruses compared to viruses isolated in MDCK33016PF cells after two passages. Egg generated or cell generated reassortant viruses gave higher HA titres compared to the homologous wild type viruses (Table 1). No amino acid changes were observed in MDCK33016PF isolated influenza viruses compared to original specimens or viruses isolated in conventional ATCC derived MDCK cells, unlike egg isolated viruses which showed a number of amino acid changes, many consistent with egg adaptation mutations (Table 1).[10] Viruses isolated in MDCK33016PF cells grouped phylogenetically with viruses isolated in conventional ATCC derived MDCK cells or viruses sequenced from original clinical samples, while egg isolated viruses grouped slightly differently (data not shown).

As a result of the poor growth of H1N1pdm viruses in MDCK33016PF cells, serial dilutions were performed over a number of passages (Figure 1). Based on the results obtained from the virus isolates, A/Victoria/2081/2009, A/Wellington/188/2009, and A/Darwin/2131/2009, a supplemental protocol was developed and used in the isolation of A/Brisbane/10/2010 (Figure 1). Only small differences in HA titer were seen between different dilutions, and copy number showed a similar trend to HA titer at each passage (Figure 1). Following the supplemental protocol for the isolation of A/Brisbane/10/2010 results showed slightly higher HA titres with little variation between passages.

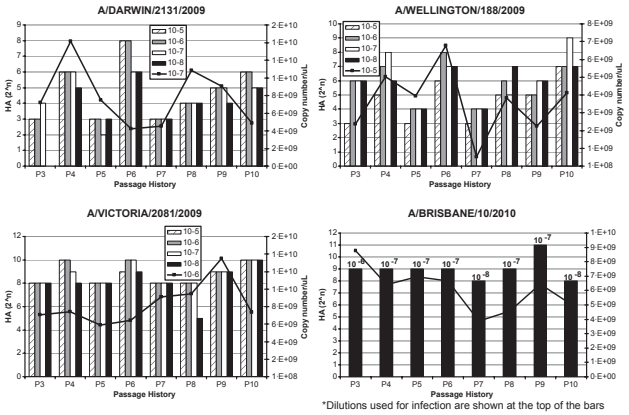

Figure 1. Haemagglutinin titre and copy number of virus isolates from MDCK33016PF cells at different passages and dilutions. These graphs show HA titres at each passage for all dilutions and the copy number for the highest HA titre at the highest dilution over time. The isolate with the best HA titre at the highest dilution was used for the subsequent passage.

The egg derived reassortants NYMC-X-179A and NYMC-X-181 were also assessed for growth in MDCK33016PF cells and were found to be superior by HA titer to other conventional reassortants (egg or cell derived), reverse genetics derived reassortants, or wild type viruses (Table 1). Two methods were used to determine the ratio of HA to other viral proteins: densiometric analysis using SDS–PAGE and reversed-phase HPLC using a subtype specific standard.[11] HA content in different vaccine seeds of influenza A subtypes demonstrated that the HA content per total virus protein from the NYMC H1N1pdm reassortants was significantly different to the seasonal influenza A subtypes. For the seasonal H1N1 the ratio of HA to

Table 1. Characterisation of influenza virus isolates from MDCK33016PF and eggs

		Host substrate					
		Cell	Egg	Cell	Egg	Cell	Egg
Virus	**Subtype**	**Passage history**		**HA titres**		*** HA gene sequence changes**	
A/VICTORIA/2081/2009	H1N1pdm	C2	No growth	64	No growth	Nil	N/A
A/WELLINGTON/188/2009	H1N1pdm	C2	E2	8	128	Nil	E21K, L1911
A/DARWIN/2131/2009	H1N1pdm	C2	E2	16	16	Nil	E21 K, G225A
A/BRISBANE/10/2010	H1N1pdm	C2	E2	256	1024	Nil	E127D
Egg reassortants							
A/CALIFORNIA/7/2009 (NYMC-X-179A)	H1N1pdm	E9/C3		1024	N/A		
A/CALIFORNIA/7/2009 (NYMC-X-181)	H1N1pdm	E12/C3		2048	N/A		
A/FIJI/2029/2009 (A/Fiji/2029)	H1N1pdm	E2/C8		512	N/A		
Cell reassortants							
A/HAMBURG/4/2009 (NVD-c-07)	H1N1pdm	C13	N/A	64	N/A		
200911025 (wt H1N1sea)	SeasonalH1N1	C6	N/A	1024	N/A		
A/FINLAND/09 (wt H3N2)	H3N2	C6	N/A	256	N/A		

N and M1 was ≥30%, for the H3N2 the ratio of HA to N and M1 was ≤30%, while for the pandemic A/H1N1, the ratio of HA to N and M1 was much lower at ≤20% (data not shown).

Discussion

The results of this study has observed the growth of a series of 2009–2010 H1N1pdm viruses in vaccine suitable MDCK33016PF cells to be generally lower than what has been seen with other seasonal influenza viruses.[11] Little improvement in virus yield was seen with extra passaging of H1N1pdm viruses isolated and passaged in MDCK33016PF cells. Passaging up to 10 times in MDCK33016PF cells using dilutions ranging from 10^{-5} to 10^{-8} resulted in supernatants with viral HA titres ranging from 8 HA/25 μl to 512 HA/25 μl. The isolation rate of H1N1pdm viruses was higher in MDCK33016PF cells (89%) compared to allantoically inoculated (and passaged) eggs (66%), a trend also seen in previous work with seasonal influenza viruses.[12] In contrast a study by Hussain and colleagues [13] found similar rates of isolation and replication of seasonal influenza viruses in MDCK cells and eggs.

The virus load as determined by matrix gene copy number showed a similar trend to HA titers. Two of the isolates exhibited small rises and falls in HA titer during passaging, while a third, A/Victoria/2081/2009 gave consistently higher titers. Interestingly this virus was unable to be isolated in eggs. The HA sequences of all strains were assessed at p1, p2, p3, p4, p10 and when available compared to the original clinical sample HA sequence. MDCK33016PF-isolated viruses had few if any changes in their HA amino acid sequence, while the majority of egg isolates showed 1-2 amino acid changes compared to the clinical sample, with an egg adaption change (L191I) evident in a number of them. The HA sequence of one of the better growing viruses, A/Victoria/2081/2009, was found to have a G155E change compared to the A/California/7/2009 reference virus. This change was also seen in the virus isolated in conventional, adherent MDCK cells. These viruses with G155E change when tested by HAI have shown reduced reactivity with ferret antisera to A/California/7/2009-like viruses, but normal reactivity with ferret antisera to H1N1pdm A/Bayern/69/2009-like viruses. Despite this mutation all MDCK33016PF derived viruses appeared to be A/California/7/2009-like by HAI.

The H1N1pdm egg-derived reassortants (NYMC X-179A and NYMC X-181) when grown in MDCK33016PF cells were superior to wild type H1N1pdm viruses, reverse genetics derived reassortants, and other egg-derived reassortants. The yields of haemagglutinin from the NYMC H1N1pdm reassortants were still below those seen with seasonal H1N1 reassortants as was also seen in eggs. This trend has also been noted in other studies.[10]

In summary, attempts to improve growth and yield of the H1N1pdm wild types for MDCK 331016PF cells by extended passaging were not successful, and reassortants did not perform as well as seasonal H1N1 reassortants have in the past. However, using higher dilutions for the passaging of H1N1pdm viruses in MDCK33016PF cells did result in higher HA titres (A/Brisbane/10/2010). Further work is therefore required to generate pandemic H1N1 seed viruses that grow well in a variety of cell culture and egg based vaccine production systems.

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Detection of anti-neuraminidase antibody in preclinical and clinical studies of live influenza vaccine

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Keywords Anti-neuraminidase antibodies, influenza virus, live influenza vaccine, neuraminidase.

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Abstract

The aim of this study is to evaluate antibody response to influenza virus neuraminidase (NA) following immunization with live attenuated influenza vaccine (LAIV). We adjusted the peroxidase-linked lectin micro-procedure previously reported by Lambre, *et al.* (1990) to assay neuraminidase inhibition (NI) antibody in sera taken from immunized mice and from human subjects in a clinical trial. For the assay, we prepared the A(H7N1) reassortant virus containing the NA of A/California/07/2009 (H1N1) and the hemagglutinin (HA) of A/equine/Prague/1/56(H7N7). In addition, we used an NA-specific IgG ELISA assay to test sera from immunized mice and volunteers. In mice, one dose of LAIV induced NI antibody of a geometric mean titer (GMT) of 31·7, compared to 10·6 in the control group. GMT of NI from human subjects who received two doses of pandemic A(H1N1) were significantly higher than pre-vaccination titers. In unvaccinated human subjects, NA-specific cross-reactive antibodies to pandemic A(H1N1) were detected more often than cross-reactive antibodies to HA.

Background

Antibody response to influenza virus NA contributes to the overall immune response to influenza and may provide partial protection against influenza infection and reduce severity of disease in the host.[1] A number of preclinical studies using purified or recombinant NA have shown that various two–dose vaccine regimens in mice may significantly reduce pulmonary virus titers following viral challenge.[2–4] A plasmid DNA-vaccine model demonstrated cross-reactive antibodies to human N1 in mice could provide partial protection against a lethal challenge against H5N1 or recombinant PR8 bearing the avian N1.[4]

Immunogenicity of current influenza vaccines, including LAIVs, is measured primarily as a level of strain-specific hemagglutination inhibition (HI) antibodies.[5] However, the WHO meeting on the role of NA in inducing protective immunity against influenza infection (2008) specified a need to develop suitable assays for anti-NA antibody detection to enhance influenza vaccine evaluation in preclinical and clinical studies.[6] The aim of the current study was to evaluate anti-NA antibodies to pandemic A(H1N1) 2009 influenza virus following LAIV immunization.

Materials and methods

Viruses

The RN1/09-swine A(H7N1) reassortant influenza virus containing the NA of A/California/07/2009(H1N1) and the HA of A/equine/Prague/1/56(H7N7) generated by classical genetic reassortment in embryonated chicken eggs (CE). Parental A/equine/Prague/1/56(H7N7) influenza virus was obtained from the Center for Disease Control and Prevention, Atlanta, GA, USA. Viruses were propagated in 10 day old CE and purified by sedimentation out of the allantoic fluid, followed by ultracentrifugation on 30–60% sucrose step gradient.

Immunization and sera samples

For the mouse studies, 10 week old CBA mice were inoculated intranasally with one dose 10^7 EID_{50}/0·05 ml of A/17/California/09/38(H1N1) vaccine strain or received 0·05 ml PBS. Blood samples were collected on day 15 post inoculation. Healthy young adults were immunized twice, 10 or 21 days apart in the fall 2009 with A/17/California/09/38(H1N1) LAIV manufactured by Microgen, Irkutsk, Russia.

For the human studies, peripheral blood specimens were collected from volunteers before vaccination, 21 days after the first vaccination, and 21 days after the second dose of vaccine. Sera from five subjects diagnosed with influenza A(H1N1) were collected in December 2009, 3 to 4 weeks post infection and kindly provided by E. Voĭtsekhovskaia from Biotechnology Laboratory, Institute of Influenza, RAMS. Also, sera obtained in 2005 from unvaccinated vol-

Table 1. A/California/07/09(H1N1)-specific antibodies in mice 2 weeks after administration of intranasal LAIV

Vaccine groups	HI (GMT)	NI (GMT)	ELISA IgG (log_{10}, M ± σ)
LAIV (n = 9)	17·4	31·7	1·7 ± 0·3
PBS (n = 6)	7·6	10·6	1·0 ± 0·0

unteers were tested for presence of cross-reactive antibodies to A/California/07/2009 (H1N1).

Serum antibody evaluation

Sera were treated with a receptor-destroying enzyme from *Vibrio cholera* (Denka-Seiken, Tokyo, Japan) and then were tested in duplicates for hemagglutination-inhibition (HI) H1 specific antibodies by standard procedures[7] using A/17/California/09/38(H1N1) test antigen. The peroxidase-linked lectin micro-procedure previously reported by Lambre, *et al.* [8] was adjusted to assay NI antibody. Briefly, 96-well plates (Sarstedt, Inc., Nümbrecht, Germany) were coated overnight with 150 μl of 50 μg/ml fetuin. The purified A(H7N1) reassortant virus was diluted in PBS with 1% BSA and 10 mm Ca^{2+} to give a four times higher optical density at 450 nm (OD_{450}) compared to control wells not containing virus. Fifty-microliter volumes of serially diluted serum samples were incubated with an equal volume of prediluted virus for 1 hour at 37°C. After incubation, the plates were washed and neuraminidase activity was measured by subsequently adding peroxidase-labeled lectin (2 μg/ml; Sigma, St. Louis, MO, USA), incubating for 1 hour at room temperature, washing the plates, and adding 100 μl of peroxidase substrate (TMB). The reaction was stopped after 5 minute by adding 100 μl of 1N sulfuric acid. OD values were measured at 450 nm using the universal microplate reader (EL_x800; Bio-Tek instruments, Inc., Winooski, VT, USA). The NI titers were expressed as the reciprocal dilution that gave 50% OD of positive control (virus, no serum control). In addition we used an IgG ELISA assay[9] with 0·5 μg/ml of purified NA from A/California/07/09(H1N1) to test sera from immunized mice and volunteers.

Statistical analysis

Data were analyzed with Statistica software (version 6·0) (StatSoft, Inc. Tulsa, Oklahoma, USA). Geometric mean titers (GMT) were calculated and used to represent the antibody response. The comparisons were made within groups between pre- and postvaccinated titers (expressed as log_2) after first and second vaccination using Wilcoxon matched pairs test. To compare multiple independent groups we used a Kruskal–Wallis anova with subsequent multiple pairwise comparison based on Kruskal–Wallis' sums of ranks. A P-value of <0·05 was considered to be statistically significant.

Results

In mice, one dose of LAIV induced antibody responses to both HA and NA components of the A/California/07/2009(H1N1) influenza virus vaccine (Table 1). Geometric mean titers of NI antibody levels from vaccinated mice were 31·7 and were significantly higher compared to those in unvaccinated control animals ($P < 0·02$). ELISA IgG titers expressed as log_{10} were 1·7 compared to 1·0 in control group. There was good correlation between antibody rises obtained using NI or ELISA tests (r = 0·7).

In a study during the fall of 2009, 85% of 60 examined unvaccinated subjects were negative to pandemic A(H1N1) (HI titers ≤1:10). Serum HI antibody titers to pandemic A(H1N1) ≥1:40 were considered to be protective against

Table 2. Antibody response to HA and NA of A/California/07/09(H1N1) in volunteers before and after vaccination with LAIV

	HI data			NI antibodies	
		GMT			
Groups	% with ≥4-fold antibody rises	Before vaccination	After vaccination	Before vaccination	After vaccination
LAIV 2 doses (n = 31)	32·3	6·7*	11·4*	13·3**	17·2**
LAIV 1 dose (n = 14)	7·1	6·4	8·6	15·3	18·9
Placebo (n = 6)	0	6·3	6·3	9·1	9·4
Convalescents (n = 5)	nd	nd	183·8	nd	177·4

*The postvaccination GMTs of HI antibodies after revaccination were higher than respective prevaccination titers (P = 0·04)
**The postvaccination GMTs of NI antibodies after revaccination were higher than respective prevaccination titers (P = 0·02)

influenza infection and were detected only in three subjects (5·0%), whereas NI antibody titers ≥1:40 were found in eight subjects (13·3%). In a study during the fall of 2005, 100% of 56 examined clinical trial subjects were negative to pandemic A(H1N1) according to HI test, however, NI antibody titers of ≥1:20 were found in 18 (32·1%), including 2 (3·6%) with NI antibody titers ≥1:40 (data not shown).

Serum HI and NI antibodies to A/California/07/09(H1N1) after one or two doses of pandemic LAIV were evaluated in subjects who had pre-vaccination HI titers ≤1:10 (Table 2).

Post-vaccination GMTs of A(H1N1)-specific antibodies were significantly higher than pre-vaccination titers only among subjects who received two doses of LAIV (Table 2). The frequency of subjects with ≥ fourfold rises in HI antibody titers was higher after two doses (32·3%) compared to responses after one dose (7·1%) although the differences were not statistically significant (Table 2). The highest antibody titers of HI and NI antibodies were achieved after natural infection ($P < 0{\cdot}01$ compared to all post-vaccination groups). All five subjects with confirmed influenza also had high levels of N1-specific IgG measured by ELISA using purified NA as the coating antigen (data not shown).

Influenza HA and NA surface proteins are primary targets of neutralizing antibodies that provide protection against influenza infection. The correlation of strain-specific HI antibody titers ≥1:40 to protection of 50% of the subjects against influenza infection is based on a number of reports published in 1980s.[10] Serum antibodies against viral NA as result of influenza infection or vaccination also can neutralize the virus from infecting cells; however, little is known about protective levels of such antibodies. To evaluate NI antibodies directed against pandemic A(H1N1) we used the reassortant A(H7N1) influenza virus with mismatched HA to avoid non-specific inhibition. We demonstrated LAIV immunization effectively increased levels of NI antibody, although in smaller amounts compared to influenza infection. Our data suggest that an antibody to neuraminidase, resulting from an earlier infection of the circulating seasonal influenza A(H1N1), evidently cross-reacted with the N1 of pandemic influenza virus, perhaps due to the previously reported 17% of conserved NA epitopes in pandemic A(H1N1).[11]

Conclusions

The peroxidase-linked lectin test using the reassortant A(H7N1) influenza virus was shown to be a sensitive and time effective means of revealing homologous and cross-reactive anti-NA antibodies after LAIV immunization or influenza infection. This could be a useful method for influenza vaccine evaluation. Significant levels of anti-NA antibodies detected in peripheral serum from subjects infected with wildtype H1N1 virus or with H1N1 LAIV.

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Cross-reactive antibody response to pandemic H1N1 2009 in mainland China

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Abstract

Background: We measured the cross-reactive antibody response to pandemic H1N1 2009 in children and adults before and after vaccination with 2007–2008, 2008–2009 influenza season vaccines as part of the rapid public health response to the emergence of pH1N1 and to provide evidence for pH1N1 vaccination policy development in mainland China. Materials and methods: Archived serum specimens from previous vaccine studies were detected by Hemagglutination inhibition assay. Results: Limited cross-reactive antibody response to pH1N1 had been detected among participants of all age groups before and after they had been vaccinated with 2007–2008, 2008–2009 influenza seasonal vaccines. Vaccination with seasonal influenza viruses resulted in limited seroconversion to pH1N1 in all age groups, compared with 59–94% of seroconversion to seasonal influenza viruses. But similar to recent studies, a peak of cross-reactive antibody response to pH1N1 was observed in 23% and 39% of participants born from 1915 to 1925 before and after vaccination. Conclusions: In order to protect our populations in China, our study strongly suggests vaccination with pH1N1 is required in all age groups and that older populations born before 1925 may be associated with a lower infection rate of pH1N1.

Introduction

On April 15 and April 17, 2009, cases of pH1N1 were identified in specimens obtained from two epidemiologically unlinked patients in the United States and soon thereafter in Texas and Mexico.[1] Since that time, the virus has spread across the globe. Assessment of cross-reactive antibody response to the pH1N1 after vaccination with seasonal influenza vaccine was first reported from US Centers of Disease Control and Prevention (US CDC). According to their results, the seasonal influenza vaccines provided little or no protection against the pH1N1, but some degree of preexisting immunity to the virus existed, especially among adults aged ≥60 years.[2] In this study, using archived serum samples from previous vaccine studies, we measure the level of cross-reactive antibody response to pH1N1 in children and adults vaccinated intramuscularly with trivalent inactivated vaccine developed for the northern hemisphere 2007–2008, 2008–2009 influenza season in mainland China.

Materials and methods

Serum specimens were collected and provided by Provincial Centers for Disease Control and Prevention of China as a public health response to the emergence of pH1N1 exempt from human-subjects review. A total of 1308 serum samples were collected from Xinjiang Uygur Autonomous Region, Yunnan, and Shandong Provinces. All the serum specimens were grouped by the age of subjects (0–7, 8–17, 18–59, ≥60 years) and by different influenza seasons.

Hemagglutination inhibition assay was performed according to standard procedures in this study.[3–5] As with H1N1 components of the vaccine, the seasonal influenza viruses used in this study were A/Solomon Islands/3/2006 and A/Brisbane/59/2007. The pH1N1 influenza virus used in this study was A/California/07/2009 provided by US CDC. All the viruses were propagated in specific pathogen-free embryonated chicken eggs and inactivated by 1‰ paraformaldehyde. The criteria[6] recommended by the European Agency for the Evaluation of Medical Product was applied for the assessment of seasonal influenza vaccine

and the cross-antibody response to pH1N1. For calculation of geometric mean titer (GMT), a titer of <10 was assigned a value of 5. Statistical significance was determined by paired *t*-test.

Results

Cross-reactive antibody response to pH1N1 in vaccinated populations of seasonal influenza virus

Table 1 shows the antibody response to seasonal influenza viruses and pH1N1 of participants. Before vaccination, no or little antibody response to pH1N1 had been detected in all age groups. Vaccination with seasonal influenza vaccines resulted in seroresponse in over 70% of subjects, except children aged 0–7 years (68%) and subjects aged of 18–59 years (69%) vaccinated with 2007–2008 season influenza vaccine and adults aged ≥60 years (59%) vaccinated with 2008–2009 season influenza vaccine. Seroconversion was detected in over 40% of subjects of all ages. Postvaccination to prevaccination GMT ratios for response to seasonal influenza viruses was more than 2·5-fold. In contrast, seroresponse to A/California/07/2009 after vaccination with 2007–2008 and 2008–2009 seasonal influenza vaccines were detected in only 3% and 4% of those aged 0–7 years, 3% of those aged 8–17 years, 7% and 3% of those aged 18–59 years, 7% and 5% of those aged ≥60 years, respectively. Seroconversion in all participants ranged from 2% to 4%, and postvaccination to prevaccination GMT ratios were <2·5-fold.

Preexisting antibody response to pH1N1 among subjects born before 1920s in China

According to a recent report, people who were born from 1910 to 1929 had a preexisting immunity to pH1N1.[7] Although only a very low level of cross-reactive antibody response to pH1N1 had been observed among older subjects aged more than 60 years old in China, we further analyzed these data by different age distribution of subjects, which can trace back to the previous infection that is genetically and antigenically more closely related to this new pH1N1 influenza virus. The proportion of seroresponse to pH1N1 with the titer of 40, 80, and 160 (Highest titer detected from participants of all ages in this study) and the value of GMT were analyzed according to the birth decade of subjects from 1915. Similarly, a peak of antibody response and the value of GMT occurred both in subjects born from 1915 to 1925 and sharply decreased afterward (Figure 1). The seroresponse of subjects born in and before 1925 is significantly higher than subjects born afterward ($P < 0{\cdot}05$).

Table 1. Cross-reactive antibody response to pH1N1 in children and adults

Influenza seasons	Virus	Age Group (year)	No. of subjects		% with HI titer of ≥40			GMT		
								Pre	Post	
			Pre	Post	Pre	Post	% Rise	(95%CI)	(95%CI)	Ratio
2007–2008	A/Solomon Is/3/2006	0–7	65	60	3	68	68	9 (7–10)	102 (62–166)	11
	A/California/07/09				2	3	2	6 (5–6)	6 (5–8)	1
	A/Solomon Is/3/2006	8–17	66	63	9	100	94	12 (10–15)	238 (195–288)	19
	A/California/07/09				3	3	0	6 (5–6)	7 (6–8)	1
	A/Solomon Is/3/2006	18–59	76	71	24	87	69	13 (10–17)	96 (74–126)	7
	A/California/07/09				1	7	4	6 (5–6)	7 (6–9)	1
	A/Solomon Is/3/2006	≥60	93	89	8	83	75	7 (6–9)	87 (66–115)	12
	A/California/07/09				3	7	3	6 (5–7)	7 (6–9)	1
2008–2009	A/Brisbane/59/2007	0–7	74	74	9	85	74	10 (7–12)	186 (127–273)	19
	A/California/07/09				3	4	1	5 (5–6)	6 (6–8)	1
	A/Brisbane/59/2007	8–17	71	71	15	86	73	11 (8–14)	282 (192–414)	26
	A/California/07/09				0	3	3	5 (5–6)	7 (6–8)	1
	A/Brisbane/59/2007	18–59	123	123	9	76	71	8 (7–9)	86 (66–109)	11
	A/California/07/09				3	3	2	5 (5–6)	6 (6–7)	1
	A/Brisbane/59/2007	≥60	93	93	12	67	59	9 (7–10)	61 (44–83)	7
	A/California/07/09				2	5	2	6 (5–6)	7 (6–8)	1

GMT, geometric mean titer; HI, hemagglutination inhibition.

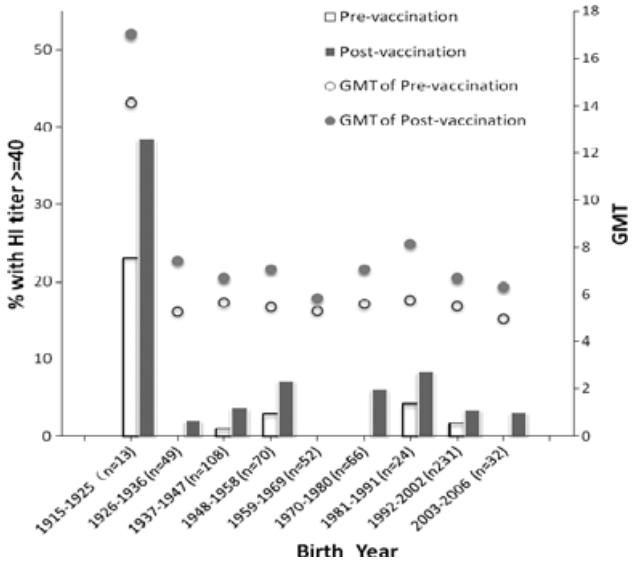

Figure 1. Antibody response to pH1N1 among subjects born since 1915.

Discussion

Similar to recent studies in some Asia countries (Guangxi Province of China and Singapore), limited antibody response to pH1N1 had been detected in children and adults.[8,9] But, some other studies from European countries (Finland, Germany, the United Kingdom) and the United States reported a high proportion of older individuals aged >60 years with pre-existing cross-reactive antibodies to pH1N1, which may possibly ba a result of previous exposure to antigenically related H1N1 influenza viruses circulating in earlier decades or a lifetime of exposure to influenza A, which has resulted in broad heterosubtypic immunity among older individuals in those countries. Previous infection and vaccination with A/New Jersey/76 may also contribute to the high level of cross-reactive antibody response to pH1N1 among adults older than 60 years in the US.[10,11]

The peak of the antibody response to pH1N1 in subjects born between 1915 and 1925, which is consistent with recent reports, may suggest the previous viral infections of 1918 Spanish flu or closely related influenza viruses, which is before and little after the year of 1918. Recent antigenic report of new pH1N1 viruses indicated that they are antigenically homogeneous among historical viruses, which are most similar to classical swine A(H1N1) viruses. A number of reviews[12–15] confirmed that the 1918 virus is the likely ancestor of all four of the human and swine H1N1 and H3N2 lineages, as well as the 'extinct' H2N2 lineage. In 1930, A(H1N1) influenza viruses were first isolated from swine. They have been shown to be antigenically highly similar to the recently reconstructed human 1918 A(H1N1) virus.[16] The cellular responses may contribute to the sustaining and long term antibody response. Probably, boosting by persisting antigenically related viruses in the early decades of the 20th century, may have contributed to the ability of these subjects to sustain memory B cells,[17] and it is well established that a subset of plasma cells is long-lived, and these cells contribute to durable humoral immune responses,[18] such as that observed after childhood smallpox vaccination. Furthermore, T cells that recognize cross-reactive epitopes are preserved and might be enriched in the memory population; the course of each infection is influenced by the T-cell memory pool that has been laid down by a host's history of previous successive infections.[19]

Our study indicated that wide transmission of this new virus or any antigenically close related influenza A(H1N1) viruses may not have circulated among populations in China before the outbreak of pH1N1. Our data also suggests the need for vaccination with pH1N1 vaccine in all age groups.

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Pandemic influenza vaccination of hypogammaglobulinemic patients induces normal or elevated influenza-specific CD4+ Th1-cell responses

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Keywords Clinical trial, hypogammaglobulinemia, influenza vaccine, pandemic H1N1, Th1 cells.

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Introduction

Hypo- and agammaglobulinemia patients have an impaired immune system and are particularly susceptible to bacterial infections that are normally defended against by antibodies. Therefore, patients routinely receive replacement therapy with immunoglobulins isolated from healthy blood donors.[1] These patients are also prone to get viral infections, possibly due to defects in toll-like receptors 8 and 9.[2] Because these patients lack an antigen specific humoral immune response, they are rarely vaccinated. The ability of hypogammaglobulinemic patients to produce a specific cell-mediated immune response upon vaccination has only been sparsely investigated. In contrast to local mucosal antibodies, vaccine-induced cell-mediated immunity is not believed to protect against pathogen entry per se, but may be sufficient to provide protection against severe disease and death following transmission of some microbes.[3,4] The aim of this pilot study was to investigate if influenza vaccination of hypogammaglobulinemic patients can induce an influenza-specific cell-mediated immune response. We therefore vaccinated hypogammaglobulinemic patients and healthy controls with pandemic H1N1 virus vaccine and subsequently investigated the B-cell and T-cell responses. The percentages of IFN-γ, IL-2, and TNF-α cytokine producing CD4+ Th1-cells were determined, as these cytokines are important indicators of cell-mediated immunity.

Materials and methods

Patients

Five a- or hypogammaglobulinemic patients were classified based on the Freiburg classification[5]: Patient #1 is diagnosed with X-linked agammaglobulinemia, patient #2 and #4 are in group Ia, Patient #3 is in group Ib and patient #5 is in group II.

Vaccine and adjuvant

The monovalent egg grown split virus vaccine adjuvanted with AS03 was manufactured by GlaxoSmithKline (GSK), Belgium. The vaccine strain was produced by reassortment

between influenza A/California/7/2009 (H1N1) and A/PR/8/34 (H1N1) to produce A/California/7/2009-like virus (X179a). The vaccine was mixed with adjuvant to contain 7·5 μg haemagglutinin (HA) of A/California/7/2009-like virus (H1N1), squalene (21·38 mg), DL-a-tocopherol (23·72 mg), and polysorbate 8 (9·72 mg) per ml.

Vaccination and sampling

Healthy controls and hypogammaglobulinemia patients were vaccinated by intramuscular (IM) injection. Hypogammaglobulinemia patients received one or two vaccine doses 21 days apart. The intention was to vaccinate the hypogammaglobulinemic patients with two doses of 3·75 μg HA, but 7·5 μg HA was inadvertently administered to the patients as the first dose. For patient #4 this was the second dose as he had received an initial dose of 3·75 μg HA 3 months prior to the study. Patient #1, #2, and #5 received a second dose of 3·75 μg HA. Four healthy controls were immunised with one dose of 3·75 μg HA according to Norwegian national guidelines. Peripheral blood mononuclear cells (PBMCs) were harvested and washed in PBS with 10% FBS. The PBMCs were resuspended in lymphocyte medium (RPMI 1640 with l-glutamine, 0·1 mm non-essential amino acids, 10 mm Hepes pH 7·4, 1 mm sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml fungizone and 10% FBS) prior to use in the Enzyme-linked immunospot (ELISPOT) and influenza-specific CD4+ T-cell assays.

Haemagglutination inhibition

Serum haemagglutination inhibition antibodies were tested by a standard method using 8 HA units and 0·7% Turkey erythrocytes. All samples were tested in duplicate and the test was repeated at least two times. Titres <10 were assigned a value of 5 for calculation purposes.

Influenza-specific antibody producing cells

For numeration of antibody-secreting cells (ASC), an ELISPOT assay was conducted as previously described[6] with the following modifications. Ninety-six well ELISPOT plates were coated with 2 μg/ml of A/California/7/2009-like (X179a) H1N1 virus diluted in PBS overnight at 4°C. After blocking with RPMI (10% FBS), 10^5 PBMCs were added and incubated (37°C, 5% CO_2) for 16 hour. Secreted antibodies were detected with biotinylated goat anti-human IgG, IgA and IgM specific antibody (Southern Biotech, Birmingham, Alabama, USA), incubated for 2 hour at room temperature and developed with extravidin peroxidase and AEC substrate. The numbers of spots were counted using an ELISPOT reader (Immunoscan™) and ImmunoSpot® software.

Influenza-specific CD4+ T-cell responses

The influenza-specific CD4+ Th1-cell response was measured by intracellular cytokine production of IFN-γ, IL-2, and TNF-α. Peripheral blood mononuclear cells (10^6 per well) were incubated for 16 hour (37°C, 5% CO_2) in 200 μl lymphocyte medium containing 1 μg/ml anti-CD28, 1 μg/ml anti-CD49d, 0·7 μg/ml Monensin, 1 μg/ml Brefeldin A, (BD Biosciences, Franklin Lakes, New Jersey, USA), and the H1N1 influenza split virus vaccine X179a (either 2·5 μg/ml or 10 μg/ml HA). Basal cytokine production was determined by incubating PBMCs in lymphocyte medium without influenza virus, and the percentage of cytokine positive cells without influenza stimulation were subtracted from influenza-stimulated cells. Cells were stained for CD3, CD4, CD8, IFN-γ, IL-2, and TNF-α (BD Biosciences) as previously described.[7] Finally, cells were resuspended in PBS containing 5% FBS and 0·1% sodium azide and analysed by BD FACSCanto flow cytometer (300 000–500 000 cells acquired). FlowJo v8·8·6 (Tree Star, Ashland, Oregon, USA) was used for data analysis.

Results

Haemagglutination inhibition antibody response

Five to six fold lower GMTs were found in the patient group as compared to the healthy controls throughout the study (Figure 1A). The lowest HI titres were obtained in patients #1, #2, and #3, whilst patients #4 and #5 and all healthy controls fulfilled two of three European Medicines Agency Committee for Medicinal Products for Human use (CHMP)[8] seasonal influenza vaccine licensing criteria, by obtaining an HI titre >40 and a mean geometric increase of 2·5 between pre- and post-vaccination. Thus, the HI data indicate that two vaccine doses was sufficient to induce a protective HI antibody response in two out of five of the hypogammaglobulinemia patients tested in this study.

The influenza-specific antibody-secreting cell response

The numbers of influenza-specific IgA, IgG, and IgM ASC were tested pre-vaccination and 7 days post-vaccination with the h X179a virus. Few or no ASCs were detected pre-vaccination (data not shown). At 7 days post-vaccination the patient's IgA, IgG, and IgM ASC levels were significantly lower ($P < 0·01$) compared to the healthy controls (Figure 1B). But, the post-vaccination ASC numbers in the patients were generally higher than at pre-vaccination stage (0–2 ASCs). Patient #3 had the highest IgA and IgG ASC numbers, followed by patients #2 and #5, whilst patient #4 and #1 had few or no ASC's. These results confirm that the patients are indeed hypogammaglobulinemic and that some of the patients (#1 and #4)

could be agammaglobulinemic in the context of producing influenza-specific antibodies. The ASC levels of patients #2, #3, and #5 were lower than those of the healthy controls, but could possibly be adequate for reducing the severity of influenza disease.

Influenza-specific Th1-cell response

The influenza-specific Th1-cell response was evaluated by stimulating PBMCs with the influenza X179A virus 21, 42, and 90 days post-vaccination. Stimulation of healthy control PBMCs with X179A 21 days after vaccination, induced IFN-γ, IL-2, and TNF-α production by an average of 0·10%, 0·09%, and 0·03% CD4+ T-cells, respectively. Patient #1 and #2 had higher responses than the healthy controls and stimulation with X179A induced 0·45%, 0·34%, and 0·05% of T-cells from patient #2 to produce IFN-γ, IL-2, and TNF-α, respectively (Figure 2A). The response of patient #2 was further boosted by a second vaccine dose, which resulted in 0·73%, 0·54%, and 0·25% CD4 T-cells producing IFN-γ, IL-2, and TNF-α, respectively at day 42 (Figure 2B). These results show that the hypogammaglobulinemia patients studied here did not have a common impaired influenza-specific CD4+ Th1 cytokine response. Rather, there was a tendency towards increased responses, suggesting that the diminished antigen specific B-cell responses could induce a compensatory antigen specific Th1-cell response.

Discussion

The results from this pilot study suggest that some hypogammaglobulinemia patients may benefit from influenza vaccination. We found very different patient responses to influenza vaccination, but some of the patients (Patient #2 and #3) did mount low influenza-specific ASC responses. In addition, the vaccine-induced HI antibody titres above the protective level in patient #4 and #5. These results are in accordance with previous publications, which described that polypeptide vaccines induce humoral responses in subgroups of common variable immunodeficiency patients.[9–11] In this study, we also investigated cell-mediated immunity and found the percentages of homologous and cross-reactive influenza-specific CD4+ Th1-cells to be in the same range (for patient #3, #4, and #5) or higher (for patient #1 and #2) in the a- or hypogammaglobulinemic patients compared to the healthy controls. The higher response is probably due to the patients having received a vaccine dose of 7·5 μg HA, whilst the controls received 3·75 μg HA. In addition, the patients received a second booster dose, which influences the day 42 and 3 months responses. Nonetheless, these results are the first to demonstrate that proliferation of pandemic influenza antigen specific Th1-cells can be induced in hypogammaglobulinemic patients. In addition, vaccination induced influenza-specific ASC's in some patients. The findings are promising and provide hope that hypogammaglobulineamic patients could be vaccinated against influenza and other diseases preventable by

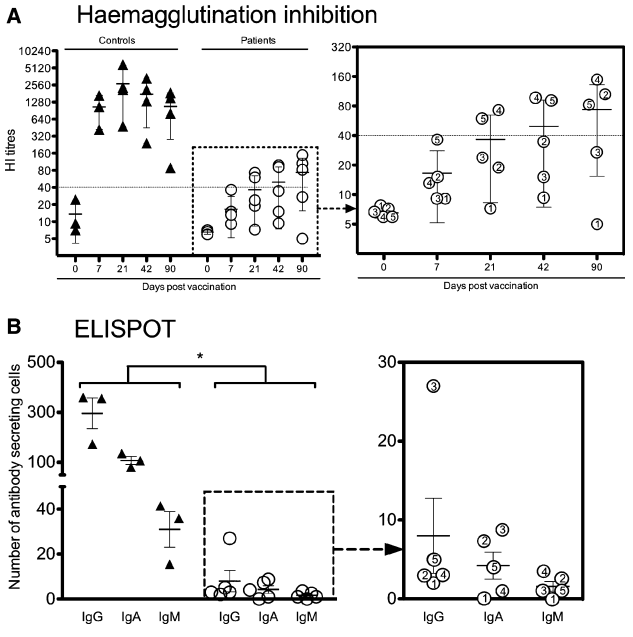

Figure 1. Serum samples were tested for haemagglutination inhibition antibodies 0, 7, 21, 42, and 90 days post-vaccination (A). Titres are presented as the geometric mean titre ± 95% confidence interval. ELISPOT data (B) are presented as the mean number of influenza-specific IgA, IgG, and IgM ASCs per 100 000 Peripheral blood mononuclear cells ± SEM. Data for the hypogammaglobulinemia patients are additionally presented by a number for each patient. *Significantly higher numbers of ASCs were detected in the healthy controls as compared with the hypogammaglobulinemia group (Students *T*-test, $P < 0{\cdot}01$).

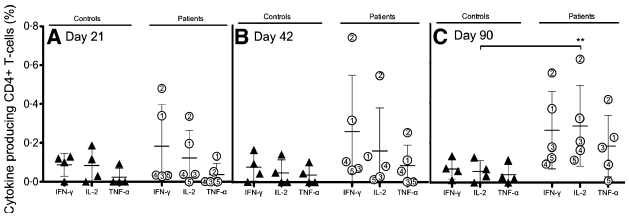

Figure 2. Peripheral blood mononuclear cells s from patients and healthy controls were isolated at day 21 (A), 42 (B), and day 90 (C) and stimulated for 17 hour with X179A virus before staining and flow cytometric analysis. The figure shows the mean ± SD frequency of influenza-specific CD4+ cytokine producing cells (%) where the basal cytokine production from unstimulated cells has been subtracted. Data for the hypogammaglobulinemia patients are additionally shown as a number for each patient. **Significantly higher frequency of IL-2 producing CD4+ T-cells in the patients compared to the healthy controls (students *T*-test $P < 0{\cdot}05$).

vaccination. However, this hypothesis should be tested in larger clinical studies.

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Roles of adjuvant IgV combined with influenza M2e based virus like particle in the immune response and cross protection

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The aim of this study is to use IgV as adjuvant combined with fused M2e to HBC as a vaccine and to evaluate the immune response. To determine the IgV effect on influenza M2e-HBC virus like particle (VLP) particles, we expressed the recombinant IgV protein in *Escherichia coli* and immunized mice with different doses of IgV plus the M2e-HBC particles. Results showed that IgV as adjuvant can induce Th1-biased cellular immune response. T cell proliferation *in vitro* showed cross reaction was produced between different subtypes. These results are the first to suggest recombinant IgV protein may serve as a potent adjuvant in the development of new influenza virus vaccines.

Introduction

The influenza virus undergoes antigenic evolution under intense immune selection pressure from herd immunity in humans through the process called antigenic drift and shift.[1,2] Because of antigenic drift, yearly updating of vaccine strain is needed. A mismatch between the circulating

strains and the vaccine strain in the subsequent season is often encountered, resulting in reduction of vaccine effectiveness and lack of protection from the circulating strain.[3] In order to address this, a universal influenza vaccine based on a more conserved part of the influenza virus, which is not affected by antigenic change and that is conserved across all strains, remains the ultimate goal to afford cross-protection to drifted strains as well as to other subtypes of influenza which may arise from antigenic shift.[4,5] Previous studies have investigated the potential of the M2e.[6,7] M2e has remained highly conserved since it was first isolated in 1933.[6] Several studies have examined the use of M2e as a vaccine component, using various approaches including proteins, peptides, DNA vectors, and attenuated viral vectors.[6,8–13] Although M2e is a weak antigen, by linking the protein to a carrier hepatitis B virus core particle, protection against influenza has been achieved in mice particularly when administered with an adjuvant.[3] Some articles found that vaccination with M2e coupled to HBC induces protective antibodies, whereas the contribution of T cells to protection was negligible. Protection induced by vaccination with M2e-HBC was weak overall and failed to prevent weight loss in vaccinated infected animals, and mice succumbed to high dose infection.[14] We aimed to address the poor immunogenicity of M2e-HBC by using IgV as adjuvant. IgV domain is common and conserved in the TIM family. Ligand binding sites of T cell immunoglobulin mucin (TIM) located at IgV domain.[15–17] TIM function is done by anti TIM antibody which recognized the ligand binding sites of IgV domain.[18] TIM family members share a common motif, including an IgV domain. They are differentially expressed on Th1 cells and Th2 cells with the ability to regulate the immune system.[19,20] The IgV domain of human B7-2 is sufficient to co-stimulate T lymphocytes and induce cytokine secretion.[21] Soo Hoo *et al.* vaccinated with TIM-1 antibody and inactivated influenza and found enhanced vaccine-specific immune response.[22]

We report here for the first time the use of IgV recombinant protein as adjuvant to immunize mice with influenza M2e-HBC. Results indicated that IgV can induce the strong cellular immune response and cross reaction with different subtype influenza virus antigen. Target IgV may be used to develop the new method for vaccination strategies.

Materials and methods

Expression and purification of recombinant IgV protein

RNA was extracted from healthy human PBMC. One-step RT-PCR (Qiagen, Valencia, CA, USA) was done for the amplification IgV gene. The PCR product was purified and cloned into Pet32a (Novagen, Madison, Germany). The resultant construct Pet32a-IgV has a histidine (His) tag (6His) at the N terminus. DNA sequence of the insert was determined by sequencing. IgV. Recombinant protein was expressed in *Escherichia coli* and was purified on a Ni column (Novagen). The purified protein was examined by SDS-PAGE and Western Blotting.

Mice and immunization

Six-eight weeks female Balb/C mice (Institute of Zoology Chinese Academy of Sciences, China) was used for the study. Mice were immunized twice intradermally with 10 ug M2e-HBC (provided by CNIC, China) combined with different doses of recombinant IgV protein 5, 10, 50 ug, respectively, or without IgV as control. The area proximal to the tibialis anterior muscle was sterilized with 70% ethanol and different groups of mice were injected bilaterally with 5, 10, 50 ug IgV plus 10 ug M2e-HBC in 50 ul phosphate buffer saline per mouse using a 1 ml syringe with attached 1/2″ 30G needle. The immunization was given at 3 weeks intervals. Four blood samples were obtained from every mouse: before immunization, after the first and second immunization, and after virus challenge by retro-orbital plexus puncture. After clotting and centrifugation, serum samples were collected and stored at −80°C prior to use for assays.

Purification and inactivation of virus antigen

Mouse-adapted A/PR/8/34 (H1N1), A/Brisbane/1/10 (H3N2), A/Xinjiang/1/2006 (H5N1), and A/Guangzhou/333/1999 (H9N2) were provided by Chinese National Influenza Centre. Nine to eleven days old embroynated specific pathogen free (SPF) chicken eggs were inoculated with virus, and the eggs were incubated at 35°C for 2–3 days. The allantoic fluid was collected and purified by sucrose density gradient centrifugation, and the virus was inactivated by formaldehyde at 4°C overnight.

Analysis of production antibodies production against M2e

To identify IgG, IgG1, IgG2a against M2e, ELISA assays were used. In brief, 96-well (Nunc, Brunei, Denmark) were coated with 100 ul/well of M2e recombinant protein (provided by Gene Lab of IVDC, XuanWu District, Beijing, China) in carbonate buffer (pH 9·6) overnight at 4°C. Immediately before use, the coated plates were incubated with blocking solution (2% BSA in PBS) for 2 h at 37°C and washed four times with PBS containing 0·05% Tween 20 (PBS-T). The serum samples were serially diluted and added in the plates. The detection color was developed by adding HRP-labeled goat anti-mouse IgG, IgG1, or IgG2a

(Rockland, Philadephia, PA, USA) 1·5 h at 37°C. Tetramethylbenzidine (TMB) was applied, and the optical density was read at 492 nm.

Analysis of T cell proliferation between different subtypes

Three weeks after boosting immunization, spleens were harvested from immunized and control mice. Splenocytes were prepared by lymphocyte separation media (EZ-Sep™, Shen Zhen, China). The cells were washed and resuspended in complete RPMI-1640 containing fetal bovine serum (Hyclone, Logan, UT, USA), GlutaMAX, 5 um β-ME. Splenocytes were cultured *in vitro* in the presence of inactivated H1N1, H3N2, H5N1, and H9N2 influenza virus antigen for 96 h. Quick cell proliferation Assay kit (Biovision, San Francisco, CA, USA) was used to detect the cell proliferation. The 420–480 nm absorbance was read on a plate reader.

Statistical analysis

All experiments have been repeated at least three times. Results are presented as mean standard error of the mean (SEM). Comparison of the data was performed using the Student's *t*-test. Significance was defined as a *P* value of $<0{\cdot}05$.

Results

Recombinant IgV produce Th1 biased immune response

To evaluate the adjuvant effect of recombinant IgV, the anti M2e antibody subclasses was measured. IgG1 and IgG2a were detected after the first and second immunization (Table 1). The ratio of IgG2a/IgG1 was calculated. Immunization with only M2e-HBC showed a lower IgG2a/IgG1 ratio <0·5. IgV combined with M2e-HBC led to a high IgG2a/IgG1 ratio of up to 5–15 after first and second immunization. These IgG subclass distributions indicated that IgV can induce a Th1 immune response.

Recombinant IgV generate cross-strain immune response

To determine whether the splenocytes were stimulated *in vitro* with different subtypes of inactivated influenza antigen after the IgV plus M2e-HBC antigen immunization, H1N1, H3N2, H5N1, H9N2 inactivated antigen was used

Table 1. The serum IgG, IgG1, IgG2a, and IgG2a/IgG1 ratio were measured by ELISA after first and second immunization. M2e were coated on the 96 wells plate overnight, and serial dilution sera of day 7, 14, 21 after first and second immunization were added 0, 2·5, 5, 10, 20, 40 ug/ml of IgG, IgG1, IgG2a purified antibody were also added for obtaining the standard curve. HRP-labeled goat anti-mouse IgG, IgG1, or IgG2a was then added, washed, and the optical density was read at 492 nm. The results were showed at mean ± SEM.

	Day after first immunization			Days after second immunization		
Antibody class	**7**	**14**	**21**	**7**	**14**	**21**
IgG (ug/ml)						
50 IgV/M2e-HBC	11·5 ± 3·6	125·0 ± 7·9	224·2 ± 12·6	620·4 ± 17·7	936·4 ± 20·6	959·5 ± 32
10 IgV/M2e-HBC	9·4 ± 5·8	121·4 ± 10·6	194·7 ± 14·7	600·2 ± 11·3	926·9 ± 23·7	946·0 ± 24·5
5 IgV/M2e-HBC	10·8 ± 2·1	117·9 ± 5·7	198·5 ± 11·8	606·7 ± 23·6	942·1 ± 25	976·2 ± 19·8
M2e-HBC	4·9 ± 4·3	43·6 ± 5·1	61·4 ± 10·3	176·7 ± 14·8	295·6 ± 19	349·7 ± 21·4
IgG1 (ug/ml)						
50 IgV/M2e-HBC	6·3 ± 2·5	18·6 ± 11·2	34·3 ± 18·0	73·0 ± 32·1	75·3 ± 42	90·2 ± 21·9
10 IgV/M2e-HBC	4·2 ± 2·1	15·9 ± 6·5	20·6 ± 14·3	57·8 ± 40·2	68·3 ± 25·1	82·1 ± 33·1
5 IgV/M2e-HBC	5·4 ± 3·5	9·6 ± 4·7	19·0 ± 9·7	53·9 ± 35·0	63·3 ± 26·3	78·6 ± 16·9
M2e-HBC	2·7 ± 1·8	9·1 ± 8·1	15·1 ± 2·9	35·3 ± 18·4	43·0 ± 15·4	61·4 ± 7·6
IgG2a (ug/ml)						
50 IgV/M2e-HBC	7·0 ± 2·0	130·0 ± 20·0	210·0 ± 20·0	616·0 ± 42·7	921·3 ± 65·0	978·6 ± 30·4
10 IgV/M2e-HBC	6·0 ± 2·0	114·0 ± 12·5	185·6 ± 15·5	556·6 ± 32·1	893·3 ± 20·2	954·3 ± 30·1
5 IgV/M2e-HBC	6·6 ± 1·5	111·3 ± 11·7	194·1 ± 18·5	552·6 ± 36·1	914·3 ± 35·0	983·0 ± 8·38
M2e-HBC	3·0 ± 1·0	46·0 ± 3·6	55·3 ± 4·9	166·6 ± 20·8	262·0 ± 21·6	303·0 ± 19·0
IgG2a/IgG1 ratio						
50 IgV/M2e-HBC	1·2 ± 0·11	9·3 ± 0·21	6·5 ± 0·10	9·3 ± 0·06	13·2 ± 0·19	11·3 ± 0·07
10 IgV/M2e-HBC	1·7 ± 0·05	11·4 ± 0·17	12·2 ± 0·15	9·8 ± 0·15	14·5 ± 0·24	12·7 ± 0·14
5 IgV/M2e-HBC	1·3 ± 0·12	13 ± 0·08	11·8 ± 0·13	10 ± 0·23	14·8 ± 0·14	12 ± 0·26
M2e-HBC	1·1 ± 0·2	7·9 ± 0·13	4·3 ± 0·07	5 ± 0·19	5·3 ± 0·05	4·7 ± 0·18

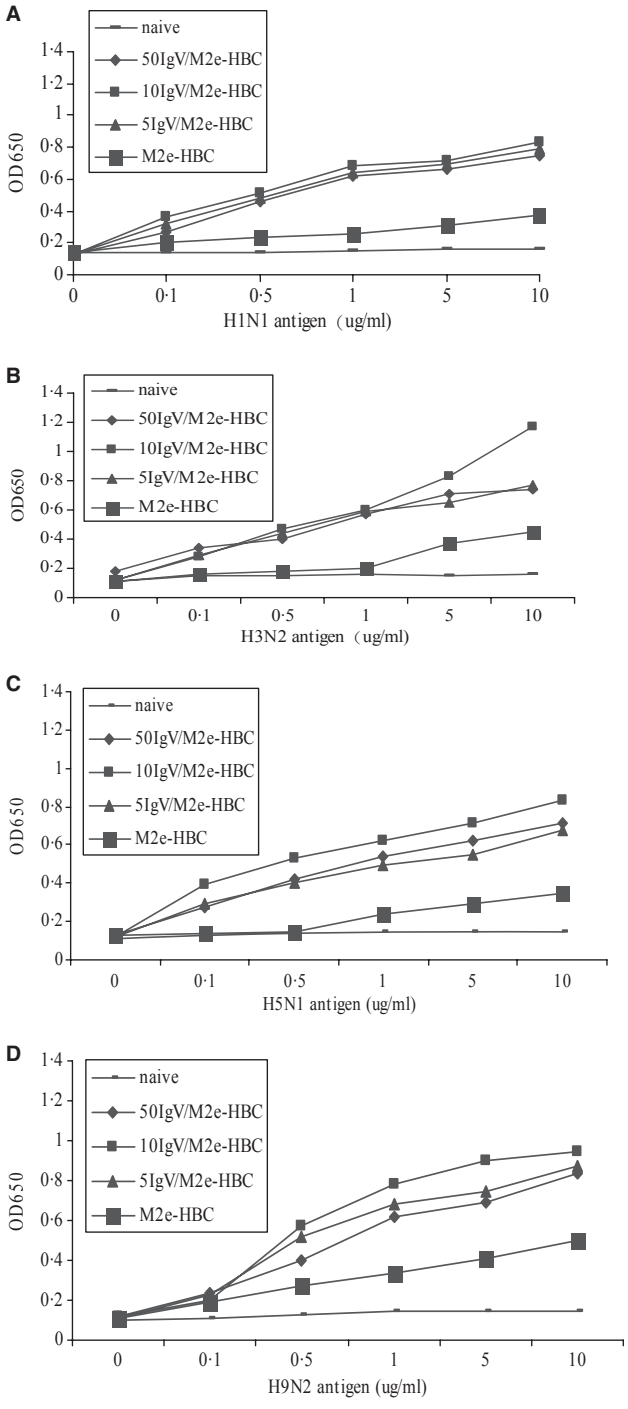

Figure 1. T cell proliferation assay. Mice were immunized twice with 50, 10, 5, 0 ug/ml IgV plus M2e-HBC, respectively, and naive group was immunized with PBS. Three weeks after a boosting immunization, spleens were harvested from immunized and naive mice. Different subtypes of inactivated virus antigen (A) H1N1, (B) H3N2, (C) H5N1, (D) H9N2 were added and cocultured with different group splenocytes for 96 h. Quick cell proliferation Assay kit was used to detect the cell proliferation. The 420–480 nm absorbance was read on a plate reader. Data were showed were shown as mean values. The difference between naive group and different doses IgV plus M2e groups was determined using the student's *t*-test. All significance level is $P < 0{\cdot}05$.

(Figure 1). No cross-strain response was observed in the control group. The IgV adjuvented groups show splenocytes stimulation with seasonal H1N1, H3N2, H5N1, and H9N2 antigens. M2e-HBC immunization without IgV showed splenocyte stimulation, but the extent was lower than animals immunized in the presence of the IgV adjuvent. These data suggested that IgV had enhanced effect on priming against the conserved viral antigen matrix protein and generation cross-strain immune response.

Discussion

Influenza is a respiratory disease causing epidemics every year. H5N1 viruses and swine-origin H1N1 have also infected humans in recent years. Seasonal influenza vaccine cannot cope with significant antigenic drift or with the emergence of pandemic viruses of different subtypes not contained in the vaccine. The high extent of conservation of the M2e makes it a promising immunogen. A vaccine based on coupling of the M2e peptide to an appropriate carrier may provide a universal vaccine with effectiveness and safety. M2e based vaccination induces protective antibodies not only in mice, but also in ferrets and monkeys. The carrier hepatitis B core as carrier with M2e forms a virus like particle (VLP). Vaccination with M2e coupled to HBC induces protective antibody,[11] whereas the contribution of T cell protection was negligible. Protection induced by vaccination with M2 coupled to HBC was weak overall. In order to improve the vaccination effect of M2e-HBC, new adjuvant IgV was evaluated in combination with the M2e-HBC. The TIM molecules are a recently discovered class of proteins with the ability to regulate the immune system. Crystal structures of the TIM molecules has revealed a unique, conserved structure with ligand-binding sites in the IgV domain. To determine the potential immunostimulatory molecular properties of IgV, we have evaluated immune response of the IgV in combination with M2e-HBC VLP.

Previous papers reported that VLP immunized mice can induce the Th1 and Th2 immune response.[23] Different adjuvant combined VLP can produce biased immune response Th1/Th2 mixed immune response, or Th1-preferred Th1/Th2 profile.[24] Thus, the response following the use of IgV as a new adjuvant combined with M2e-HBC VLP needs to be evaluated. Results indicated that IgV combined groups showed Th1 biased immune response and enhanced cross reactive T cell immune responses. This may show that IgV immunized the mice and antiIgV antibody can cross link the IgV on T cells and enhance the cell

response. We also evaluated the cross-protection produced by IgV combined M2e-HBC. We challenge with mouse-adapted strain PR8 and prove the cross protection via reaction between the cells from the immunized animal and different subtypes of virus antigen. Some subtypes of virus cannot infect the mice naturally, and therefore, virus challenge cannot be used to evaluate the effect. We co-cultured the T cells with inactivated antigen H1, H3, H5, and H9, and T cell proliferation was measured. Results indicated that after immunization with IgV plus M2e-HBC, the T cells show cross-protection with other subtypes. This provides evidence that IgV can enhance the cross protection across subtypes.

The results of this study demonstrated that recombinant IgV can be useful as an adjuvant and polarize the M2e-HBC VLP immune response to a Th1 profile. IgV induced the M2e-HBC VLP to induce T cell proliferation and cross-reactive responses to different influenza virus subtypes. This finding represents a new direction for the promotion of cell mediated immunity in M2e based vaccine against influenza.

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Systematic random versus *ad hoc* non random sampling strategy: does it modify estimations of influenza vaccine efficiency?

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Abstract

The French GROG (Groupes Régionaux d'Observation de la Grippe) early warning network collects more than 5000 specimens yearly from cases of acute respiratory illness (ARI), using two sampling methods: systematic randomized and non systematic ''*ad hoc*'' sampling. The circulation of pandemic influenza 2009 viruses in France gave an opportunity to compare Influenza vaccine effectiveness (IVE) estimates using these two sampling methods. An influenza case was defined as an influenza like illness (ILI) case with a respiratory sample positive for influenza during the study period. Controls were cases of ILI having a swab negative for influenza during the study period. The outcome of interest is laboratory confirmed influenza. Analysis was done for the two sampling groups on cases/controls following the European method proposed by Epiconcept. Conclusion: Influenza early warning networks can estimate IVE, taking into account many covariates. From a stakeholders and patients point of view, during the 2009–2010 influenza pandemic wave, there were no major discrepancies between IVE estimated with an *ad hoc* sampling strategy, based on sentinel practitioners instinct, and IVE estimated with a systematic random sampling strategy, whatever the multivariable analysis methodology. Although, in *ad hoc* sampling, GPs were more likely to swab vaccinated flu cases (because selection based on specific sign (headache) and vaccine status).

Introduction

A core European protocol, I-Move, describing the methods to estimate influenza vaccine effectiveness (IVE) was proposed by the European Centre for Disease Prevention and Control (ECDC) and Epiconcept for the 2009–2010 season. It includes a case control method for pooled analysis based on a randomized ''systematic'' sample of swabs.[1,2]

Collection of swabs using a non randomized, i.e., ''*ad hoc*,'' sampling strategy, left at the appreciation of sentinel practitioners, provides a greater number of cases and controls for IVE estimation more easily than using a systematic randomized sampling strategy.

The French GROG (Groupes Régionaux d'Observation de la Grippe) early warning network collects more than 5000 specimens yearly from cases of acute respiratory illness (ARI), using both sampling methods.[3,4] During the circulation of pandemic influenza 2009 viruses in France, it gave an opportunity to compare IVE estimates using systematic randomized versus non systematic ''*ad hoc*'' sampling.

Materials and methods

Influenza vaccine effectiveness was estimated by a case–control methodology according to ECDC I-MOVE protocol, using on the one hand a systematic random sampling, on the other hand ''*ad hoc*'' non random sampling.

Participants

The study was proposed to 608 primary care practitioners of the GROG network (493 general practitioners and 115 pediatricians) trained to collect data and swabs.

Study population

The study population was patients from the community of all ages consulting a GROG practitioner for an influenza like illness (ILI) and having a nasal or throat swab taken within an interval of <5 days after symptom onset. ILI was defined according to the European Union (EU) case definition as sudden onset of symptoms with at least one of the following four systemic symptoms: fever or feverishness, malaise, headache, myalgia; and at least one of the following three respiratory symptoms: cough, sore throat, shortness of breath. Swabs were performed through usual surveillance. No ethical approval was needed, but an oral informed consent was requested. Cases were excluded if they refused to participate in the study or if they were unable to give informed consent or to follow the interview in native language because of aphasia, reduced consciousness, or other reasons.

Vaccination status
An individual was considered as vaccinated against pandemic influenza if he or she reported having received a pandemic influenza vaccination during the current season, and if at least one vaccine dose occurred more than 14 days before ILI onset.

Study period
The study period started with the initiation of active influenza surveillance by the GROG network, i.e., 15 days after the beginning of the influenza vaccination campaign, and finished at the end of the influenza period defined as the last week with at least one swab positive for influenza within the GROG network.

Sampling

"*Ad hoc*" sampling
Patients from which swabs were taken were selected by the GROG practitioners during the study period.

Systematic random sampling
During the same period, patients were selected at random as follows. An age-group 0–4 years (GPs and pediatricians); 5–14 years (GPs and pediatricians); 15–64 years (GPs); 65 years or more (GPs) was assigned to each practitioner, who was requested to swab the first patient of the week presenting with an ILI within the pre-assigned age-group.

Swabs were collected in appropriate transport medium (Virocult®, ViralPack®, UTM Copan®) and sent by post to the laboratory in triple packaging following the international guidelines for the transport of infectious substances (category B, classification UN 3373). Laboratory confirmation of influenza was by RT-PCR to detect currently circulating influenza A (subtypes H3, seasonal and pandemic H1) and B viruses.

Case definitions
An influenza case was defined as an ILI case with a respiratory sample positive for influenza during the study period. Controls were cases of ILI having a swab negative for influenza during the study period.

Outcome
The outcome of interest is laboratory confirmed influenza.

Confounding factors and effect modifiers
Confounding factors and effects modifiers identified during the I-Move preliminary study[5] were registered: risk factors, chronic diseases, severity of underlying conditions, smoking history, former vaccinations, and functional status.

Data
Data on cases and controls were collected by the practitioners using a standardized questionnaire adapted from the I-Move study. Questionnaires were sent by the practitioners with the swab to the virology laboratory, and sent to the GROG national coordination. Data entry and validation were ensured by Open Rome through the Vircases computing tool. Validation steps included control of exhaustiveness of centralization of questionnaires, comparison of data entered by the labs and the national GROG coordination, coherence control, and identification of missing data.

Analysis
Analysis was done for the two sampling groups (systematic and *ad hoc*) on cases/controls following the European method proposed by Epiconcept, using Excel 2007© (Microsoft Corp. Redmond, Washington, USA) and Stata©. Baseline characteristics of cases and controls in unmatched studies were compared using the chi-square test, Fisher's exact test, or the Mann–Whitney test (depending on the nature of the variable and the sample size). The association between vaccination status and baseline characteristics was assessed for both case and control groups. The vaccine effectiveness was computed as IVE = 1−OR (Odds Ratio). An exact 95% confidence interval (CI) was computed around the point estimate. Analysis was stratified according to age groups, time (month of onset), presence or absence of chronic disease, and previous influenza vaccination. Effect modification was assessed comparing the OR across the strata of the baseline characteristics. Confounding factors were assessed by comparing crude and adjusted OR for each baseline characteristic. A multivariable logistic regression analysis was conducted to control for negative and positive confounding factors using a complete case analysis (with records with missing data dropped) and using multiple imputation with chained equations. The complete model included age group, number of GP visits, onset week, seasonal vaccination, previous seasonal influenza vaccination, presence of chronic disease and associated hospitalizations in the previous 12 months, gender, and smoking status. Variables were tested for multi-colinearity. Interactions were tested using the Likelihood Ratio test (or Wald test) and included in the model if significant at 5% level. A model with fewer variables (age group, number of GP visit, onset week, and seasonal vaccination) was also tested.

Results and discussion

Several models were applied to both the "*ad hoc*" and systematic sampling groups of cases and controls. As shown in Table 1, whatever the analysis method used, the

Table 1. *Ad hoc* sampling vs systematic random sampling. Comparisons of number of patients (cases or controls), estimation of influenza vaccine effectiveness and confidence interval during the 2009–2010 influenza season (Sources: GROG network, Open Rome)

	***Ad hoc* (non random)**				**Systematic random**			
	N	**IVE**	**95% CI**		***N***	**IVE**	**95% CI**	
Model 1: missing data dropped – fewer covariates								
Crude	1097	88·7	68·0	96·0	1072	93·2	71·9	98·4
Adjusted	1097	73·9	20·7	91·4	1072	87·5	45·2	97·1
Model 2: analysis of vaccine effectiveness using imputed dataset – fewer covariates								
Crude	2690	88·7	75·8	94·7	1782	94·8	78·5	98·7
Adjusted	2690	71·8	36·6	87·4	1782	89·2	53·3	97·5
Model 3: missing data dropped								
Crude	623	69·2	0	91·6	612	92·4	43·2	99·0
Adjusted	623	43·9	0	86·8	612	90·0	14·5	98·8
Model 4: analysis of vaccine effectiveness using imputed dataset								
Crude	2690	88·7	47·1	75·8	1782	94·8	78·5	98·7
Adjusted	2690	71·8	36·3	87·5	1782	90·0	55·7	97·8

Models 1 and 2 are adjusted by age group, number of GP visits, onset week and seasonal vaccination. Models 3 and 4 are adjusted by age group, number of GP visits, onset week, seasonal vaccination, previous seasonal influenza vaccination, presence of chronic disease and associated hospitalizations in the previous 12 months, gender and smoking status.
IVE, influenza vaccine effectiveness.

"*ad hoc*" sampling strategy led to a slightly lower estimate of IVE. The CI were extended when data were missing and reduced when using multiple imputations with chained equations. However, from a statistical point of view, comparison of "*ad hoc*" versus systematic strategies is not straightforward, because "*ad hoc*" sampling is not randomized and does not allow comparisons with statistical tests using statistical distribution laws.

Missing data

There are more missing data with the *ad hoc* sampling method. This is mainly due to our validation procedure: in the case of missing data in the systematic sampling group, as required by the I-Move study protocol, queries were sent to sentinel practitioners using mail and phone calls. This specific heavy workload is not usually performed during routine surveillance and has not been achieved for the "*ad hoc*" sampling group given the great number of cases and controls (2690). Within the framework of the I-Move study, several items were added to the GROG's usual clinical form accompanying swabs (hospitalizations, number of GP visits, smoking status, help needed for bathing or walking). In 2009–2010, GPs explained that this added workload was not compatible with their daily additional workload due to the pandemic situation. Therefore, many of them refused to fill these new items systematically and threatened to leave the network. We thus obtained that the "I-Move items" would be filled in for the clinical forms linked to systematic sampling, but were not in a position to obtain that for "*ad hoc*" sampling.

Laboratory results

The weekly distribution of systematic swabbing is not similar to that of *ad hoc* swabbing. The percentage of *ad hoc* swabs was higher than systematic swabs during the pandemic wave (mid-November to end of December) during which time the percentage of swabs positive for influenza was also higher (Figure 1). This could explain the higher rate of positive swabs within the "*ad hoc*" samples.

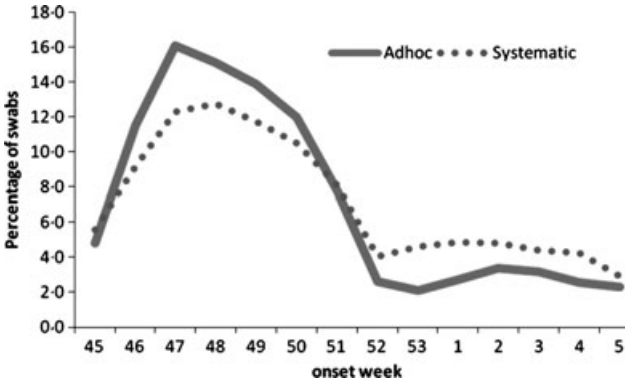

Figure 1. Percentage of swabs. Comparison of systematic random versus *ad hoc* sampling protocol. Distribution by week of swabs during 2009–2010 influenza season. Source: GROG network.

Vaccination coverage

The vaccination campaign was launched by the Ministry of Health on October 20, 2009,[6] and vaccination coverage increased during the surveillance period. In February, the vaccination coverage was 10·2% [3·4–22] in patients swabbed in the systematic group (9·6% on imputed data) and 11·5% [4·4–23·4] in the *ad hoc* group (11·9% on imputed data). At the national level, vaccine coverage is estimated at 7·95%.[7] Due to the over-mediatisation of pandemic vaccination and to rumors about its poor effectiveness, over-consultation of vaccinated patients and over-swabbing of vaccinated patients in the *ad hoc* group are not surprising.

Age groups

Age distribution is significantly different between our two samples ($P < 0{\cdot}0001$): the rate of 5–14 years old is lower in the systematic sampling group (25·5%) than in the *ad hoc* sampling group (35·7%). This can be explained by the fact that for the systematic sampling procedure, each GROG practitioner had to swab the first ILI patient in his assigned age group, whereas for "*ad hoc*" sampling, every GROG practitioner could swab any ILI patient irrespective of age. Given the emphasis by Health Authorities and media on the burden of pandemic influenza among children and teenagers, one can hypothesize that when they were able to, sentinel practitioners focused on these age groups.

Clinical symptoms

GPs in the *ad hoc* sampling scheme seem to have been more likely to select cases and further, to select vaccinated cases. Those patients may have consulted earlier with specific symptoms (strong headache being more prevalent among cases). Over-swabbing of patients having these symptoms in the *ad hoc* group is likely.

Distinctive features of the 2009–2010 pandemic season

The 2009–2010 pandemic influenza season was markedly different from previous ones: vaccination rate increased during and mainly after the pandemic peak; behaviors were strongly modified by unusual media hype; clinical features and risk factors might be different. It will be necessary to see if similar results are observed during a regular influenza season during which the vaccination rate increases before the epidemic peak with usual messages about vaccination and usual clinical influenza features.

Conclusion

Influenza early warning networks can estimate IVE, taking into account many covariates. From a stakeholders and patients point of view, during the 2009–2010 influenza pandemic wave, there were no major discrepancies between IVE estimated with an *ad hoc* sampling strategy, based on sentinel practitioners instinct, and IVE estimated with a systematic random sampling strategy whatever the multivariable analysis methodology.

Although from a statistical point of view, comparison of the two strategies is not readily feasible because of the non random nature of *ad hoc* sampling. This latter strategy seems to result in slightly lower IVE estimates, which could potentially be attributed to sentinel practitioners swabbing behavior.

The ability to avoid missing data is a key point to decide which sampling method must be adopted, because CI extent depends greatly on the proportion of missing data among covariates. To match IVE evaluation to surveillance networks practicality, selection of only those data essential for the study endpoint and easily collected by sentinel practitioners is paramount.

It will be necessary to determine if results similar to those observed during the 2009–2010 pandemic season are found during a regular influenza season.

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NS deletions convert the 2009-H1N1 pandemic virus into a live attenuated vaccine

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Keywords H1N1, live attenuated, NS, pandemic, vaccine.

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Abstract

Although vaccines against influenza A virus are the most effective method by which to combat infection, it is clear that their production needs to be accelerated and their efficacy improved. A panel of recombinant live attenuated human influenza A vaccines (LAIVs), including NS1-73, NS1-126, NSΔ5, were generated by rationally engineering mutations directly into the genome of a pandemic-H1N1 virus. The vaccine potential of each LAIV was determined through analysis of attenuation, immunogenicity, and their ability to protect mice and ferrets. The data indicate that the novel NSΔ5-LAIV was ideally attenuated and elicited strong protective immunity. This study also shows that attenuating mutations can be rapidly engineered into the genomes of emerging/circulating influenza A viruses in order to produce LAIVs.

Introduction

Influenza A viruses are important pathogens which remain a major cause of morbidity and mortality worldwide, and large numbers of the human population are affected every year. The first influenza pandemic in this century broke out in humans in March 2009, and it was declared to be pandemic by mid-June. As of 1 August Jul 2010, the pandemic virus had caused more than 18 449 deaths worldwide, according to the World Health Organization (http://www.who.int/csr/don/2010_08_06/en/index.html). The infection and spread of the pandemic influenza was reduced in part due to the use of vaccines. However, the lack of H1N1pdm vaccine early in the pandemic illustrates the need to improve vaccine production and to generate vaccines that induce stronger cross-protection.

Inactivated split vaccines or live attenuated influenza virus vaccines (LAIVs) against H1N1pdm viruses were approved for human use by the United States Food and Drug Administration. Both the inactivated vaccines and LAIVs are produced by creating reassortant viruses that generally contain six vRNAs (PB2, PB1, PA, NP, M, and NS) from a master donor strain, plus the two glycoprotein vRNAs (HA and NA) from a virus that antigenically matches the strain predicted to circulate in upcoming influenza season (e.g. A/CA/07/2009). The reference viruses containing inactivated split virus vaccines are produced in embryonated chicken eggs, and primarily result in the production of antibodies that recognize the viral glycoproteins. Both of these vaccine approaches require significant lead time for vaccine production, and modern approaches to speed preparation of vaccines and improve their efficacy is a global priority.[1,2]

The NS1 protein of influenza A virus is a multifunctional protein that plays important roles in virus replication and as potent type I IFN antagonist.[3,4] Mutations and/or deletions in NS1 typically induce stronger IFN responses by the host; those in turn suppress the replication of influenza virus[3–7] and can enhance immune recognition.[8–11] In this study, we created a panel of experimental H1N1pdm NS-LAIV candidates that have different deletions in the NS vRNA and analyzed the vaccine potential of each NS-LAIV in mice and ferrets to identify the best candidate(s).

Materials and methods

Generation of recombinant viruses

WT H1N1pdm influenza A virus A/New York/1682/2009 (NY1682) was created by reverse-genetics directly from a human swab specimen collected in New York state in April 2009.[12] Deletions were introduced into the NY1682 NS plasmid to create three mutant NS segments: NS1-73, NS1-126, and NSΔ5. Nucleotides 246–482 (cDNA of NS segment) and 405–482 were replaced by stop codons to generate NS1-73 and NS1-126; nucleotides 612–626 were deleted to generate NSΔ5, whose open reading frames for NS1 and NEP were maintained. Recombinant viruses were generated by co-transfection of eight reverse-genetics plasmids carrying the cDNA of each gene segment into 293T/MDCK co-cultured monolayer adapted from Hoffmann *et al.*[12,13]

Mouse studies

Experiments were performed in a biosafety level 3 laboratories approved by the U.S. Centers for Disease Control and Prevention and the U.S. Department of Agriculture, and were conducted under approved animal care and use protocols. Groups of 6-week-old female BALB/cJ (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized with isoflurane and inoculated intranasally with 10^5 $TCID_{50}$ of each recombinant virus in 20 μl of PBS diluent, or PBS as controls. Body weights and clinical symptoms of the mice were monitored daily for 10 days. Nine mice in each group were euthanized on 1, 2, and 5 days post inoculation (dpi), and nasal washes and lungs were collected for virus titration by $TCID_{50}$ assay in MDCK cells.

At 30 dpi, 12 mice per group were challenged intranasally with 5×10^4 $TCID_{50}$ (100 LD_{50} in 6-week-old mice) of a mouse-adapted variant of NY1682 (A/NY/1682/2009-MA7) (accepted, Journal of Virology). Disease symptoms and weights of the vaccinated mice were monitored for 10 days, and four mice from each virus group were euthanized at 3 and 6 days post challenge. Lungs were removed and homogenized for virus titration by $TCID_{50}$ assay. The mice that became moribund or lost >25% of their starting body weight were euthanized for humane reasons.

Ferret experiments

Male Fitch ferrets (Triple F Farms, Sayre, PA, USA), 7–10 months of age and serologically negative by hemagglutination inhibition (HI) assay for currently circulating influenza viruses were used in this study. Groups of 3 or 4 ferrets were inoculated intranasally with $10^{6.5}$ $TCID_{50}$ of one of the viruses: NY1682 WT ($n = 4$), NS1-73 ($n = 3$), NS1-126 ($n = 4$), or NSΔ5 ($n = 4$). Ferrets were monitored for clinical signs through 14 dpi as previously described.[14] Nasal washes were collected on 1, 3, 5, and 7 dpi and were titrated in MDCK cells by $TCID_{50}$ assay. Serum was isolated from blood collected 6·5 weeks after immunization and used for neutralization assays. The ferrets were challenged with 10^6 PFU of A/Mexico/4482/2009[15] 6·5 weeks post-immunization and monitored for clinical signs of disease through 14 dpi. Nasal washes were collected on 1, 3, 5, and 7 dpi, and were titrated in MDCK cells by plaque assay.

Results

Generation of experimental live attenuated influenza vaccines (LAIVs)

Using reverse genetics, we created three LAIV candidates (NS1-73, NS1-126, and NSΔ5) based on the recombinant virus A/New York/1682/2009 (NY1682). To confirm the desired mutations were present in the panel of recombinant viruses, we used M-RTPCR to amplify the genome of each virus and then analyzed the amplicons by agarose gel electrophoresis. M-RTPCR amplicons of NS-vRNA from the WT and NSΔ5-LAIV migrate similarly (~890 bp), whereas the deletions present in NS-vRNA of NS-73 and NS1-126 lead to accelerated migration of the NS amplicons. The NS vRNAs of the viruses were further confirmed by sequencing of the M-RTPCR amplicons.

Attenuation and protection in mice

Weight loss of WT virus inoculated mice became evident at 3 dpi, and the mice did not recover until 8 dpi (Figure 1A). In contrast, mice inoculated with any one of the vaccine candidates had no clinical signs of disease and continued to gain weight at the same rate as did the mock-

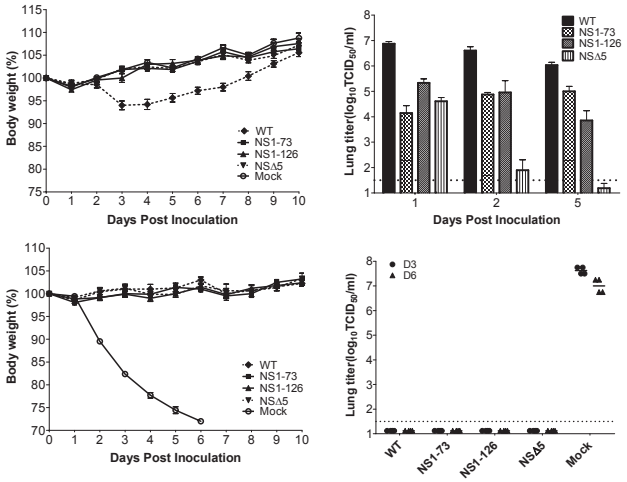

Figure 1. NS-LAIVs protect mice from lethal challenge. (A) Body weight of mice immunized with recombinant viruses. (B) Viral titers in the lung of immunized mice on 1, 2, and 5 dpi. (C) Body weight of mice immunized with a lethal mouse adapted variant. (D) Viral titers in the lungs of challenged mice on 3 and 6 dpc.

inoculated mice (Figure 1A). Viral titers in the lungs of NS1-73, and NS1-126 infected mice were ~100-fold lower than titers from WT virus-infected mice at all the time points analyzed (1, 2, and 5 dpi) (Figure 1B). Notably, the NSΔ5 LAIV was cleared from the mouse lungs very rapidly, and the mean titers were ~100-fold and 50 000-fold lower than the titers of the WT virus at 1 and 2 dpi, respectively (Figure 1B).

The vaccinated mice were challenged with a mouse-adapted variant of NY1682 (accepted, Journal of Virology) on 30 dpi. No disease symptoms were observed in the mice immunized by any of the NS-LAIV candidates or the WT control. In contrast, disease symptoms including ruffled fur, hunched posture, and weight loss were observed in the mock-immunized mice as early as 2 days post challenge (dpc); the symptoms progressed to severe disease, and the animals showed dramatic weight loss, became moribund, and succumbed to infection by 5 dpc (Figure 1C). High titers of virus (~10^7 $TCID_{50}$/ml) were present in the mock-immunized mice at 3 dpc and at 6 dpc (Figure 1D). In contrast, virus was not detected in the lungs of immunized mice (Figure 1D). This challenge data demonstrates that all of the NS-LAIV candidates, including the highly attenuated NSΔ5, induced sterilizing immunity that protected mice from a lethal NY1682 H1N1pdm variant.

Attenuation and protection in ferrets

Groups of ferrets were intranasally immunized with $10^{6.5}$ $TCID_{50}$ of each vaccine candidate or the WT virus. The titer of viruses recovered from nasal washes ranged from $10^{6.8}$ to $10^{4.5}$ $TCID_{50}$/ml through day 5 in the WT virus-infected group, while the NS-LAIVs showed various degrees of attenuation (Figure 2A). The viral titer of all of the NS-LAIVs is at least 100-fold lower than that of WT in the nasal wash collected at 1 dpi. The NS1-73 LAIV was the least attenuated in ferrets, and its replication was similar to that observed in mice. Relative to the WT virus, the NS1-126 LAIV showed 100-fold reduction in titer, and the NSΔ5 LAIV was below the limit of detection (at least 1000-fold reduction) at 3 dpi.

Sera from blood collected 6·5 weeks after immunization was analyzed for the presence of neutralizing antibodies by micro-neutralization assays. The NS-LAIV candidates all induced very strong neutralizing antibody responses (1280-5120) that were similar to the titer elicited by WT virus infection (Figure 2B).

The ferrets were challenged with 10^6 PFU of A/Mexico/4482/2009 (H1N1pdm) 6·5 weeks post immunization. Little disease or weight loss were observed in the naïve ferrets, and the ferrets immunized by infection with WT virus or the NS-LAIV candidates didn't show any disease symptoms or weight loss. In contrast to the high titer of virus

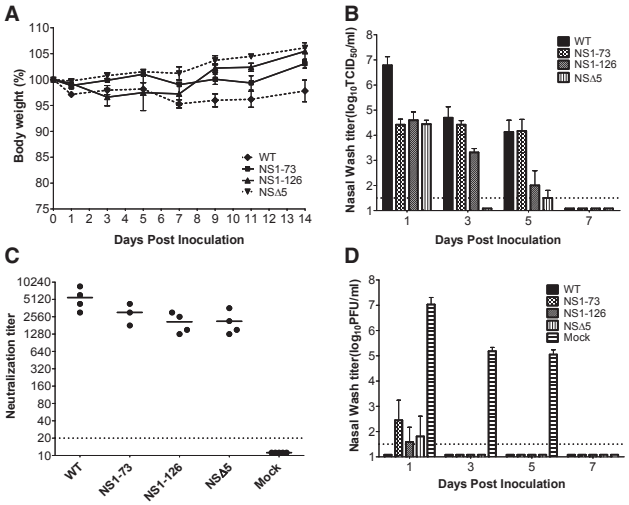

Figure 2. NSΔ5 LAIV is attenuated and protective in ferrets. (A) Body weight of ferrets immunized with recombinant viruses. (B) Virus shedding from the nasal airway of the infected ferrets on 1, 3, 5, and 7 dpi. (C) Neutralizing antibodies levels in serum isolated 6·5 weeks post immunization. (D) Virus shedding from the nasal airway of immunized ferrets on 1, 3, 5, and 7 days post challenge.

detected in the naïve ferrets through 5 dpc, the NS-LAIV immunized ferrets had very low levels of A/Mexico/4482/2009 in their nasal washes at 1 dpc (Figure 2C). The ferrets immunized with the NY1682 NS-LAIVs had ~10 000- to 100 000-fold lower viral titers than did the naïve animals (Figure 2D). In summary, the NS-LAIV candidates dramatically inhibited initial replication of the H1N1pdm virus under stringent challenge conditions (10^6 PFU), and that the vaccinated animals rapidly cleared the infection (to below the limit of detection, by 3 dpc).

Discussion

Our results demonstrate that all of the NS-LAIV candidates are attenuated compared to the WT H1N1pdm virus, and the degree of attenuation is dependent on the specific NS mutation. NS1-73 was the least attenuated and does not represent a good vaccine candidate; whereas, NSΔ5 and NS1-126 were highly attenuated in both the mouse and ferret models. Although they were markedly attenuated, they elicited strong neutralizing antibody responses and protected mice and ferrets from subsequent challenge. NSΔ5 has a subtle in-frame deletion (15 nt) that affects both the NS1 (residues 196–200) and NEP (residues 39–43), and is analogous to a naturally attenuated variant of a normally highly pathogenic H5N1 virus (A/SW/FJ/03).[16] The analogous NS1 deletion in A/SW/FJ/03 (residues 191–195) was shown to reduce binding to host cleavage and polyadenylation specificity factor (CPSF), reduce NS1 protein stability,

and enhance the type I IFN response of this H5N1 virus.[16] Our study indicates that deletion of these 15nt in the NS vRNA of the H1N1pdm also stimulates the host IFN response, specifically, IFN-ß, IFN-λ1, IP10, and MxA (data not shown). The role of the deletion of residues 39–43 from NEP has not been elucidated, but the induction of IFN and ISGs by NSΔ5 was similar to, or slightly lower than, their induction by NS-126, suggesting that the NEP mutation also has an attenuating effect that warrants future investigation.

In summary, we have generated a panel of LAIVs directly from a swab specimen containing a new pandemic virus and analyzed their attenuation and immunogenicity in two animal models. Our study demonstrates that NSΔ5 is a novel NS-LAIV that could be used to create LAIVs for diverse influenza A viruses. This study also validates the use of NS-LAIV candidates, which are not only highly attenuated, but they also elicit strong innate and adaptive immune responses, resulting in protection of mice from subsequent challenge with a lethal mouse-adapted variant of NY1682, and ferrets from challenge with A/Mexico/4482/2009 (H1N1pdm).

Disclosure

Data presented in this manuscript were previously published in Zhou, B., Y. Li, J. A. Belser, M. B. Pearce, M. Schmolke, A. X. Subba, Z. Shi, S. R. Zaki, D. M. Blau, A. Garcia-Sastre, T. M. Tumpey, and D. E. Wentworth. 2010. NS-based live attenuated H1N1 pandemic vaccines protect mice and ferrets. *Vaccine* 2010;28(50):8015–25.

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Development of the influenza B reassortant NYMC BX-35 for use as seed virus for influenza B vaccine production

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Keywords Classical reassortment, influenza B reassortant, NYMC BX 35, NYMC BX-33B.

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Background Since influenza virus human isolates grow poorly in embryonated chicken eggs, it is essential to develop high yield seed viruses for large scale production of influenza vaccines. Objectives We aimed to investigate the usefulness of B/Panama/45/90 as a donor to generate high yield B reassortants. B/Brisbane/60/2008, a recommended vaccine virus for 2009/10–2010/11 seasons, was used as a wild type (wt) ''target'' strain for potential use as a seed virus for influenza B vaccine production. We also incorporated B/Lee/40 NP gene (a previously known ''high yield gene'') into one of the resultant high yield reassortant for further enhancement of the virus yield. Materials and methods B reassortant viruses were prepared by the classical reassortment method. The anti- B/Panama/45/90 HANA antibodies were used for the selection of wt surface antigen carrying viruses. Restriction Fragment Length Polymorphism (RFLP) was used for genotyping of resultant reassortants. Results Reassortants with significantly higher yield than the parental vaccine strain were produced in this reassortment between B/Panama/45/90 and B/Brisbane/60/2008. RT-PCR/RFLP analysis revealed that all the high yield reassortants contained the PB2 of B/Panama/45/90, suggesting that the high yield property of B/Panama/45/90 was donated to the reassortant viruses by PB2. One of the resultant high yield reassortants, NYMC BX-33B, was further reassorted with B/Lee/40 to introduce its NP gene for further enhancement of virus yield. The resultant triple reassortant, NYMC BX-35, has been utilized as a seed virus for influenza B vaccine production for the 2010/11 season. Conclusions A Yamagata lineage strain, B/Panama/45/90, was useful for developing high yield reassortants of B/Brisbane/60/2008. NYMC BX-33, BX-33B, and BX-35 developed in this study were candidate viruses for the 2010/11 Northern Hemisphere vaccine.

Introduction

Currently, a total of approximately 300 million doses of inactivated influenza vaccine are being produced worldwide each year. One of the limitations in vaccine production is poor growth of human isolates in embryonated chicken eggs. This is essential to develop high yield seed viruses for large scale production of influenza vaccines.

Influenza A vaccine production utilizes high yield reassortants carrying HA and NA genes from a wild type (wt) strain with generally 5–6 internal genes from the A/PR/8/34 (PR8) strain, an highly egg adapted high growth donor strain.[1] Influenza B vaccines, however, have been produced directly from wt strains, partly because no high yield donor analogous to PR8 has been identified.

In recent years, reverse genetics has been used as an alternative means of developing high growth vaccine viruses.[2,3] Since in this plasmid-based technology, a 6:2 reassortant (six internal genes from a donor strain and two surface antigen genes from wild type strain) can be directly rescued, reverse genetics-derived reassortant viruses were expected to grow as efficiently as those derived from classical reassortment. However, reverse genetics reassortants have not produced the expected high growth for several reasons: (i) the 6:2 configuration is not always the best for virus yield, (ii) there is no process included for positive selection of adaptive mutants from quasispecies, and (iii) cell-derived viruses are not readily adapted to grow efficiently in eggs.

Our laboratory at New York Medical College has been preparing B reassortants for several years by classical reassortment using B/Lee/40 as a donor. It has been possible to develop B reassortants, which produce higher virus yields than wt strains in eggs, and it was found that the NP gene of B/Lee/40 was important in producing high yield B

reassortants.[4] However, B/Lee/40 is inconsistent in providing high yield properties to B reassortants.

In this study, in an attempt to find an alternative donor, we investigated the usefulness of B/Panama/45/90 for developing high yield B reassortants. As a wt strain, B/Brisbane/60/2008 was used, which is one of the recommended influenza B virus vaccine strains for the 2009/10 and 2010/11 seasons. We found that B/Panama/45/90 is a useful donor, and some of the resultant reassortants were considered as vaccine candidates.[5]

Materials and methods

B reassortant viruses were prepared by the classical reassortment method described by Kilbourne.[1] The antiserum to B/Panama/45/90 hemagglutinin and neuraminidase (HANA) was raised in this study by immunizing rabbits with HANA isolated from B/Panama/45/90; purified IgG was used for antibody selection. The yields of the reassortants and their corresponding parent viruses were assessed by hemagglutination assay. Viral RNA was extracted directly from the allantoic fluid and amplified by RT-PCR to produce cDNA for analyzing the gene composition. Restriction Fragment Length Polymorphism (RFLP) analyses were performed to determine the origin of each gene segment of the high yield reassortants. Restriction enzyme sets for each gene segment are available upon request.

Results

In this study we investigated the usefulness of B/Panama/45/90 as a donor for transferring high yield phenotype. B/Panama/45 is a Yamagata lineage strain with high growth phenotype (HA titer: 1024–2048). B/Panama/45/90 itself was a recommended B virus vaccine strain for 1989/90–1994/95 seasons. As a wt virus, a Victoria lineage strain, B/Brisbane/60/2008, was used, which is a recommended B virus vaccine strain for use in the 2009/10 and 2010/11 seasons.

Reassortants were prepared according to classical reassortment protocol. After co-infection of B/Panama/45/90 and B/Brisbane/60/2008, progeny viruses carrying surface antigens (HA and NA) of the vaccine strain were negatively selected by anti-B/Panama HANA antibodies, followed by passages without antibodies for positive selection of egg-adapted viruses and finally limited dilution cloning. NYMC BX-33, BX-33B, BX-33D, and R-3A are representative of resultant reassortants, which have significantly higher HA titers than the wt strain. The complete gene compositions of these reassortants were determined by RT-PCR/RFLP analyses. As shown in Table 1, all of these reassortants contained the PB2 of B/Panama/45/90. Other genes of B/Panama/45/90 (NP of BX-33, M of BX-33B, and PB1 of BX-33D) may not be involved in the high virus yield, since no significant growth difference among these reassortants

Table 1. Gene composition of BX-33,-33B,-33D and R-3A from reassortment between B/Panama/45/90 and B/Brisbane/60/2008. Genes derived from the donor strain are highlighted in shaded box

Reassortant	PB1	PB2	PA	HA	NP	NA	M	NS
BX-33	Brisbane	Panama	Brisbane	Brisbane	Panama	Brisbane	Brisbane	Brisbane
BX-33B	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Panama	Brisbane
BX-33D	Panama	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane
R-3A	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane	Brisbane

Table 2. Gene composition of viruses from reassortment between B/Lee/40 and BX-33B. Genes derived from B/Panama/45/90 and B/Lee/40 are highlighted in shaded box and bold, respectively

Reassortant	PB1	PB2	PA	HA	NP	NA	M	NS
R-2B	Brisbane	Panama	Brisbane	Brisbane	Brisbane	Brisbane	**Lee**	Brisbane
R-4C	Brisbane	**Lee**	**Lee**	Brisbane	**Lee**	**Lee**	Panama	Brisbane
BX-35	Brisbane	Panama	Brisbane	Brisbane	**Lee**	Brisbane	Panama	Brisbane
R-7B	Brisbane	Panama	Brisbane	Brisbane	Brisbane	**Lee**	Panama	Brisbane
R-10B	Brisbane	Panama	Brisbane	Brisbane	Brisbane	**Lee**	**Lee**	**Lee**

in eggs was found as assayed by hemagglutination test. Accordingly, the PB2 of B/Panama/45/90 is considered to be the sole factor involved in the high yield phenotype donated to the vaccine strain.

We previously found that the B/Lee/40 NP gene was important in producing high yield B reassortants. It was of interest to examine whether B/Lee/40 NP and B/Panama PB2 could work together to produce even higher yields. To test this possibility, BX-33B (2:6 reassortant: PB2 and M genes from B/Panama and the rest of the genes from B/Brisbane) was selected and further reassorted with B/Lee/40. Despite some difficulty in removing the NA gene of B/Lee/40 (R-4C, 7B, 10B in Table 2), by monitoring HA and NA genes of resultant viruses after each antibody selection passage with anti B/Lee/40 HANA antibodies, we were able to isolate and clone a triple reassortant, NYMC BX-35, which contains the NP gene from B/Lee/40 and PB2 and M genes from B/Panama; the remaining genes are from B/Brisbane/60/2008 (Table 2). In comparison with BX-33B, no significant growth enhancement (nor reduction) in eggs was found for BX-35 over that seen for BX-33B. Nevertheless, BX-35 stably produces high virus yield and has been utilized as a seed virus for influenza B vaccine production for the 2010–2011 season by one or more vaccine manufacturers.

Discussion

There are contradictory reports [6–8] about the usefulness of reassortment for high yield influenza B viruses. However, we have been preparing B reassortants for several years by classical reassortment [1] using B/Lee/40 as a donor, and have been able to generate higher virus yield than wt strains.[4]

In this study, we found that B/Panama/45/90 serves as an efficient donor in providing the high growth capacity to B/Brisbane/60/2008 (a recommended vaccine virus of Victoria lineage for 2009/10–2010/11 seasons), and that the PB2 of B/Panama/45/90 is associated with the high yield phenotype. This particular strain from Yamagata lineage might be useful to prepare high yield reassortants for other Victoria lineage vaccine viruses.

We noticed in this study that there may be segment incompatibilities between B/Panama/45/90 and B/Brisbane/60/2008. As shown in Table 1, the PA and NS genes of all the high yield reassortants examined are derived from wt, B/Brisbane/60/2008, not from the donor, B/Panama/45/90. This indicates that in this reassortment, the PA and NS genes are not replaceable with that of the donor to obtain high yield viruses.

This degree of incompatibility might be common in B reassortment, resulting in low donor/wt reassortants, such as 2:6 and even 1:7 reassortants that we obtained in this study. If this is the case, reverse genetics based on 6:2 configuration may not result in generating high yield B reassortants unless a variety of donor/wt combinations are designed.

One can speculate that in influenza B viruses, the surface glycoproteins (HA and NA) and some of the internal proteins are functionally more closely related than in influenza A virus, as was seen in that PA and NS genes of B/Brisbane/60/2008 reassort together with the HA and NA genes of the same parent (Table 1). In our recent study on a reassortment between B/Lee/40 and B/Panama/45/90, it appeared that HA shapes overall gene constellations of the resultant reassortants, namely the reassortants tend to have more internal genes from the same parent of HA, no matter which parent's HA is selected by antibodies against the surface antigens of the other parent (data not shown).

Because of success in influenza A virus reassortment with PR8, it is generally believed that reassortant with 6:2 or 5:3 configuration is optimal for virus yield. This may be the case in most instances of influenza A reassortment, but is not necessarily so in B reassortment. As shown in this study, only a single donor gene is capable of improving the yield of vaccine strain by reassortment.

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Pandemic influenza in Russia: detection and molecular characterization of the H1N1v virus

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Introduction

Influenza A/H1N1v has spread rapidly in all parts of the world in 2009 as a true pandemic.[1] Epidemic events in Russia occurred during the last week of September 2009 starting from Far East region (Yuzhno-Sakhalinsk). Kaliningrad (the western most Russian city) was the second starting point of the epidemic. During October 2009 the epidemic spread over the whole Russian territory. In a short period the new virus started to change genetically as it began to adapt to human populations during this pandemic (http://www.who.int; http://www.euroflu.org).

In the period from May to December 2009, 1558 clinical samples (nasopharyngeal swabs and postmortem materials) of patients with influenza-like illness from different regions of Russian Federation were analyzed to confirm the diagnosis using real-time reverse transcription PCR (rRT-PCR).

Materials and methods

Clinical nasopharyngeal swabs and bronchoalveolar lavage and post mortal fragments of trachea, lungs, bronchi, spleen from Saint Petersburg hospitals and 49 basic laboratories of Federal Influenza Center were included in this study. All specimens were taken from patients with influenza-like illness or viral pneumonia.

Specimens were tested by rRT-PCR according to CDC Protocols, i.e. using SuperScript III Platinum One-step qRT-PCR System (Invitrogen) with primers and probes for InfA, H1 seasonal, and H1Sw (Biosearch Technologies). In addition, the test-systems 'AmpliSense Influenza virus A/B-FL' and 'Influenza virus A/H1-swine-FL' for PCR-detection, typing and subtyping of influenza viruses were also used. These test-systems are produced by Central Institute of Epidemiology, Moscow, Russia and recommended by Russian Ministry of Health as tests for influenza diagnosis.

Sequencing was carried out on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, USA) with BigDye Terminator Cycle Sequencing kit.

Phylogenetic analysis was performed using programs Vector NTI 10.0 (Invitrogen) and MEGA 4.1 (PSU, USA) by maximum likelihood with the TIM+I+G model for HA, and – HKY+I+G model for NA. Evolutionary model was selected by Akaike information criterion (AIC) in ModelTest (Posada, Crandall, 1998). Statistical reliability of tree branches was evaluated by bootstrap test (100 replications).

Immunohistochemical study was performed using Novalink antibodies to HA and NP with Novocastra visualization system.

Results

Influenza virus A/H1N1v RNA was detected in 409 patients with severe form of influenza-like illness and 163 fatal cases. Out of PCR-confirmed flu recovered cases 58% were patients under 14 years of age, 41% were aged 15–64 years, and 1% were older than 65 years. Mean age of recovered patients was 15·7 years (from 1 month to 77 years).

Viral RNA in postmortem materials was detected mostly in lung tissue (86% of specimens) and trachea fragments (64%), and less commonly in spleen (17%). Mean age of the deceased with confirmed flu (H1N1v) infection was 39·3 years with age ranging from 7 months to 75 years. In 84% of fatal cases, influenza was complicated by viral or secondary bacterial pneumonia. Median time from the onset of illness until death was 10 days. According to our data, 4% of patients died had diabetes, 4·5% were obese, and 4% were pregnant women in the 2nd or 3rd trimester.

HA and NP were detected by immunohistochemical assay in lung tissue of dead patients with confirmed influenza virus A/H1N1v infection. HA and NP was revealed in the endothelium of different sized blood vessels (capillaries and arterioles). These influenza virus proteins were also detected in some tissue macrophages apart from epithelium and endothelium. The localization of the two proteins was different: HA is mostly localized in cell membrane and cytoplasm, and NP – mostly in the nucleus.

Here we present data on molecular genetic characteristics of 51 strains of pandemic virus, 40 strains obtained from clinical specimens, and 11 from post mortal ones isolated in the Research Institute of Influenza. All the strains studied contain the S31N substitution in M2 protein, which indicates resistance to the adamantane antivirals, and have no H275Y substitution in the neuraminidase, which indicates resistance to oseltamivir.

The phylogenetic analysis showed that Russian viruses were similar to influenza viruses A/Texas/05/2009 and A/California/07/2009 (HA similarity 98·9%). All Russian viruses could be divided in two clusters: the first one includes viruses similar to the reference strain A/California/07/2009, and the second one, which is the majority of viruses analyzed includes strains with substitutions HA S203T, NA N248D, V106I, and NS I23V (Figure 1). Bootstrap support was 59. The isolates with HA S203T substitution can be classified in one of the five minor genome variants of A/H1N1v viruses found in the United States and Mexico in 2009.[2] Several viruses had strain-specific substitutions in antigenic sites Sb and Ca and the mutation D222G in HA receptor-binding site.

The substitution of amino acid residue Asp to Gly at position 222 of HA was found in eight of eleven isolates (73%) from postmortem lung and trachea samples and two of forty isolates (5%) from nasopharyngeal swabs of patients with severe course of the disease. Appearance of amino acid substitutions in the HA receptor-binding site (D187E and D222G/E) could be associated with influenza virus passaging on eggs.[3] Five strains that contained G at position 222 of HA were isolated from post mortal specimens on MDCK cells in this study, thereby excluding the possibility of substitution appearance hence to virus adaptation on eggs.

In order to reveal genome changes in A(H1N1)v, strains isolated on the territory of Russian Federation during the 2009 pandemic, 67 full genome sequences from GenBank, and Research Institute of Influenza database were analyzed comparing two groups of viruses (isolated before and after 22 Sept 2009). Nine amino acid changes observed predominantly in late pandemic strains were found. Five of them (S128P, S162N, D222G, V234I, V321I) reside in HA, two in NA (I263V, N386K), two in PB2 (K340N, T588I), and one in PA (F35L). Towards the end of the epidemic the viral population had demonstrated statistically certain rise in number of strains containing mutations in four genes. Difference between groups was statistically significant (Chi-square test, $P = 0{\cdot}05$). If $\chi^2 > 3{\cdot}84$, than difference between early and late strains is statistically significant.

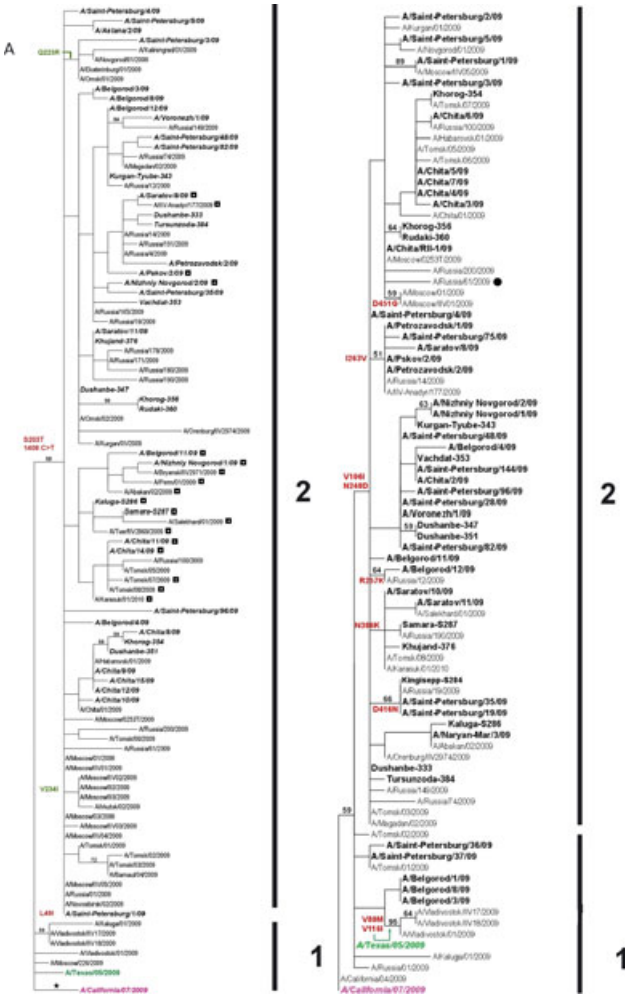

Figure 1. Phylogenetic tree of influenza virus A/H1N1v strains HA (A) and NA (B). (Strains isolated in the Institute of Influenza are in bold, ■ marks the strains with D222G in HA, ● marks the strain with H275Y in NA).

Additionally Fisher's test determined whether 'early strains' and 'late strains' differ significantly in the proportion of 'no mutation event' and 'mutation event' attributed to them in each particular position. All calculations were performed in Fisher_TK freeware by Vladimir Belyaev similar to CalcFisher (Haseeb, 2003) fully described here (http://www.jstatsoft.org/v08/i21/paper). We have selected positions with statistically significant amino acid changes in late strains (*P*-value 0·05).

According to full genome analysis of influenza virus A/H1N1v 2009 strains, seven clades were distinguished, but the divergence between representatives of different clades remained small.[4] To make prognosis of the 2010 H1N1v epidemic season in Russia, full genome sequences of 41 strains isolated in Southern hemisphere from GenBank and EpiFlu (GISAID) databases were analyzed. A/California/04/2009 (clade 1) strain was used as an outside group and strains A/Mexico/4108/2009, A/Texas/05/2009 (clade 2), A/Arizona/01/2009 (clade 3), A/Korea/01/2009 (clade 4), A/Wisconsin/629-D01505/2009 (clade 5), A/Rhode Island/02/2009 (clade 6), A/Moscow/01/2009 (clade 7) were included as representatives of other clades.

The phylogenetic analysis showed that the majority of strains (97·6%) belonged to clade 7 and contained characteristic substitutions S220T (HA), V100I (NP), V106I, N248D (NA), I123V (NS1), and only one strain A/Tallinn/INS431/2010 (05.02.2010) belonged to clade 3. Formation of a new phylogenetic clade can be noticed inside clade 7 confirmed by bootstrap analysis. This subclade includes Australian strains of influenza virus A/H1N1v isolated in June-August, 2010 as well as strains A/Perth/12/2010, A/Victoria/05/2010, A/South Auckland/05/2010, A/South Auckland/10/2010 and has seven characteristic substitutions in PB2, PB1, NP, NS1, and HA antigenic site Sa (Figure 2). Besides the strain A/Perth/12/2010 also contains substitution S181F in the same HA antigenic site.

Discussion

According to data obtained, the 2009 epidemic in Russia was caused only by influenza virus A/H1N1v. Unlike the previous epidemic periods when most severe influenza cases were registered among the children under 5 years and among elderly people aged over 65 years, the first wave of pandemic due to influenza virus A (H1N1)v resulted in increased level of mortality mainly among the people aged 18–53 years. Though all pandemic viruses showed comparative genetic homogeneity, some evolutionary trends could be outlined. For clarification of the exact pathogenic role of mutation D222G in HA receptor binding site, further studies are necessary.

Full-genome analysis of influenza virus A/H1N1v strains circulating in the Southern hemisphere in the new epidemic season 2010 revealed the phylogenetic subgroup distinguished by seven substitutions in inner proteins (PB2, PB1, NP, NS1) and Sa antigenic site of HA (N142D). The changes revealed could be caused by adaptation of the virus to an immunized human population.

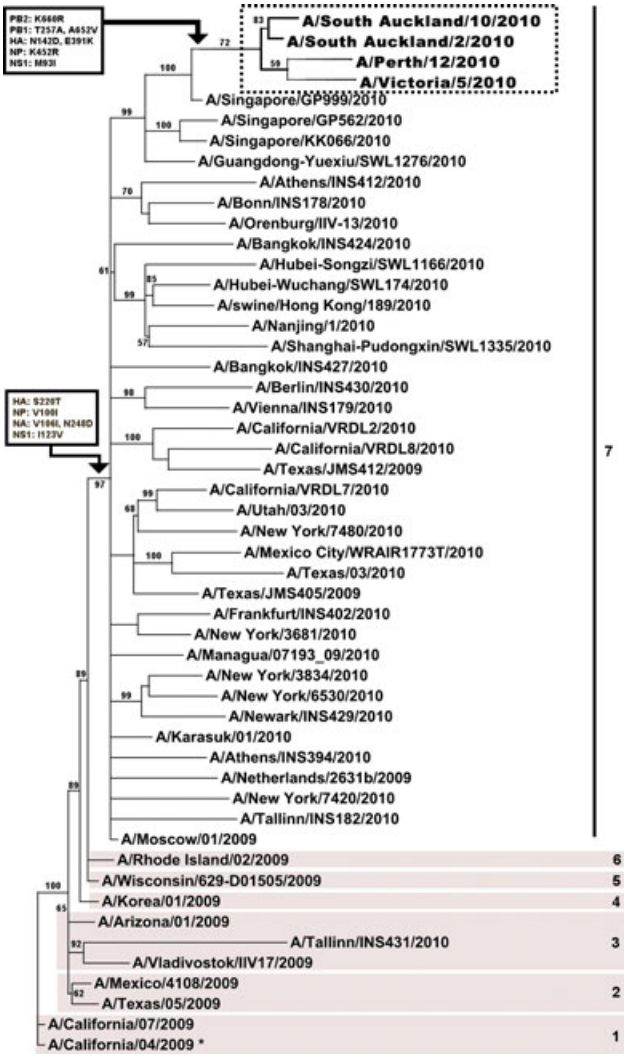

Figure 2. Full-genome phylogenetic tree of A/H1N1v strains isolated in 2010.

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Evolutionary studies of 2009 H1N1 pandemic via next generation sequencing (NGS): circulating influenza A from the worldwide locations are the variants of original 2009 H1N1

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Keywords 2009 H1N1, evolution, next generation sequencing.

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Abstract

Pandemic 2009 influenza A virus (A/H1N1/2009) continues to cause worldwide influenza outbreaks beyond its 1st anniversary. Full genomic sequence analysis of 2009 pandemic infections might provide an important role on preparation for future influenza outbreaks. We investigated the potential of ultra-deep pyrosequencing (Roche 454 FLX) to determine and assemble >100 full genome sequences of novel 2009 H1N1 viruses isolated worldwide since the beginning of the 2009 pandemic. Phylogenetic studies of the eight influenza A gene segments were generated and were found to display higher genetic diversity for HA and NA segments as compared to the mutational rates for other five RNA segments. Analysis of full influenza genomes containing all 8 concatenated RNA segments revealed the existence of two distinctive genetic groups, Clades 1 and 2. It was concluded that 2009 H1N1 influenza A currently causing large scale global infections all belong to the genetic Clade 2. The initial 2009 H1N1 isolates of Mexican/California origins isolated at the beginning of 2009 pandemics are probably no longer in circulation.

Introduction

Novel 2009 influenza A (H1N1) is a flu virus of swine origin that was first detected in Mexico and the United States in March and April 2009.[1–3] Since its initial identification and the announcement of the unusual human outbreaks in Mexico, the 2009 swine H1N1 virus quickly spread north into the United States, mostly via spring break tourists traveling to and from Mexico (MMWR).[4] Following Mexico and US reported cases, confirmed outbreaks of 2009 swine H1N1 were reported throughout Europe, Asia, Africa, and South America, most probably via global airline travel.[5,6] Based on up-to-date viral sequence comparisons (GenBank) from various laboratories worldwide, it was clear that not all deposited 2009 swine H1N1 sequences were 100% identical.[7,8] It was uncertain if specific and significant mutations had occurred in viral genomes since the initial infections in Mexico.[9] In this study, we utilized a robust and constant 2009 H1N1-specific RT-PCR amplification scheme in conjunction to Roche 454 FLX system to accomplish a large number of full genome sequences of various clinical samples containing moderate to low viral (2009 H1N1) concentrations collected worldwide. Constructions of phylogenetic trees for various 2009 full genome H1N1containing concatenated 8 RNA segments since the beginning of 2009 pandemic reveals evolutionary changes of 2009 H1N1 viruses.

Materials and methods

Viral sample collection and culturing

Nasal and throat swabs (placed in 3 ml MEM and frozen at −80°C until use for viral RNA extraction and tissue culture inoculation) were collected from patients with febrile illness, i.e., >38·0°C. Samples were received from clinics in US Embassies and US military laboratories located throughout the world since the initial WHO declaration of 2009 novel H1N1 outbreaks as a global pandemic on June 11, 2009. Viral isolates were obtained from inoculating cultures of MDCK cells with 0·1–0·2 ml viral suspensions collected in MEM originated from patients after 2–7 days incubation.[10–13] Due to low viral titers in normal clinical samples, most of full viral genome sequences were derived

from viral stocks obtained by tissue culturing passages (MDCK, 1–2 times).

Viral RNA extractions

Viral RNA was extracted from clarified supernatant fluid of nasal/throat swabs or MDCK cultures using the 'ChargeSwitch' RNA extraction system based on the user manual protocol from the manufacturer (Invitrogen Inc., CA, USA). Total RNA was eluted into volume equal to original sample volume, i.e., 100 μl starting viral supernatant used to yield final 100 μl RNA in molecular grade water (Invotrogen Inc.) and stored at −80°C until tested.

Generating/preparing overlapped cDNAs for full genome coverage of 2009 Novel H1N1 viruses by multiple RT-PCR amplifications

The first step in the high-throughput sequencing pipeline for full influenza genome sequences was to establish a robust RT-PCR amplification scheme consisting 67 different RT-PCR primer pairs covering all 8 RNA segments to ensure 100% amplification coverage of full viral genomes of all the incoming targeted viruses (Houng, HS. 2011, submitted for publication). Extracted viral RNA (5 μl), derived from mostly MDCK culturing stock or clinical sample containing sufficient viral load (>100 infectious units per ml) was added to primer-free RT-PCR total master mixture (10·5 μl) for each virus followed by adding primer pair (2 μl, 3 pmole/μl per primer). RT-PCR was then performed: RT reaction through two hold-steps (50°C, 15 minutes and 94°C, 2 minutes); 35 cycling amplifications (94°C for 15 seconds, 53°C for 15 seconds, 72°C for 2·5 minutes). Specific cDNA amplicons corresponding to each individual primer pair were routinely monitored and visualized by agarose gel electrophoresis.

Sequencing pooled Influenza genomic cDNAs by 454 Roche FLX pyrosequencing

Pooled 67 cDNA products (2–5 μg) from each viral RT-PCR amplification run were used as sequencing substrates according to the Roche 454 FLX user manual and bulletins by incorporating adaptors containing individually Multiplex Identifier [MID]-key assigned to each individually pooled viral cDNA. Up to 12 different MID-keyed viral cDNA were further pooled together to be clonally amplified on capture beads in water-in-oil emulsion micro-reactors (em amplifications), and pyrosequenced using one of two regions of a 40 × 75 mm PicoTiterPlate. For each individual viral genome containing multiple assemblies (8 RNA segments), we obtained SFF file(s) containing raw sequencing reads from which nucleotide sequence data and phred-like quality scores were extracted. On average, 80·0–90·0% of 3–9 million MID-key specific nucleotides were extracted and mapped for consensus genome sequences.

2009 H1N1 full genome assemblies, annotations, and phygenetic analyses

Roche 454 gsMapper (v. 2.0 and 2.3) software was used to assemble all sequencing raw data and SFF files into consensus sequences. New reference mapping projects were created to assemble each individually MID- keyed viral cDNA into consensus viral sequences. One of the earliest 2009 H1N1 genomes of California origin, A/California/04/2009(H1N1), deposited in GenBank, was routinely employed as a reference genome sequence for most of gsMapper projects. The resultant consensus sequences obtained were further verified and validated through the NCBI annotation utility check and ultimately deposited to the NCBI influenza database, GenBank. Nucleotide sequences specific to each individual RNA gene were aligned by the Geneious Pro 4.7.5 software (http://www.geneious.com).[14] Trees were built based on the Tamura-Nei genetic distance model using the Neighbor-Joining method with no outgroup used via Geneious Pro 4.7.5. Phylogenetic trees of the 2009 H1N1 genomes were constructed by importing FASTA files containing specific concatenated target sequences of PB1, PB2, PA, HA, NP, NA, MP, and NS from each individual virus into the Geneous Pro software and going through the sequence assembling and tree building steps.

Results

High-throughput pyrosequencing of pooled 2009 novel H1N1 cDNAs by Roche 454 FLX system

Up to 24 viral cDNAs could be routinely sequenced to completion for 24 different full viral genomes from a single Roche 454 FLX PicoTiter plate by utilizing the combination of Pico-Titer Plate's two distinct regions as well as 12 different MID-keyed adaptors. The 'shotgun' sequencing approach employed in this study is a feasible method to

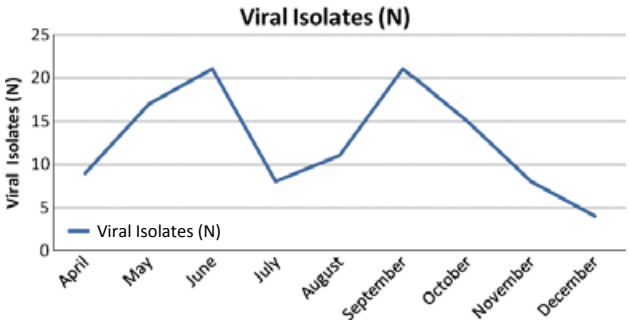

Figure 1. Distribution of isolation dates for total 114 sequenced 2009 H1N1 originated from worldwide locations. Walter Reed Army Institute of Research (WRAIR) had sequenced representative viral isolates mostly from two waves of pandemic infections, late spring-early summer 2009 and fall 2009.

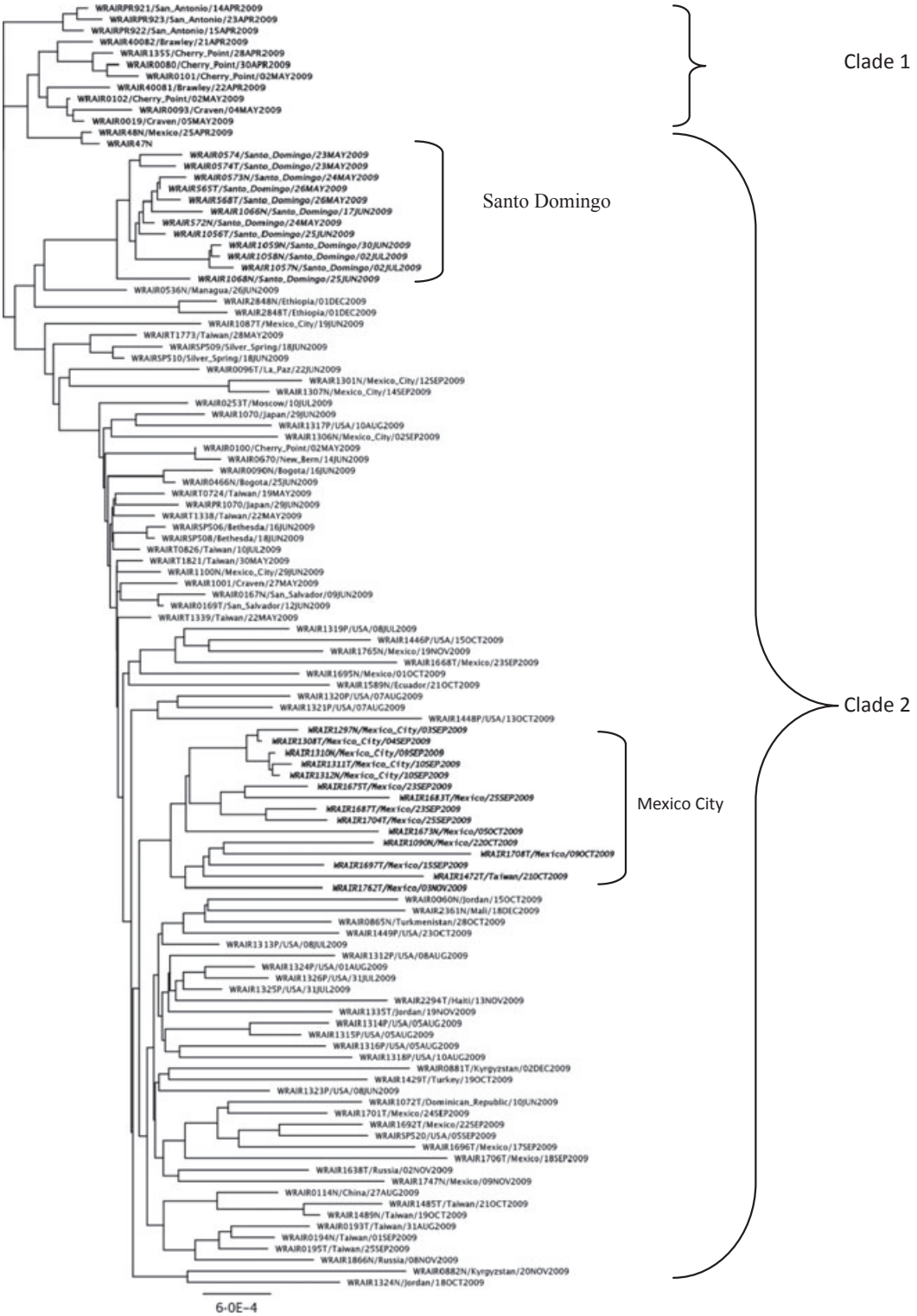

Figure 2. Phylogenetic tree of 114 concatenated full 2009 H1N1 genomes derived from worldwide origins since the beginning of 2009 Pandemics till the end of December, 2009. Scale bar indicates number of nucleotide substitutions per site. Those 2009 H1N1 isolated from Mexico and California during the early phase of pandemics, April through May of 2009 clustered nicely to form a distinctive genetic group, Clade 1 shown as blue labeled group at the top of figure. All the other 2009 H1N1 isolated throughout the world were all belong to the 2nd genetic group, Clade 2. It was also noticed that viral genome sequences of Santo Domingo and Mexico City origins form nice geographic dependent sub-clades.

sequence multiple pooled 2009 H1N1 viral genomes. For each pyrosequencing experiment, approximately 800 000–1 000 000 passed key reads (single fragment per bead) were obtained that yielded readable nucleic acid sequences. Among those close to a million passed key reads, only 500 000–600 000 passed key reads had an average sequencing read length of >220 bps, defined as 'long reads' (220 bps × 500 000 reads = total of 110 million bases of nucleic acid sequences) that were used to assemble into influenza genome sequences. Mathematically, 110–130 million bases of raw sequencing data from each single Roche 454 FLX experiment would provide sufficient sequencing bases to cover 24 full genome sequences with approximate 3–400 × of sequencing depth coverage of influenza A with average genome size of 13 500 bps for the total of eight segmented RNAs. So far, more than 100 full 2009 H1N1 genomes sampled worldwide have been successfully sequenced and deposited in the NCBI database by Division of Viral Diseases, Walter Reed Army Institute of Research (WRAIR).

Preliminary phylogenetic trees of the 2009 H1N1 isolates through the course of 2009 Pandemic infections

The bioinformatics derived from unique viral genome sequences generated from this study based on constant RT-PCR amplification scheme and identical Roche 454 pyrosequencing protocols provide a reliable data set in predicting the evolutionary patterns of pandemic viruses. WRAIR received clinical samples from US Embassies and military personnel throughout the world since the initial WHO announcement of 2009 novel H1N1 outbreaks. Nearly equal distributions of sequenced viruses derived from three broadly categorized geographic regions, North America, Central/South America, and Asia/Europe/Africa (data not shown). Besides the geographic distribution pattern of viral isolates, Figure 1 displays the viral isolation time lines of all the sequenced viruses reflecting two peaks that coincided with two waves of 2009 pandemic infections, early-mid summer and fall of 2009.[15] Phylogenetic trees of the eight influenza A segments of all sequenced viruses were tentatively generated. It was found that the substitution frequencies per site for the HA, NA, and NS genes are at much higher rate than the other five genes, PB2, PB1, PA, NP, and MP genes (data not shown). The observed higher genetic variations for HA and NA genes of 2009 H1N1 are consistent with the historical genomic and epidemiological dynamics data of human influenza A revealing higher temporal fluctuations in HA and NA genes.[16–19] Analysis of full influenza genomes containing concatenated eight complete RNA segments revealed the existence of two distinctive genetic clades in circulation since the beginning of 2009 pandemic, as shown in Figure 2. It is noteworthy that all viruses of Mexico and California origins (Clade 1 shown at the top of Figure 2) were isolated at the beginning of 2009 pandemic prior to the isolation of all other viruses belonging to the second genetic Clade 2.[18,20]

Discussion

During the past decade, the advance of DNA sequencing technology, such as development of NGS, in making full viral genome sequences readily available have enabled study of far broader and more detailed aspects of evolutionary change for any new emergent infectious pathogen. The massive sequencing capacity of Roche 454 FLX system allows simultaneously process and sequence millions of individual cDNA molecules, in contrast to processing and sequencing individual cDNA fragments by conventional Sanger sequencing method. Within a short period of few months since the beginning of the 2009 pandemics, WRAIR accomplished large number of representative 2009 H1N1 full genomes of worldwide origins via Roche 454 FLX system. Sequencing data derived from this study illustrates a much higher genetic variation rate for HA and NA genes of 2009 H1N1 that is compatible to the higher temporal fluctuation rate for HA and NA genes of seasonal Influenza A derived from decades of intensive monitoring and comparison studies and analyses.[16–19] Following the Mexican and US reported cases, confirmed outbreaks of 2009 swine H1N1 rapidly proliferated and spread throughout Europe, Asia, Africa, and South America, most probably via global airline travel.[5,6] It seemed that new cases in the US and most cases throughout the world had been clinically mild relative to the initial reported cases in Mexico.[21–24] Here we demonstrate through the phylogenetic relationship of sequenced 2009 H1N1 full genomes that the clinical isolates could be divided into two different clades of viruses, i.e., the Clade 1 genetic group contains only viruses isolated at the beginning (March/April 2009, Mexico and California) of 2009 pandemics and the rest of other viruses all belong to the 2nd genetic group, Clade 2. Thus, it's likely that the currently circulating 2009 H1N1 of Clade 2 causing worldwide infections is genetically different from the initial 2009 H1N1 isolates that caused the early infections in Mexico and California.[18,20]

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Prediction of the antigenic changes of the pandemic (H1N1) 2009 influenza virus hemagglutinin

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Keywords antigenic change, bioinformatics, molecular evolution, prediction.

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Introduction

A pandemic influenza virus (2009 H1N1) was recently introduced into the human population. The hemagglutinin (HA) gene of 2009 H1N1 is derived from 'classical swine H1N1' virus, which likely shares a common progenitor strain with the human H1N1 virus that caused the pandemic in 1918.[1] Since antigenic changes of influenza virus HA occur more slowly in swine than in humans,[2] we hypothesized that 2009 H1N1 might still retain an antigenic structure similar to that of 1918 H1N1 or the early isolates of its descendants.

In this study, we compared HA antigenic structures of 2009 H1N1 and human H1N1 viruses by a molecular modeling approach to demonstrate the existence of shared epi-

topes for neutralizing antibodies. We found that HAs of 2009 H1N1 and the 1918 H1N1 virus shared a significant number of amino acid residues in known antigenic regions. From this observation, we hypothesize that the 2009 H1N1 HA antigenic sites will be targeted by antibody-mediated selection pressure in humans in the near future. We further discuss possible directions of antigenic changes in the evolutionary process of 2009 H1N1.

Materials and methods

Sequence data of HA genes

Nucleotide sequences for HA genes of A/South Carolina/1/1918 (SC1918) (AF117241), A/Brisbane/59/2007 (BR2007) (CY030230), A/California/04/2009 (CA2009) (FJ966082), A/Puerto Rico/8/1934/Mount Sinai (AF389118), A/Bellamy/1942 (CY009276),A/Albany/4836/1950 (CY021701), A/USSR/90/1977(DQ508897), A/Singapore/6/1986 (CY020477), A/Texas/36/1991 (AY289927), and A/Hong Kong/1035/1998 (AF386777) were obtained from Influenza Virus Resource at the NCBI.

Molecular modeling

MODELLER 9v6 was used for homology modeling of HA structures. After 100 models of the HA trimer were generated, the model was chosen by a combination of the MODELLER objective function value and the discrete optimized protein energy statistical potential score. After addition of hydrogen atoms, the model was refined by energy minimization with the minimization protocols in the Accelrys Discovery Studio 2.1 software package using a CHARMm force field. Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square gradient was less than or equal to 0·01 kcal/mol/A. The generalized Born implicit solvent model was used to model the effects of solvation. The HA model was finally evaluated by using PROCHECK, WHATCHECK, and VERIFY-3D.

Table 1. Amino acid similarity in the HA antigenic sites among recent seasonal H1N1 (BR2007), 2009 H1N1 (CA2009), and 1918 H1N1 (SC1918)

Antigenic sites	No. amino acids involved	No. of amino acids identical to SC1918	
		BR2007	CA2009
Sa	13	8	12
Sb	12	4	10
Ca	19	13	13
Cb	6	2	5

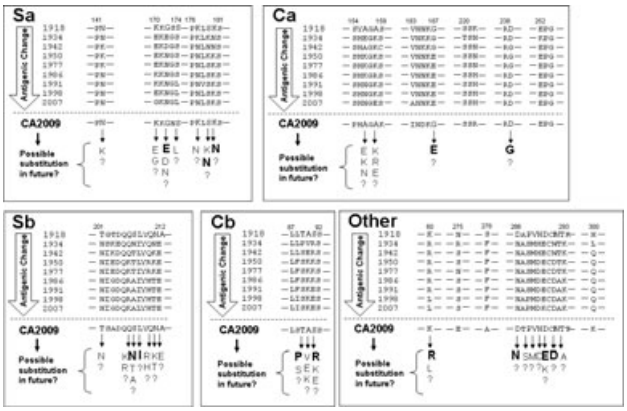

Figure 1. Prediction of the future amino acid substitutions on the antigenic sites of 2009 H1N1 HA. Amino acid residues of 1918 H1N1 (SC1918), its descendant strains, and 2009 H1N1 (CA2009) are shown. Amino acid residues indicated by arrows represent the predicted substitutions which might be associated with antigenic changes of 2009 H1N1 in the near future. The amino acid substitutions which have already been found in the recent variants of the 2009 H1N1 (as of March 24, 2010) are shown in bold letters.

Sequence data analyses for *N*-Glycosylation sites

Custom-made programs were developed with the Ruby language and used for investigating the numbers of potential *N*-glycosylation sites and candidate codons (Cand1) in HA sequences.

Results

It is known that the H1 HA molecules have four distinct antigenic sites: Sa, Sb, Ca, and Cb.[3,4] As a result, these sites consist of the most variable amino acids in the HA molecule of the seasonal human H1N1 viruses that have been subjected to antibody-mediated immune pressure since its emergence in 1918, although it was absent in humans from 1957 to 1976. To investigate the structures of these antigenic sites of 2009 H1N1, 3D structures of the HA molecules of SC1918, the recent seasonal human H1N1 virus (BR2007), and 2009 H1N1 (CA2009) were constructed by a homology modeling approach, and compared by mapping all the amino acid residues that were distinct from those of SC1918 HA (data not shown). We found that most of these antigenic sites of BR2007 HA predominantly contained altered amino acid residues if compared with SC1918. By contrast, amino acid residues at these positions were relatively conserved in CA2009 HA when compared with SC1918 HA. Notably, the Sa and Sb sites, which contain many amino acids involved in neutralizing epitopes near the receptor binding pockets, remain almost intact (Table 1), suggesting that antibodies raised by natural infection with SC1918 or its antigenically related

descendant viruses play a role in specific immunity against CA2009.

These observations lead us to hypothesize that such antigenic sites involving the conserved amino acids will soon be targeted by antibody-mediated selection pressure in the human population. Based on this hypothesis, we speculated that 2009 H1N1 would undergo patterns of amino acid substitutions in HA similar to those seen in seasonal human H1N1 viruses during its epidemic period (i.e. those that have been substituted since 1934) (Figure 1). We then predicted possible amino acid substitutions of 2009 H1N1 from the sequence similarity of the antigenic sites. For example, both SC1918 and CA2009 had an Asn residue at position 142 in the Sa site. For SC1918, the residue at this position has altered from Asn to Lys since 1942. Combining these two facts, it seems reasonable to hypothesize that CA2009 will also undergo an amino acid substitution from Asn to Lys at position 142 in the future. Interestingly, we found that some of the recent variants of the 2009 H1N1 virus have indeed undergone substitutions identical to those predicted in Figure 1. It is important to monitor whether such variants will be selected and survive in sustained circulation in humans.

Next, we analyzed the acquisition of potential *N*-glycosylation sites associated with antigenic changes. Previously, we reported that Cand1 sites, a set of three codons that require single nucleotide substitution to produce *N*-glycosylation sequons, were important motifs to rapidly acquire *N*-glycosylation sequons.[5] Therefore, we investigated the number and location of potential *N*-glycosylation sites and Cand1 sites in 2009 H1N1 HA. We found that CA2009 also had a single *N*-glycosylation sequon at the same position in the globular head region of HA, and lacked the multiple *N*-glycosylations that have been observed in the antigenic changes of the human H1N1 virus during the early epidemic of this virus. We also found that CA2009 HA possessed three Cand1 sites that were present at the same position in SC1918 HA (positions of the first Asn residue, 177, 179, and 184). Of these, the Cand1 sites with positions at 177 and 179 had actually become potential *N*-glycosylation sites in human H1N1 viruses. This result suggests the likelihood of additional *N*-glycosylation at these sites during future antigenic changes of 2009 H1N1 HA. Notably, some of the recent 2009 H1N1 variants (as of March 24, 2010) have an additional *N*-glycosylation sequon at position 179, where the 1918 H1N1 virus readily acquired an *N*-glycosylation site during its circulation.

Conclusions

The present study suggests that the antigenic structure of 2009 H1N1 HA is similar, at least in part, to that of the 1918 H1N1 HA. The 2009 and 1918 H1N1 HAs share unique three-codon motifs that are important to readily acquire *N*-glycosylation sequons in their globular head region. Based on these similarities, we predicted possible amino acid substitutions that might be associated with future antigenic changes of 2009 H1N1, and confirmed that such substitutions occurred in some of the recent variants of this virus. The present study provides an insight into likely future antigenic changes in the evolutionary process of 2009 H1N1 in the human population.

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Evolution of influenza viral neuraminidase (NA) revealed by large-scale sequence analysis

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Keywords : divergence time, influenza, lineage and sub-lineage assignment, molecular phylogeny, neuraminidase.

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Abstract

The influenza virus exhibits complicated evolutionary dynamics due to multiple reasons, such as diverse hosts, high mutation rates, and rapid replications. In this study, large-scale analyses of 11 454 influenza neuraminidase (NA) sequences revealed influenza A and B NA genes diverged first around 2641 years ago, and subsequently the NA subtypes of influenza A emerged around 1125 years ago. All nine NA subtypes of influenza A were genetically distinct from each other, with a total of 23 lineages identified. In addition, five and three sub-lineages were further identified in lineage 1A of NA1 and lineage 2B of NA2, respectively. The majority of lineages and sub-lineages were found to be host or geographic specific. This study provides not only a better understanding of influenza NA evolution, but also a database of lineages and sub-lineages that can be used for early detection of novel genetic changes for improved influenza surveillance.

Introduction

Influenza viruses are classified into three types, A, B, and C, based upon the antigenic properties of nucleoproteins and matrix proteins. Influenza A virus infects a wide range of hosts, including human, bird, swine, equine, and marine mammal species, while influenza B and C are less pathogenic than influenza A and are mainly found in humans, although there is evidence that they can also infect other species. Influenza A has evolved in association with its various hosts on different continents for extended periods of time.[1]

To survive as a successful pathogen, the influenza viruses have developed a number of mechanisms, including antigenic mutation and genome reassortment, to continuously evolve and evade the surveillance of the host immune systems. Antigenic and genetic analyses have provided important insights into the molecular dynamics of influenza virus evolution.[2] However, a comprehensive understanding of influenza viral genetic divergence and diversity remains lacking.

Neuraminidase (NA) is a major surface glycoprotein of influenza A and B, but is absent in influenza C. It plays a key role in virus replication through removing sialic acids from the surface of the host cell and releasing newly formed virions. Influenza A viral NA genes are classified into nine subtypes (NA1–NA9) based upon their antigenic properties, while NA genes of influenza B are not classified into subtypes. Furthermore, most NA subtypes of influenza A have evolved into distinct lineages and sub-lineages, which correspond to specific hosts or geographical locations. In this study, we conducted large-scale analyses of influenza NA sequences in order to infer their evolution and to identify lineages (or sub-lineages) of influenza A viruses.

Materials and methods

A total of 11 454 NA sequences that excluded laboratory recombinant sequences were downloaded from GenBank. Sequences were aligned with MUSCLE and MAFFT. The alignments were adjusted manually using TranslatorX,[3] based upon corresponding protein sequences.

Phylogenetic analyses were conducted using the maximum-likelihood (ML) method in RAxML.[4] A set of Perl scripts were written by us to facilitate this computational analysis. Lineages and sub-lineages were determined based on the topology of the ML trees. Additional information such as hosts, geographical regions, and circulation years were also considered in the classification.

We used the same lineage nomenclature as described in,[5] but with the following modifications: a single digit is used to represent one of the nine subtypes and a letter is used to represent a lineage; a sub-lineage is also represented using a digit; a dot is used to separate a lineage and a sub-lineage. For example, 2A.2 means NA2 subtype, lineage A, and sub-lineage 2.

The time of most recent common ancestor (TMRCA) was estimated using the Bayesian MCMC method in BEAST.[6] In all cases, we employed the GTR + Γ4 nucleotide substitution model, in which the first and second

codon positions are allowed different rates relative to the third codon position. All data sets were analyzed under a relaxed molecular clock and the Bayesian skyline population coalescent prior. The Maximum Clade Credibility (MCC) tree across all plausible trees was computed from the BEAST trees using the TreeAnnotator program, with the first 10% trees removed as burn-in.

Results

Phylogenetic analysis based upon NA sequences revealed two large groups corresponding to influenza A and B, respectively (Figure 1A). Within influenza A, two subgroups were found, one consisting of NA1, NA4, NA5, and NA8 and the other consisting of the remaining five subtypes. Subtype NA9 was found to be a sister subtype of NA6, NA1 being a sister subtype of NA4, and NA8 a sister subtype of NA5. Finally, each NA subtype forms a distinct cluster, indicating its genetic uniqueness.

Influenza A and B viral NA were estimated to have diverged around 2641 years ago (Figure 1B). However, it had large 95% HPD values which ranged from 1259 years to 4299 years ago. The NA subtypes of influenza A diverged from more than 1000 to several hundred years ago. The time of most recent common ancestor (TMRCA) of each subtype of influenza A virus was generally recent and ranged from the calendar years 1767 to 1928 (Figure 1B). In addition, the TMRCA for influenza B viral NA was dated back to 1936.

A total of 23 lineages were identified in influenza A (Table 1). Three lineages, 1A, 1B, and 1C, were identified for NA1 based upon the tree topology. Linage 1A originated from avian viruses and was further divided into 5 sub-lineages: 1A.0, 1A.1, 1A.2, 1A.3, and 1A.4. Linage 1B consists of North American swine influenza viruses whereas 1C is a human lineage. Two large lineages, 2A and 2B, were identified in NA2. Lineage 2A is a human-specific lineage. Interestingly, five major swine clades were observed within this lineage. Lineage 2B is an avian-specific lineage, and consists of 3 sub-lineages, 2B.0, 2B.1, and 2B.2.

Three lineages were found in NA3. Lineage 3A was found in North American avian, 3B in Eurasian/Oceanian avian, and 3C also in avian, but it does not show any geographical pattern. For NA4, NA5, and NA6, each was classified into 2 lineages, one found in North American avian (4A, 5A, 6A) and the other in Eurasian/Oceanian avian (4B, 5B, 6B). Three lineages identified respectively in NA7 and NA8 are North American avian (7A, 8A), equine (7B, 8B), and Eurasian avian (7C, 8C). NA9 was also found to have 3 lineages: North American avian (9A), Eurasian/Oceanian avian I (9B), and Eurassian/Oceanian avian II (9C), respectively.

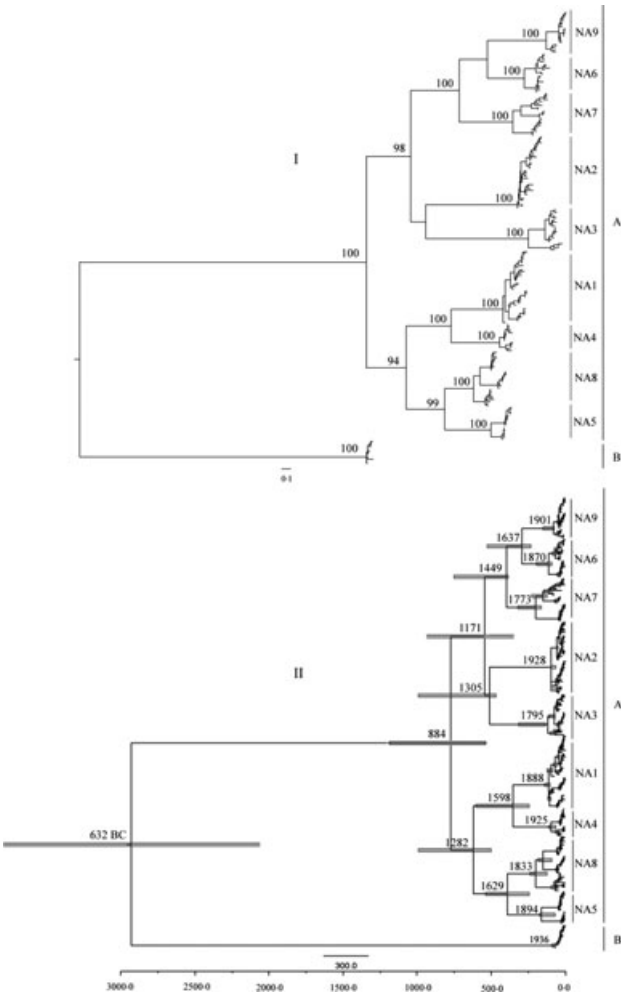

Figure 1. Phylogenetic relationship and divergence times of influenza types and subtypes. (A) The Maximum-Likelihood (ML) tree is scaled according to the numbers of nucleotide substitutions per site, with bootstrap values shown at the major nodes. (B) The Maximum Clade Credibility (MCC) tree is scaled to the number of years before the present. The divergence times (calendar year) of influenza NA are shown on the major nodes, with 95% HPD values shown as node bars.

Discussion

In this study, we conducted large-scale phylogeny and evolutionary analyses using influenza viral NA sequences. The results showed that divergence between influenza A and B viruses occurred earlier than between any influenza A subtypes. This observation was consistent with previous findings based upon phylogenetic analysis of the HA gene, one of the most important genes related to host infection.[7] Within influenza A, two sub-groups were found, one consisting of NA1, 4, 5, and 8 and the other consisting of the rest of five subtypes (NA2, 3, 6, 7, 9). This observation does not agree with the result described by Liu *et al.*,[8] where NA subtypes 1, 3, 4, 5, and 8 formed one group and

Table 1. Lineages and sub-lineages assigned in each influenza A viral NA subtype

Subtype	Lineage/sub-lineage	Annotation	Isolation period
NA1	1A	Major avian	1934–2010
	1A.0	North American avian	1934–2008
	1A.1	Eurasian (avian-like) swine	1979–2010
	1A.2	2009 H1N1 pdm	04/2009–03/2010
	1A.3	Eurasian avian	1979–2007
	1A.4	H5N1 avian	1996–2010
	1B	North American swine	1957–2009
	1C	Major human	1918–2010
NA2	2A	Major human	1957–2009
	2B	Major avian	1965–2009
	2B.0	North American avian	1972–2008
	2B.1	Eurasian avian	1965–2009
	2B.2	H9N2 avian	1994–2009
NA3	3A	North American avian	1971–2008
	3B	Eurasian/Oceanian avian	1961–2009
	3C	Other avian	1980–2002
NA4	4A	North American avian	1968–2008
	4B	Eurasian/Oceanian avian	1979–2006
NA5	5A	North American avian	1976–2008
	5B	Eurasian/Oceanian avian	1972–2009
NA6	6A	North American avian	1976–2008
	6B	Eurasian avian	1976–2007
NA7	7A	North American avian	1977–2008
	7B	Equine	1956–1977
	7C	Eurasian avian	1927–2008
NA8	8A	North American avian	1963–2009
	8B	Equine	1963–2009
	8C	Eurasian avian	1963–2009
NA9	9A	North American avian	1974–2008
	9B	Eurasian/Oceanian avian I	1979–2009
	9C	Eurasian/Oceanian avian II	1978–1999

the remaining four subtypes (NA2, 6, 7, and 9) formed the other group. This difference is apparently caused by the fact that an outgroup was not used in their phylogenetic analyses. In the present study, both influenza A and B viral NA sequences were included in the analysis. High bootstrap values were obtained for major groups, indicating that the inferred evolutionary relationship should be highly reliable.

Classification and designation of the lineages and sub-lineages within the influenza A virus are essential for studies of viral evolution, ecology, and epidemiology. A total of 23 lineages were identified within nine influenza A viral NA subtypes and with the majority of the identified lineages found to be host or geographic specific or both. Our results demonstrated a comprehensive view for the evolution of NA genes and provided a framework for the inference of evolutionary history of pandemic viruses and for further exploring of viral circulations in multiple hosts. For example, the global pandemics of human H1N1 in 1918, H2N2 in 1957, the pandemic of human H3N2 virus in 1968,[9] the crisis of H5N1 HPAI in Hong Kong in 1997,[10] and swine-origin H1N1 influenza in 2009,[11] all can be mapped onto the lineages and sub-lineages identified in this study. Such information will facilitate not only identification of known genetic origins but also early detection of novel influenza A viruses.

Acknowledgements

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Phylogenetic analyses of pandemic influenza viruses

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Keywords novel influenza viruses, oseltamivir resistance, phylogenetic comparisons, sequencing.

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Introduction

Influenza viruses constantly evolve to avoid the human immune pressure in the process of antigenic drift.[1] Through sequencing of viral genomes, the rates and direction of virus evolution can be observed. Moreover, comparison of protein sequences allows us to determine amino acid substitutions that are related to immune pressure and antigenic drift. The creation of global influenza genetic databases, along with concurrent development of analytical tools, allows the comparison of multiple influenza virus strains.[2] The main aim of this study was to perform antigenic and genetic comparison of pandemic influenza viruses (H1N1) isolated during the 2009–2010 pandemic in Ukraine and in other countries.

Materials and methods

Nasopharyngeal swabs and autopsy materials collected from infected patients were received from the areas of Ukraine. In addition, field isolates of influenza viruses from the 2009/10 season and strain specific serum were used for identification by hemagglutinin inhibition assay. Influenza viruses were identified and subtyped using real-time RT-PCR analyses using CDC primers and adopted protocols.[3] Sequencing was performed in two World Health Organization (WHO) Influenza Collaboration Centers (Centers for Disease Control and Prevention, Atlanta and National Institute for Medical Research, London). Hemagglutinin inhibition assay was conducted using chicken and guinea pig red blood cells following standard WHO protocols.[4]

The isolates A/Rivne/214/2009, A/Cherkassy/346/2009, A/Dnipro/267/2009, A/Dnipro/268/2009, A/Kiev/n1/2009, A/Lviv/411/2009, reference-viruses A/California/7/09, A/Lviv/n6/09, and their serum were taken for antigen characterization of field isolates.

Phylogenetic analysis was conducted in the computer program MEGA 4 program using Neighbor Joining with a Kimura model and statistical support calculated with 1000 bootstrap replicate.[5] Nucleotide sequences of HA and NA genes of Ukrainian isolates A/Ternopil/n11/2009, A/Ternopil/n10/2009, A/Lviv/n2/2009, A/Lviv/n6/2009, A/Kiev/n1/2009 were included in analysis along with segments of viruses from other countries available from Genbank (http://www.ncbi.nlm.nih.gov/Genbank).

Results

All 6 Ukrainian isolates of influenza viruses, which were isolated in Ukraine during August–November 2009, were identified as A/California/7/2009(H1N1)-like strains (Figure 1). The phylogenetic comparison of HA and NA genes of the Ukrainian pandemic influenza viruses and corresponding segments of viruses from other countries confirmed that one virus A/Kiev/N1/2009, isolated in mid August 2009, was genetically most similar to isolates from European countries, USA, and Australia. The other viruses (A/Lviv/N2/2009, A/Lviv/N6/2009, A/Ternopil/N10/

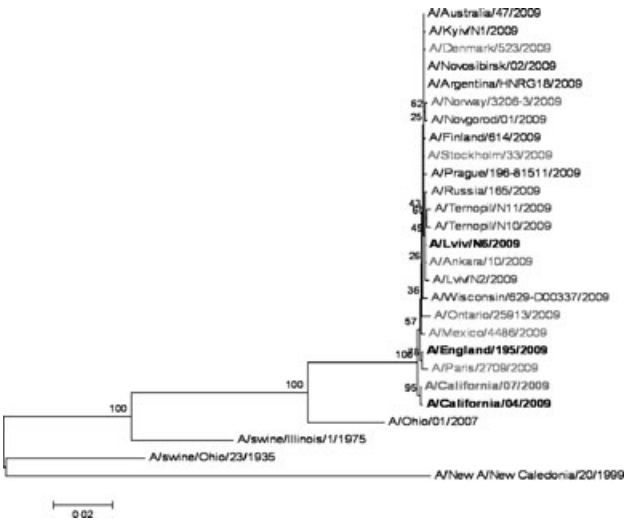

Figure 1. Phylogenetic tree of HA segments of viruses from Ukraine and other countries.

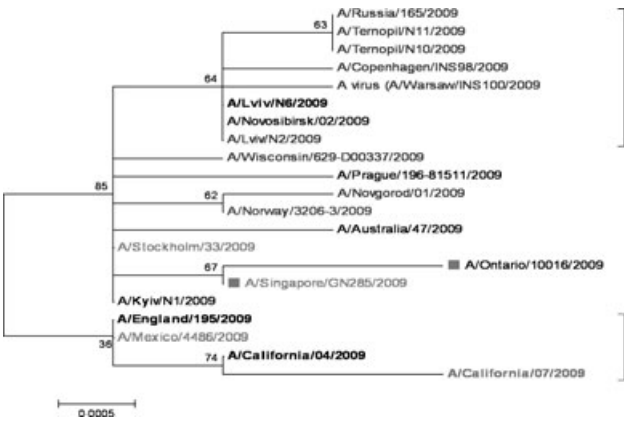

Figure 2. Phylogenetic tree of HA segments of A(H1N1)2009 pandemic viruses.

2009, and A/Ternopil/N11/2009) isolated from samples collected in October–November 2009 demonstrated higher similarity with viruses from Russia and Turkey. All Ukrainian isolates included in this study have mutation D222G in the HA gene, but do not have mutation H275Y in the NA gene, indicating they are susceptible to oseltamivir.[6]

Discussion

The phylogenetic analyses confirmed the evolutionary relationship between Ukrainian isolates and viruses from other countries, which were isolated during the first wave of the pandemic. High genetic and antigenic conservation of pandemic influenza viruses from Ukraine and other countries also were demonstrated. Considering that the emergence of the novel pandemic influenza strain occurred in countries of Northern Hemisphere during summer, it was very interesting and significant tracking the dynamics of genetic changes in influenza viruses, which were isolated at the beginning of epidemic and those isolated during the rise of the epidemic in Ukraine.

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Large-scale sequence analysis of influenza A hemagglutinin (HA): insights into a unified system for lineage assignment

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Keywords hemagglutinin, lineage and sub-lineage assignment, model-based clustering, molecular phylogeny.

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Abstract

Although phylogenetic approaches are commonly used and often found to be powerful, how to accurately identify lineages or sub-lineages of a gene segment of the influenza A virus remains a challenging issue. In this study, we address this issue by analyzing hemagglutinin (HA) sequences using a combination of statistical and phylogenetic methods. Following a hierarchical nomenclature system that uses a letter to represent a lineage and a digit for a sub-lineage, we identified 41 distinct lineages and 81 sub-lineages in all 16 HA subtypes through large-scale analyses of 11 821 influenza A hemagglutinin sequences. The majority of the lineages or sub-lineages were host or geographic specific or both. Further analysis of other segments will allow us to construct a comprehensive database for influenza A lineages and genotypes, facilitating early detection of new viral strains and genome reassortments and hence improve influenza surveillance.

Introduction

Influenza A virus causes moderate to severe epidemics annually and catastrophic pandemics sporadically. Due to the evasiveness of the influenza virus and the nature of its genome (eight single-stranded and negative-sense RNA segments), it is essential to understand the evolution of this important pathogen.[1] Influenza virus evolves by two major mechanisms: mutation and reassortment. Antigenic and genetic analyses have revealed partially the molecular dynamics of influenza virus evolution.[2,3] However, important questions, such as how many genotypes in the influenza A virus, remain unanswered. One of the major issues pertaining to this genotyping problem is how many lineages or sub-lineages can be determined for a subtype and according to what criteria.

Because of the unique structure of the influenza A viral genome, the computational genotyping methods developed for other viruses cannot be applied to the influenza virus. Constructing phylogenetic trees is a powerful technique for the identification of evolutionary groupings (i.e., lineages/clades). However, for large trees, it is hard to determine how many lineages and the boundaries for each lineage. In this regard, multivariate analysis methods, such as multidimensional scaling (MDS) and model-based hierarchical clustering, both taking advantage of dimension reduction and visualization, can complement conventional phylogenetic methods.[4]

Hemagglutinin (HA), the fastest evolving segment, is recognized as the most important gene in the influenza virus that plays a key role in viral pathogenesis. However, we have only limited knowledge of lineages and sub-lineages occurring in the hemagglutinin (HA) gene of influenza A virus,[5] although much effort has been made in assigning clades or sub-clades in highly pathogenic avian influenza (HPAI) virus HA.[6] In this study, both model-based hierarchical clustering and phylogenetic methods were used for sequence analysis. One objective for this study is to explore and develop a more accurate lineage approach for further comprehensive influenza lineage and genotype analyses.

Materials and methods

A total of 11 821 hemagglutinin (HA) sequences (approximately 1778 nucleotides long), excluding laboratory recombinant sequences, were downloaded from GenBank as of March, 2010.[7] Sequences were aligned with MUSCLE [8] and MAFFT.[9] The genetic distance matrix of all pairwise sequences was computed using the K2P model under MEGA 5.0. We then used the distance matrix as input to the cmdscale module in R 2.5.1 for the MDS analysis. The principle coordinates resulting from MDS were used for the model-based hierarchical clustering analysis, again in R 2.5.1 (The R Foundation. Available at: http://www.r-project.org/). The Bayesian Information Criterion (BIC) values were computed based upon ten different statistical data

models – EII, VII, EEI, EVI, VEI, VVI, EEE, EEV, VEV, and VVV. The highest BIC value was used to determine the number of clusters in the given sequence data.

Phylogenetic analysis was conducted using maximum-likelihood (ML) in RAxML.[10] RAxML uses rapid algorithms for bootstrap and maximum likelihood searches and is considered one of the fastest and most accurate phylogeny programs for large-scale sequence analysis. All the analyses were conducted on the Supercomputer cluster (Holland Computing Center, http://hcc.unl.edu/main/index.php). The trees were visualized in FigTree (version 1.3.1).

Lineages and sub-lineages were determined based on both the topology of the ML trees and model-based clustering results. Additional information such as hosts, geographical regions, and circulation years were also considered in the classification.

We used the same lineage nomenclature as described in,[11] with the following modifications: lineage analysis was conducted for each HA subtype, which agrees with the convention of influenza virologists that 16 HA subtypes were identified in influenza A virus; HA lineages are represented with digits and letters, where the digit(s) represent one of the 16 subtypes and a letter represents a lineage; here, we present sub-lineages or sub-sub-lineages also in digits, with smaller numbers representing earlier lineages or sub-lineages within the same subtype (e.g., lineage 1 occurs earlier than lineage 2); the digit 0 is used to indicate inclusion of ancestral viruses in a lineage (or sub-lineage); a dot is used to separate lineages, sub-lineages, and sub-sub-lineages. For example, 1A.1·2 means HA1 subtype, lineage A, and sub-lineage 1, and sub-sub-lineage 2. The sub-lineage level can be extended as necessary.

Results

The model-based clustering method corroborates commonly used phylogenetic methods in lineage and sub-lineage assignment. Here we use the H12 subtype as an example to show the lineage and sub-lineage assignment. The Bayesian Information Criterion (BIC) reaches its maximum when the number of clusters for H12 equals 3, regardless of which mode we choose (Figure 1A). Therefore, based on BIC, the optimal number of clusters for the H12 subtype is 3. As a result, a total of 3 clusters based upon the VVV model were identified (Figure 1B). A significant correlation was found in lineage assignments by the phylogenetic method and the model-based hierarchical clustering method (Figure 1B,C). Lineages 12A and 12B were identified for H12, which correspond to North American avian and Eurasian avian, respectively. Lineage 12A was further divided into 2 sub-lineages, 12A.0, 12A.1, where 12A.0 is the ancestral sub-lineage in 12A.

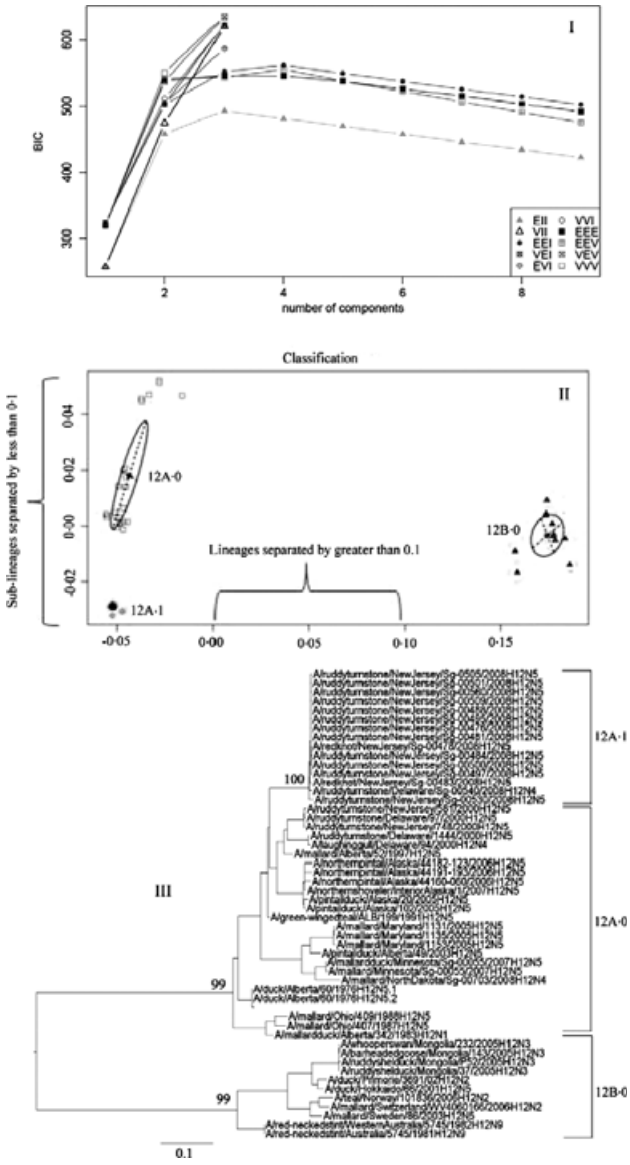

Figure 1. Lineage and sub-lineage assignment for HA12 using both statistical and phylogenetic methods. (A) the Bayesian Information Criterion (BIC) versus the number of clusters; (B) model-based hierarchical clustering results; (C) phylogenetic analysis for lineage and sub-lineage assignment.

Based on both model-based hierarchical clustering and phylogenetic analyses, a total of 41 distinct lineages were identified among 16 subtypes, averaging out to be 2·56 lineages per subtype (Table 1). The majority of the identified lineages were found to be host or geographic specific or both. For example, three lineages, 1A, 1B, and 1C, were identified for HA1. Lineage 1A was further divided into two sub-lineages, 1A.0 and 1A.1. The 1A.0 is swine-specific, whereas 1A.1 is a human pandemic 2009 H1N1 sub-line-

Table 1. Hierarchical lineage (1st level) and sub-lineage (2nd level) assignments for influenza A HA segment

Subtype	1st Level	2nd Level	Subtype	1st Level	2nd Level	Subtype	1st Level	2nd Level	Subtype	1st Level	2nd Level
HA1	1A	1A.0	HA4	4A	4A.0	HA7	7A	7A.0	HA11	11A	11A.0
		1A.1			4A.1			7A.1			11A.1
	1B	1B.0			4A.2			7A.2			11A.2
		1B.1		4B	4B.0		7B	7B.0		11B	11B.0
	1C	1C.0	HA5	5A	5A.0		7C	7C.0			11B.1
HA2	2A	2A.0			5A.1			7C.1			11B.2
		2A.1			5A.2			7C.2	HA12	12A	12A.0
	2B	2B.0		5B	5B.0	HA8	8A	8A.0			12A.1
	2C	2C.0			5B.1			8A.1		12B	12B.0
HA3	3A	3A.0			5B.2		8B	8B.0	HA13	13A	13A.0
		3A.1		5C	5C.0	HA9	9A	9A.0		13B	13B.0
	3B	3B.0			5C.1			9A.1		13C	13C.0
		3B.1			5C.2		9B	9B.0	HA14	14A	14A.0
	3C	3C.0			5C.3			9B.1	HA15	15A	15A.0
	3D	3D.0			5C.4			9B.2	HA16	16A	16A.0
		3D.1	HA6	6A	6A.0		9C	9C.0			16A.1
	3E	3E.0			6A.1			9C.1		16B	16B.0
		3E.1			6A.2			9C.2		16C	16C.0
		3E.2		6B	6B.0	HA10	10A	10A.0			
		3E.3			6B.1			10A.1			
		3E.4		6C	6C.0		10B	10B.0			

age. Lineage 1B is human-specific, with 2 sub-lineages. Sub-lineage 1B.0 includes isolates from 1939 to 2006; 1B.1 includes isolates from 2006 to 2009. Lineage 1C includes both swine and bird influenza viruses. An estimation of genetic distances within and among lineages revealed that the average p-distance was normally <0·1 within a lineage and >0·1 between lineages.

Discussion

How to accurately identify an evolutionary lineage of influenza A viruses is challenging. One commonly used approach is molecular phylogeny, where phylogenetic trees are constructed, and the tree topology is used for lineage determination. Here, we used a Bayesian model-based clustering method, along with phylogenetic methods, to decide lineages and sub-lineages of influenza A viruses based upon sequence data. The results demonstrated that the model-based clustering method corroborates phylogenetic methods and increases the accuracy of lineage assignment.

One salient feature of this study is its large-scale analysis of all available influenza A hemagglutinin sequences. A total of 41 distinct lineages and 81 sub-lineages were classified; the majority of them were found to be host or geographic specific. This observation agrees largely with previous findings.[5] We are conducting further analyses of other influenza A segments [12] and expect to identify their lineages and create a comprehensive genotypes database for all influenza A viruses. Such information will allow us to detect the genetic origin of newly found viruses, track their genetic changes, and identify potential genome reassortments.

A hierarchical nomenclature system has been proposed and adopted for HPAI HA clades and sub-clades by WHO Influenza Surveillance Centers.[6] Wan *et al.* also proposed a hierarchical approach for influenza A viral genotypes system.[13] The work presented here is one of the first steps towards the development of a nomenclature system for influenza A virus lineages (at the segment level) and genotypes (at the genome level). Whether the naming system will be accepted and used by the influenza research community is more challenging than the lineage analysis itself.

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IPMiner: a progenitor gene identifier for influenza A virus

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Abstract

Identification of the genetic origin of influenza A viruses will facilitate understanding of the genomic dynamics, evolutionary pathway, and viral fitness of influenza A viruses. The exponential increases of influenza sequences have expanded the coverage of influenza genetic pool, thus potentially reducing the biases for influenza progenitor identification. However, these large amounts of data generate a great challenge in progenitor identification. To increase computational efficiency, IPMiner is developed by integrating complete composition vector for genetic distance calculation and minimum spanning tree algorithm for progenitor identification. IPMiner is available to at http://sysbio.cvm.msstate.edu/IPMiner.

Introduction

Influenza A virus is a negative-stranded RNA virus that belongs to the *Orthomyxoviridae* family. Influenza A virus has eight genomic segments (segment 1–8) with varying lengths from about 890 to 2341 nucleotides. The subtypes of influenza A viruses are named by combining the serotypes of their surface protein hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA (H1 through H16) and 9 NA (N1 through N9) serotypes have been identified. Influenza A virus causes zoonotic diseases in various hosts, such as human, pig, bird, horse, seal, whale, and dog. As a segmented, negative-stranded RNA virus, influenza A virus is characterized by its rapid mutation and frequent reassortment. A reassortment event refers to the exchange of gene segments between co-infected influenza viruses, and it has facilitated the emergence of 1957 H2N2, 1968 H3N2, and the 2009 H1N1 pandemic strains.[1,2] Identification of the genetic origins of influenza A viruses will enhance our understanding the evolution and adaptation mechanisms of influenza viruses.

The phylogenetic analysis is the traditional approach to identify the influenza progenitor. First, the nucleotide sequences are aligned using multiple sequence alignment methods, such as ClustalW,[3] MUSCLE,[4] and T-COFFEE.[5] Second, phylogenetic analysis is performed on these aligned sequences to infer their evolutionary relationship using Neighbor-Joining (NJ),[6] Maximum Parsimony,[7] Maximum

Likelihood, or Bayesian inference.[8] Bootstrap analyses or computation of posterior probability are usually applied to estimate the phylogenetic uncertainty. However, this phylogenetic analysis is time consuming due to intensive computations in multiple sequence alignments and phylogenetic inferences. It is difficult to perform an analysis using this method on a large dataset, for instance, with more than 1000 taxa, as is the common case for influenza studies.

Alternatively, BLAST [9] is applied to identify the prototype genes in the database. BLAST determines a similarity by identifying initial short matches and starting local alignments. Since influenza viral sequences have very high similarities, especially for most conserved regions, BLAST usually generates a large number of outputs, which will not be helpful for progenitor identification. Since BLAST is a local sequence alignment, the results from BLAST may not reflect the global evolutionary information between the sequences. The BLAST scores cannot be used to define the evolutionary relations between viruses, especially in the context of the entire genetic pool.

Recently, we have developed a distance measurement method, complete composition vector (CCV), that can calculate genetic distance between influenza A viruses without performing multiple sequence alignments.[10,11] We also adapted the minimum spanning tree (MST) clustering algorithm for influenza reassortment identification.[12] The application of this approach in the analyses of PB2 genes of influenza A virus showed that the integration of CCV and MST allows us to identify the potential progenitor genes rapidly and effectively. Based on these results, here we develop a webserver called IPMiner for influenza progenitor identification. IPMiner can identify potential progenitors for a query sequence against all public influenza datasets within a few minutes.

Precomputed data matrices

In order to improve the computing efficiency, 31 distance matrices were pre-computed by CCV, and they include 16 for HA (H1 to 16), 9 for NA (N1 to N9), and one for each of the internal gene segments (PB2, PB1, PA, NP, NS, and MP). These 31 pre-computed matrices will be updated weekly. IPMiner just needs to compute the query matrices for a query sequence and sequences in the database. The standalone CCV program is also available at http://sysbio.cvm.msstate.edu/IPMiner.

Identification and visualization of influenza progenitor genes

In order to identify the influenza progenitor genes, IPMiner first integrates the query matrix and a corresponding pre-computed matrix into a full distance matrix, which is then clustered by MST clustering algorithm. We adapted the threshold we measured previously in MST, $u + n\sigma$, where u is the average distance and σ is the standard deviation of a cluster.[12] As a result, MST will generate a hierarchical structure for the clusters. In each cluster, we will randomly select 20 viruses or 10% of the cluster size if this cluster has more than 200 viruses. IPMiner will return the viruses with the smallest distances when the search reaches to the lowest level (the largest n) in this hierarchical structure. Our analyses have shown that the level 5 has generally yielded good results for influenza A viruses.

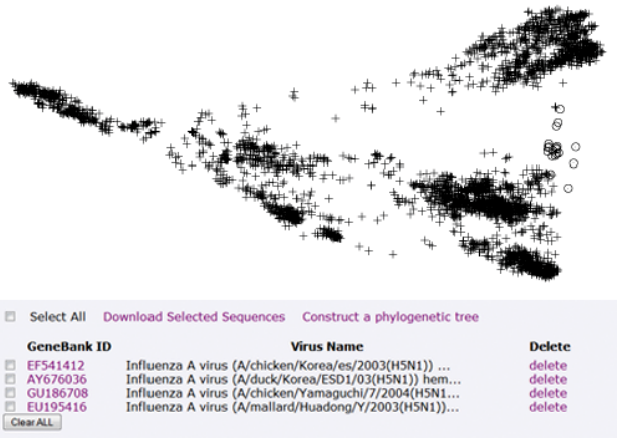

Figure 1. Visualization of the identified progenitor viruses in the influenza genetic pool. The progenitor viruses are displayed in circles.

To visualize the overall MST structure, IPMiner applies multi-dimensional scaling (MDS) method to project all the viruses in the genetic pool onto a two dimensional graph, and the precursor viruses are marked in different shapes (Figure 1). The users can select other prototype viruses from the graph for further phylogenetic analyses.

A single job with one query sequence takes <2 min. The GenBank identifiers and associated genetic distances and sequence identities are displayed. The users can download the sequences for the identified precursor viruses as well as those from the prototypes viruses. In addition, for the users' convenience, IPMiner generates a phylogenetic tree using NJ method implemented in PHYLIP [13] to illustrate the phylogenetic relationship among the query sequence(s), the identified progenitors, and the selected prototypes viruses.

Implementation and availability

The programs in this solution package are written in Java. The shell scripts are written in korn shell script in order to achieve high performance. Cascading style sheets (CSS) are

used for a consistent look across the pages. This also enables to change the overall design just by replacing the CSS definition file. PHP has been used as server side scripting and is written in Java. In order to achieve high performance for computing in a genomic scale, we apply hash function or a binary tree, which enables that the precursor identification has a time complexity of $O(n)$. For single queries, the users can visualize the results online. For batch queries of multiple sequences, the results will be sent to the users by e-mail.

IPMiner has been tested on Microsoft Internet Explorer, Mozilla Firefox, and Safari. The users need JavaScript to obtain full function of IPMiner server. The webserver is available at http://sysbio.cvm.msstate.edu/IPMiner.

Conclusions

In summary, IPMiner webserver has three major computational features for influenza progenitor identification: (i) it calculates the genetic distances through CCV and identifies the viruses with the shortest CCV distances against the query virus to be the progenitor genes; (ii) it projects influenza viruses onto a two dimensional map, which illustrates the global relationship between the progenitor genes and other viruses in the genetic pool; and (iii) it performs phylogenetic analyses between the query virus, the identified progenitor genes, and other selected prototype viruses. IPMiner provides a user friendly web service for influenza progenitor identification in real time.

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Pandemic influenza 2009 in Russia: isolation, antigenic analysis, and biological properties of viruses

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Abstract

During the 2009–2010 epidemic season in Russia, we isolated 417 strains of pandemic A(H1N1)v influenza, which comprised 92% of all strains isolated during the last season. All Russian pandemic strains were antigenically homogenous: they reacted with the WHO diagnostic antiserum as well as with antisera to the reference strain A/California/07/09 and A/St. Petersburg/56/09 up to 1–1/4 of homologous titer. The high degree of homology was also revealed concerning the ancient H1N1 strains of swine origin – A/New Jersey/8/76, A/Swine/1976/31 and A/Iowa/15/30/. Pandemic isolates reacted equally well with human and chicken red blood cells. A part of Russian A(H1N1)v strains acquired a capability to react with high titers with the thermo-inactivated equine serum, while all strains of swine origin isolated earlier as well as reference strain A/California/07/09 did not possess such a property. This feature is, for the moment, the most obvious differentiating index of the strains H1N1v isolated in Russia. Concluding, we did not find any significant antigenic drift of virus during the first pandemic wave on the territory of Russian Federation. Concerning its biological properties, the pandemic virus 2009 could be classified as moderate pathogenic, sensitive to oseltamivir, but resistant to adamantanes. In the end of epidemic season some influenza B viruses were isolated. During the season 2009–2010 neither epidemic A(H1N1) nor A(H3N2) viruses were isolated in our laboratory.

Introduction

The 2009 pandemic events in Russia started in the last week of September 2009 beginning from the Far East region (city Yuzhno-Sakhalinsk). During October to the first weeks of November, the epidemic had spread from the Far East through the Siberian region to the whole territory of Russia. In Moscow and St. Petersburg, the first laboratory confirmed cases of pandemic influenza A(H1N1)v were registered in May 2009. At the epidemic peak, the frequency of pandemic influenza in those cities confirmed by the PCR-analysis had reached 49% of examined patients.[1] The first Russian strain of the virus A(H1N1)v was isolated in the end of May 2009 from a patient who arrived from the United States.[2] The unique combination of the genomic segments of pandemic virus has led to the appearance of a principal new phenotype. The later has affected the clinical picture of disease caused by this pathogen, as well as its biological properties.

Materials and methods

Clinical (nasopharyngeal swabs) and post-mortem materials (fragments of trachea, bronchi, lungs, spleen) were obtained from clinics and/or out-patients from St. Petersburg and from 19 Base Virological Laboratories (BVLs) of the Research Institute of Influenza in different regions of the country, which cover approximately 3/4 of the territory of Russia. The informed consent for the bio-materials collection and studies was obtained from research subjects or from their relatives in cases of post-mortem materials.

Isolation of viruses was carried out in the MDCK cell culture (CDC, Atlanta, GA, USA) and in 10-day-old chicken embryos (E). Isolation was done according the standard internationally accepted methods.[3] The reaction of hemagglutination (HA) and the inhibition of hemagglutination (HAI) were performed according the WHO recommended standard method.[3]

For the identification of epidemic isolates, we used the hyperimmune diagnostic bovine or ovine antisera annually obtained from the WHO reference Center (CDC). For a detailed antigenic analysis we used the hyperimmune rat antisera against epidemic and reference influenza strains

produced in our laboratory. Normal equine serum was purchased from Sigma (USA).

Results

During the period from July 20, 2009 up to April 30, 2010, we have obtained 772 swabs from clinics and out-patients in St. Petersburg and 374 swabs from the BVLs. In this period, rather high incidence of lethality from pneumonia was observed, which developed on the background of the pandemic flu H1N1v. Thus, we received from BVLs 173 post-mortem materials from 91 deceased patients which manifested PCR+ influenza H1N1v-specific RNA. All materials were tested for a possibility of isolation of influenza virus H1N1v both in eggs and in MDCK cells. PCR-negative materials were discarded. We isolated 229 strains of pandemic influenza from the materials collected in St. Petersburg and region, which comprised 29·7% of the total number of analyzed samples. At the same time, we did not isolate any other sub-types of influenza in the season 2009–2010 except the pandemic flu. From the swabs purchased from BVLs, 47 strains were isolated, which compose 37·9% of the PCR+ samples, and 35 strains from the post-mortem materials (43·2% of the PCR+ samples).

Altogether in the season 2009–2010, we isolated, retrieved, and analyzed in HAI 453 influenza strains. 91·2% of them were pandemic strains A(H1N1)v, and only 7·9% influenza B viruses. These data together with the epidemiologic data and the results of PCR-diagnostic provide evidence in favor of nearly mono-etiological character of epidemic season 2009–2010 in Russia for pandemic influenza A(H1N1)v.

Though the isolation of pandemic viruses was fulfilled in two traditional model systems, in the case of pandemic 2009 virus, we could observe the tendency of preferential multiplication in embryos compared to MDCK, especially in cases of post-mortem material for which chicken embryos are the preferential system of isolation.

H1N1v viruses, which were isolated and passaged in MDCK, even with significant HA titers, quickly lost their HA activity provided they were kept at +4°C. Moreover, some other tested cell lines proved to be practically non-sensitive to the pandemic viruses H1N1v.

Antigenic and biological properties of the virus strains of pandemic influenza H1N1v

We used HAI reaction for the typing and antigenic characterization of isolated viruses. In the course of isolation of viruses in the reported period, we produced rat polyclonal antisera to the strains A/California/07/09 and A/St. Petersburg/56/09 (H1N1)v and the antisera to the strains A/New Jersey/8/76 – the virus isolated during the epidemic 1976 in the United States and also of the swine origin – and to the 'swine' strains A/SW/1976/31 and A/Iowa/15/30. The HAI results of representative strains are given in Figure 1.

№	Influenza viruses A(H1N1)v	Dign.serum A(H1N1)v, CDC Lot. 58845141	Rat antisera to influenza viruses					Normal equine serum, Serial N. 40 56° 30 min	Normal equine serum, Serial N. 40 80° 60 min
			A/SW/1976/31	A/Iowa/15/30	A/New Jersey/8/76	A/California/7/09	A/SPb/56/09		
1	**A/SW/1976/31**	5000	**640**	1280	80	20	20	< 20	< 20
2	**A/Iowa/15/30**	5000	640	**1280**	80	20	20	< 20	< 20
3	**A/New Jersey/8/76**	5000	320	320	**640**	80	80	< 20	< 20
4	**A/California/7/09**	5000	160	160	320	**1280**	640	< 20	< 20
5	**A/St.Petersburg/56/09**	5000	160	320	320	640	**1280**	> 1280	> 1280
6	A/St.Petersburg/44/09	2560	80	80	80	320	320	< 20	< 20
7	A/St.Petersburg/60/09	1280	80	80	80	320	320	< 20	< 20
8	A/St.Petersburg/69/09	2560	160	160	160	640	1280	640	> 1280
9	A/St.Petersburg/130/09	5000	160	320	320	1280	1280	1280	> 1280
10	A/St.Petersburg/8/10	2560	160	160	160	640	1280	1280	> 1280
11	A/St.Petersburg/21/10	2560	160	80	160	640	640	640	> 1280
12	A/St.Petersburg/22/10	2560	80	80	160	320	640	640	> 1280
13	A/Belgorod/3/09	640	80	80	80	320	320	1280	> 1280
14	A/Belgorod/6/09	1280	80	80	80	160	320	< 20	< 20
15	A/Petrozavodsk/2/09	2560	80	80	80	320	320	< 20	< 20
16	A/Vologda/6/09	2560	80	80	80	320	320	< 20	< 20
17	A/Astrakhan/60/09	1280	80	80	80	160	320	< 20	< 20

Figure 1. Antigenic structure of influenza viruses A(H1N1)v, isolated in Russia in the epidemic season 2009–2010 according to the inhibition of hemagglutination (HAI) method.

Table 1 shows that the isolated strains were homogenous in their antigenic properties and interacted with the diagnostic antiserum CDC for A(H1N1)v and also with the antisera to the strains A/California/07/09 and A/St. Petersburg/56/09 up to 1–1/4 homologous titer. Viruses that were isolated from post-mortem materials did not differ by their antigenic characteristics from those isolated from swabs of live patients. Only two strains could be attributed to the drift-variants of the strain A/California/07/09 because they reacted with the appropriate antiserum up to 1/8 homologous titer; these strains were A/Pskov/1/09 and A/Belgorod/6/09. It is interesting that the isolated strains reacted with the antisera to the strains A/New Jersey/8/76 and A/SW/1976/31 to 1/4–1/8, and some particular strains even to 1/2 homologous titer. It is even more interesting that some pandemic isolates reacted with the antiserum to the strain Iowa isolated in 1930 up to 1/4–1/8 homologous titer. Despite of the fact that since the outbreak of 'swine flu' in the USA in New Jersey 30 years had gone (and for the strain Iowa this period is nearly 70 years) the HA of these viruses and of the pandemic influenza 2009 share some common antigenic determinants as was shown in HAI.

One more interesting feature of a considerable part of isolated strains is their capability to react with high titers with normal equine serum heated to 56 and to 80°C, while all the strains of swine origin isolated earlier were inhibitor-resistant (Figure 1). Russian isolates of 2009 divided, in this respect, in two clear and approximately equal in number groups: one of them is similar to the reference strain

A/California/07/09 and did not react with the equine serum, while the other, similar to the strain A/St. Petersburg/56/09, reacted with high titers (to 1:1280 and higher); this group is marked by color in Table 1. At the present time this property proved to be the most significant differentiating indicator of Russian isolates of pandemic influenza A(H1N1)v.

Discussion

Comparative molecular-genetic analysis of predicted amino acid sequences of the HA of pandemic strains 2009 isolated in the Research Institute of Influenza, which was fulfilled in the laboratory of molecular virology and gene engineering, has shown the genetic homogeneity of strains A(H1N1)v relative to the reference strain A/California/07/09 (similarity of the HA sequence 98·9%).[4] Molecular-genetic analysis of the HA sequence of the strains A/California/07/09 and A/New Jersey/8/76 has revealed the degree of similarity between these two strains 90·2%.

Comparing to the reference strain of epidemic A(H1N1) virus of the previous epidemic season 2008–2009 A/Brisbane/59/2007, the pandemic H1N1v virus HA has 103 amino acid substitutions, among them more than 30 were disclosed in antigenic sites, so the degree of similarity to this strain is 78%. A new site of glycosylation was also discovered in the position 276 of HA.[4] Essential distinctions of the aminoacid sequence of HA and antigenic properties of the H1N1v strains as compared with actual circulating and vaccine strains is one of the factors that determine the pandemic potential of this new influenza virus.

According to the literature, the mutation in the HA gene D222G could cause a broadening of the spectrum of receptor specificity of influenza virus by the acquisition of the capacity to bind both the residues $\alpha(2 \rightarrow 6)$ and $\alpha(2 \rightarrow 3)$ of the sialic acid of cellular receptors.[5] Both types of receptors are present at the human respiratory tract, but in different parts of it, and they exist in different proportions.[6] According to the data of the European Center of Disease Control and Prevention (ECDC), the varieties G222 of the H1N1v virus were isolated in 20 countries from subjects deceased of influenza or who suffered a severe form of illness, as well as from those who sustained only a light course of influenza.[7] Concerning the strains isolated in RII, this mutation was discovered in nine cases: four were isolated from live patients and five from post-mortem materials. Thus, there are no convincing data at present that could prove a causal relationship of the given substitution and the aggravation of a disease course. This is in accordance with previous observations.[7]

Concerning the resistance of studied strains to the widely used antiviral preparations, it was shown that all tested strains possessed the substitution S31N in the M2 protein that determine the resistance to adamantanes. There was no substitution in the position 275 of neuraminidase (NA), which determines the resistance to oseltamivir (H275Y). These substitutions are the characteristic indices of the Eurasian lineage of swine influenza viruses.[8] Thus all studied Russian H1N1v isolates were resistant to adamantanes (rimantadine) and sensitive to oseltamivir.

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GISAID – a global initiative on sharing all influenza data

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Background

The GISAID initiative offers an alternative to current public-domain database models in response to growing needs of the global influenza community for the sharing of genetic sequence and associated epidemiological and clinical data of all influenza strains. GISAID's publicly accessible EpiFlu™ database is governed by a unique sharing mechanism that protects the rights of the submitter, while permitting ongoing research as well as the development of medical interventions, such as drugs and vaccines.

Materials and methods

For the GISAID Initiative, the Max Planck Institute for Informatics (MPII) Saarbrücken, Germany, has developed a web portal that is accessible at http://www.gisaid.org featuring the GISAID EpiFlu™ database that offers a unique collection of nucleotide sequence and other relevant data on influenza viruses. The database is based on software by Oracle and the dante® System by a3systems GmbH, Germany. Extensive metadata are also collected for most isolates. The database provides features for searching, filtering specific datasets for download, and user friendly upload functionality. To uphold GISAID's unique sharing mechanism, all users must positively identify themselves. While access is free of charge, all users agree that they will not attach any restrictions on the data, but will acknowledge both the originator of the specimen and the submitter of the data, and seek to undertake to collaborate with the submitter. All uploaded sequence data are submitted to rigorous curation by the Friedrich-Loeffler-Institute for Animal Health (FLI), Germany.

Results

The database has been live since September 14, 2009. Among its contributors are all five WHO Collaborating Centers for Influenza who routinely contribute data in addition to using the EpiFlu™ database for their semi-annual vaccine strain selection. To provide a complete picture of data, all data available in the public domain is routinely imported. As of October 8, 2010, the rapidly growing GISAID dataset comprises 166 989 nucleotide sequences (from 48 779 isolates) with 28 949 (from 11 857 isolates) uniquely submitted to this database. Software development is underway to continually extend the spectrum of available data analysis tools.

The intergovernmental process of the 62nd World Health Assembly specifically mentions GISAID as a publicly available database for depositing virus sequence data. Starting in 2011, Germany's Federal Ministry of Food, Agriculture and Consumer Protection will be the long-term host of the GISAID platform. The MPII will continue to develop the portal and database software and enable GISAID to act as a catalyst for the development of advanced bioinformatics software connected directly to the database.

Conclusion

GISAID has become an indispensible resource for the international scientific community on influenza. The consortium will expand its activities and offers to catalyze research and development on a wide variety of issues pertaining to risk analysis, drug development, and therapy of influenza.

Co-infecting viruses in pandemic H1N1 and seasonal influenza positive human respiratory samples

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Abstract

Respiratory clinical samples taken in 2008 and 2009 that tested positive by real time reverse transcription (RT)-PCR for seasonal influenza viruses (A and B) and pandemic H1N1 2009 respectively were assessed for other respiratory viruses using the ResPlex II Panel ver2.0 system distributed by Qiagen. Results showed that co-infections with another 16 respiratory viruses were relatively rare, with a small number of samples having another co-infecting virus present, very few samples having two other viruses detectable in their samples, and none with further viruses. This low number of co-infecting viruses and the ability of certain cell lines not to support infection with particular viruses may make primary isolation of influenza viruses in cell lines easier than might have been thought previously.

Introduction

The pandemic H1N1 virus emerged in 2009 and spread rapidly throughout the world, principally affecting children and young adults. As this virus is new to the human population, it is important to determine if these influenza infections are more commonly associated with other respiratory pathogens compared to previously circulating influenza strains.

Co-infecting respiratory viruses may cause increased morbidity in individuals with pandemic H1N1, and may also be unwanted contaminants in influenza vaccines if original clinical samples containing these adventitious viruses are used to directly inoculate certified cell lines for vaccine production.

To examine this issue, stored RNA from 300 original clinical samples (nasal swabs, nasal aspirates, throat swabs) from Australian and New Zealand subjects that were collected in 2009 that were positive for pandemic H1N1 and 300 samples collected in 2008 that were positive for seasonal influenza by real time PCR assay (using the CDC, USA kits), were subjected to a ResPlex II – Panel version 2.0 (Qiagen) pathogen screen.

The ResPlex II assay detects 18 common respiratory viruses, such as respiratory syncytial viruses (RSV A, B), influenza A and B viruses, parainfluenza viruses (PIV1–4), human metapneumo-viruses (hMPV), coxsackieviruses/echovirus (CVEV), rhinoviruses (RHV), adenoviruses (ADV B, E), coronaviruses (NL63, HKU1, 229E, OC43), and bocaviruses. ResPlex II uses a combination of multiplex RT-PCR, hybridization of PCR onto target specific beads followed by detection using Luminex-xMAP technology.

Materials and methods

Viruses

Original clinical samples were received at the center from WHO National Influenza Centers, WHO Influenza Collaborating Centers, and other regional laboratories and hospitals from Australia, New Zealand, and the Asia/Pacific region. Most samples were from Australia and New Zealand. These samples consisted of nasal swabs, nasopharyngeal swabs, nasal washes, throat washes, and throat swabs. All samples were stored at −80°C until RNA was extracted.

RNA extraction

RNA was extracted from 200 μl of clinical sample using either the MagnaPure extraction system (Roche, Australia) or the QIAxtractor system (Qiagen, Australia) according to the manufacturer's recommendations with an elution volume of 90 μl and stored at −80°C until used.

Real time RT-PCR detection

A 5 μl aliquot of RNA was used to amplify the selected influenza virus gene using specific primers and probes as supplied by CDC (Atlanta, USA)[1] along with Super-Script

III Platinum One-Step RT-PCR reagents (Invitrogen, Australia). Real time PCR detection was performed on a 7500 Fast System with SDS software (Applied Biosystems, CA, USA). A cut off of a cycle threshold (C_t) of 35 or below was considered positive.

ResPlex II Panel ver2.0 detection

The Qiagen Molecular Differential Detection (MDD) system was used, which combines QIAplex amplification (multiplex RT-PCR) with detection on the LiquiChip 200 Workstation (Luminex's xMAP microsphere based multiplexing system) and QIAplex MDD Software according to the manufacturer's instructions. A low level cutoff was used (150) to obtain maximum sensitivity.

Results

From the 300 clinical specimens that were positive for influenza from 2008 by real time PCR, there were 18 (6%) A(H1N1) seasonal influenza viruses, 106 (35·3%) A(H3N2) viruses, 174 (58%) B viruses, and 2 (0·7%) viruses which were influenza A positive, but could not be typed. Clinical samples from 2009 selected to study were all influenza A(H1N1) pandemic 2009 positive by real time PCR. Detection of influenza virus in respiratory samples was much lower with the ResPlex II assay (using a low cut off of 150 units) for pandemic influenza A virus (160/320; sensitivity 53·3%) and to a lesser extent for seasonal influenza A (116/126; sensitivity of 92·1%) and B viruses (161/174; sensitivity of 92·5%) when compared to real time PCR.

There were relatively few co-infecting respiratory viruses with either pandemic H1 infections in 2009 (5·7%) or seasonal influenza infections in 2008 (6·3%) (Table 1). The most common dual infection seen with pandemic H1N1 2009 viruses and 2008 seasonal B viruses was with CVEV (7/18; 2009 and 5/14; 2008, respectively) while for 2008 A(H3) viruses there were no dominant co-infecting viruses (Table 2). In 2009 one case was detected with three respira-

Table 1. Summary of subjects with influenza and other respiratory virus co-infections

Year	Influenza only (%)*	Subjects with one other co-infection (%)	Subjects with two other co-infections (%)	Total subjects with co-infections (%)
2008	281 (93·7)	18 (6·0)	1 (0·3)	19 (6·3)
2009	283 (94·3)	16 (5·3)	1 (0·3)	17 (5·7)

*As determined by real time RT-PCR.

Table 2. Clinical samples positive for influenza by real time PCR from 2008 and 2009 tested by ResPlex II for other respiratory virus co-infections

Virus family	Target	Co-infections with 2009 pandemic H1	Co-infections with 2008 seasonal A/B
Adenoviruses	ADVB	0	1
	ADVE	0	0
Bocavirus	BOCV	1	2
Coronaviruses	229E	4	1
	HKU1	0	2
	NL63	0	2
	OC43	0	1
Cocksackieviruses/echovirus	CVEV	7	5
Human metapneumoviruses A and B	HMPV	0	0
Parainfluenza viruses	PIV1	1	1
	PIV2	0	1
	PIV3	0	2
	PIV4	1	0
Rhinovirus	RHV	1	2
Respiratory syncytial virus	RSVA	1	0
	RSVB	2	0

tory pathogens in the same sample, a 25 year old female who had pandemic H1N1, CVEV, and RHV, and in a 2008 seasonal influenza sample, one case with a triple infection was detected (bocavirus, PIV2 and influenza B).

The median age of subjects with co-infections was younger for both pandemic H1N1 with a median age of 10 years (range: 1 months to 27 years), compared to the full 2009 sample set which had a median age of 29 years (range: 1 months to 85 years), while for the patients from 2008 with seasonal influenza viruses with co-infections they had a median age of 6·5 years (range: 1 months to 26 years) compared to all 2008 samples which had a median age of 16 years (range: 1 months to 84 years).

Discussion

There was good concordance in detecting influenza A and B in respiratory samples collected in 2008 between real time RT-PCR and the ResPlex II system (100% versus >92·1% for seasonal influenza A and B respectively). This data compares well with other studies such as Li *et al.*[2] who found that ResPlex II had 82·8% sensitivity and 100% specificity for seasonal influenza A viruses and 90·0% sensitivity and 100% specificity for influenza B viruses. In contrast, the present study found only 53·3% sensitivity for the ResPlex II detection of influenza A with the 2009 samples that were positive for pandemic H1N1 by real time RT-PCR. A recent study by Rebbapragada *et al.*[3] also showed lower sensitivity for pandemic H1N1 viruses in nasopharyngeal samples with the ResPlex II system (55% sensitivity and 100% specificity) compared to other commercial platforms Seeplex RVP (95% sensitivity and 100% specificity) and Luminex RVP (100% sensitivity and 97% specificity). Interestingly the latest version of the ResPlex system offered by Qiagen the ResPlex II Plus Panel RUO now has a separate target for the pandemic H1N1 virus (Mexico 2009).[4] In terms of detection of other respiratory viruses such as PIV-1, PIV-3, RSV and hMPV, high sensitivities (87·5%, 72·2%, 73·2%, and 80%, respectively) and specificities (99·7–100%) compared to TaqMan RT-PCR have been reported from testing of nasal wash and nasopharyngeal clinical samples.[5]

In both the seasonal influenza positive 2008 and the pandemic H1N1 positive (by real time RT-PCR) clinical specimens, few other respiratory viruses were detected. Only 18 of the 2008 samples had another virus detectable and one had two other viruses, while in 2009 16 out had another virus and one had two other viruses detected from a total of 300 influenza virus positive samples collected in each year. Enteroviruses, coronaviruses, and parainfluenza viruses were most often found with both seasonal and pandemic infections. Younger age appeared to be associated with co-infections with those subjects in 2008 with dual infections having a median age of only 10 years compared to the study groups 29 years; and similarly for 2009, the median age for subjects with dual infections was only 6·5 years compared to the study groups' median age of 16 years. A study by Chong *et al.*[6] on 298 nasopharyngeal swabs collected during 2007–2009 using ResPlex II and Luminex xTAG RVP Fast, they found dual respiratory virus infections in 27/179 (15·1%) of samples and only 2 (0·7%) with triple respiratory viral infections; however, these were from cases with any combination of multiple respiratory viruses not necessarily influenza, although influenza positive cases were the most common respiratory virus detected (37·4% of all positive samples).

Given the low level and variety of viral co-infections along with both seasonal and pandemic influenza seen in this study, it is unlikely that influenza infections predispose subjects to particular respiratory viruses, but may still allow bacterial colonization, such as has been seen with severe and fatal cases with pandemic H1N1 with various bacteria including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, or *Haemophilus influenzae*.[7,8] Low levels of other respiratory viruses along with the finding that certain cell lines (like the MDCK 33016-cells used in this study) do not propagate a number of these viruses (e.g. RSV A and B, rhinoviruses, coronaviruses), but do propagate others (e.g. parainfluenza 3)[9] should make testing for unwanted viruses that might be co-isolated with influenza viruses more focused and hence easier to detect and eliminate this isolate for future vaccine production.

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Design of an automated laboratory for high-throughput influenza surveillance

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Keywords Automated system, detection, robotics, sequencing, surveillance.

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Introduction

Global influenza surveillance is one of the most important approaches to combat spread of disease. Current laboratory methods for characterizing influenza are time-consuming and labor-intensive, and few viral strains undergo full characterization.[1] Even fewer strains from domestic poultry and swine or from wild aquatic birds are well-characterized. These strains are important for global surveillance since they are thought to be the precursors to pandemic influenza strains.[2] We have designed a high-throughput Global Bio Laboratory to address these surveillance needs. The goal of this project was to develop high-speed and high-volume laboratory capabilities for extensive surveillance and rapid and accurate detection and analysis of influenza. The workflow consists of surveillance, sample transportation, laboratory testing, data management and analysis. Five robotic systems have been designed for this laboratory: sample accessioning, biobanking, screening, viral culture, and sequencing. Sample accessioning logs barcodes, centrifuges, and aliquots samples are then sent to biobanking. The robotic biobank stores samples at −80°C and reformats tubes for screening. The screening system extracts RNA and confirms the presence and subtype of influenza. Aliquots of positive samples are sent to the viral culturing system for scale-up. Finally, cultured samples are extracted and sent to the sequencing system for full genome sequencing. The sample accessioning, sequencing, and biobanking systems have been built, delivered, and validation processes are currently being completed. Robotic screening and culturing systems have been fully designed and are ready to be built. A Biosafety Level 3-enhanced containment laboratory was built to enable the flow of samples containing highly pathogenic avian influenza viruses. In full operation, this approach to surveillance is designed to enable the sequencing of up to10 000 full virus genomes per year, more than the total of all full influenza genomes sequenced to date.

Materials and methods

The design of a robotic laboratory for influenza surveillance presents unique challenges and opportunities. Before a robotic system is built, each assay is worked out on the bench top, each movement of the plates and reagents is defined, and the laboratory information management system (LIMS) must be able to address each step of the process. Alternate assays are conceived for processes that are not automation-friendly. Waste streams, worker safety, and space constraints are considered. Each possibility is taken to reduce processes that have the potential to aerosolize or cross-contaminate influenza samples. Instruments must be

found that fit the capabilities needed. Detailed specifications for each of the robotic systems were written including all the parameters listed above. Once the systems are built, a long validation process takes place where the processes and instruments in each system are adjusted to function together properly. Finally, a validation study is performed to ensure that the system is able to produce useful data for influenza research. The entire process takes months from start to finish for each robotic system and requires complete cooperation from a diverse team of researchers.

Results

Accessioning

The accessioning system logs initial sample information with the LIMS system. Samples arrive in barcoded cryotubes. The liquid handler brings all samples up to a common volume and clarifies samples by centrifugation. Samples are then transferred from screw-cap sample vials into storage plates containing 96 individually punchable storage tubes. Each tube (0·3 ml) is individually identifiable with a 2D barcode on the bottom. Six archive aliquots are made, and tubes are individually weld-sealed for storage. Tips for aspiration are fixed and undergo a high-pressure plasma process between each use to sterilize tips and destroy nucleic acids.

Biobanking

Samples are stored at −80°C. Each module has a capacity of 600 REMP plates or ∼60 000 samples. The automated freezer system can assemble requested samples as 96-well plates while samples remain frozen.

Screening

The Screening system uses magnetic bead extraction chemistry, real-time PCR, and a liquid handling system to extract samples, confirm and quantify the presence of influenza, and reformat extracted samples for input into the sequencing system. Serotype of human influenza samples will be performed by real-time PCR.

Culturing

Many samples will not have enough material for further analysis and will need to be scaled up. The culturing system combines incubators, a liquid handling platform, plate reader, and real-time PCR to culture, monitor growth, harvest, and quantify influenza. When the system is not being used for culture and scale-up, it can be used to assay previously cultured influenza samples for drug resistance.

Sequencing

A challenge to sequencing large numbers of influenza samples is the manpower required for sample preparation. The sequencing system has the capacity to prepare up to 10 000 samples for sequencing per year for Sanger sequencing. Sanger sequencing was chosen because it is well-established for influenza surveillance, and automation-friendly. The system is designed to work with multiple primer sets (48, 96, 192).

Laboratory information management system

Robotic systems all report to the LIMS. Each process completion, plate movement, and data point are entered and checked by an online, web-based LIMS. Status updates, notification, reporting, and data analysis can be achieved without entering the BSL3 containment facility. Routine data analysis such as determining whether a cultured sample is ready to be harvested will be performed by the LIMS. Complex data analysis, while still requiring significant human input, will be made easier by the data-acquisition functions of the LIMS.

Discussion

The implementation of a high-throughput influenza surveillance laboratory will provide an influenza research and response capacity that far exceeds what is available today. With the addition of each new system, we add a new capability to the influenza community and new opportunities to foster partnerships and collaborations with government, foundations, businesses, and academic institutions. This laboratory will not only enable cutting edge research, but will also enable a more effective response of near real-time surveillance during a pandemic outbreak.

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Replication of avian and seasonal influenza viruses in human bronchus and lung

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Keywords *Ex vivo* human bronchus culture, *ex vivo* lung culture, influenza A virus, tissue tropism.

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Background

Pandemics of 1957 and 1968 were believed to arise from avian influenza viruses.[1] The tropism of avian and human seasonal influenza viruses for the human lower respiratory tract deserves investigation. The target cell types that support replication of avian influenza A viruses in the human respiratory tract in the early stages of clinical infection have not well defined. In a previous autopsy studies of human H5N1 disease, influenza A virus were found to infect alveolar epithelial cells[2] and macrophages.[3] In this study, viral infectivity and replication competence of human and high and low pathogenic avian influenza viruses were systematically investigated in the human conducting and lower respiratory tract using *ex vivo* organ cultures. We compared the replication kinetics of human seasonal influenza viruses (H1N1 and H3N2), low pathogenic avian influenza viruses (H9N2, H5N8) with that of the highly pathogenic H5N1 viruses isolated from human H5N1 disease.

Materials and methods

A range of human seasonal influenza A viruses of subtypes H1N1 and H3N2 viruses were included in this study from 1975 to 2009. Two isolates of low pathogenic avian influenza A (LPAI) (H9N2) viruses from different virus lineages isolated from poultry in Hong Kong in 1997, a low pathogenic influenza A (H5N8) virus isolate from wild ducks in Hong Kong in 2005, and two virus isolates of highly pathogenic avian influenza (HPAI) A subtype H5N1 were included.

Fragments of human bronchi and lung were cut into multiple 2–3 mm fragments within 2 hours of collection and infected in parallel with influenza A viruses at a titer of 10^6 $TCID_{50}$/ml and as control cultures were infected with ultraviolet light inactivated virus. These tissues fragments were infected for 1 hours and washed twice with PBS and incubated for 1, 24, and 48h at 37°C. The bronchial tissue was cultured in an air-liquid interface using sponge. Viral yield was assessed by titration in MDCK cells. One part of the infected tissue were fixed in formalin and processed for immunohistochemistry for influenza antigen. Other part of infected tissue was homogenized and underwent RNA extraction, and the expression of influenza virus matrix gene was measured by quantitative RT-PCR.

Results

Human bronchus *ex vivo* cultures supported human seasonal influenza virus to replicate efficiently. Avian influenza H9N2 virus replicated, although less efficiently than that of seasonal influenza viruses, whereas HPAI H5N1 did not productively replicate in *ex vivo* cultures of human bronchus. This is in agreement with our previous finding in the well-differentiated bronchial epithelial cells *in vitro*.[4] On the other hand, human lung *ex vivo* cultures supported prominent productive replication of human seasonal influenza H1N1 (Figure 1A) and HPAI H5N1 (Figure 1F) viruses. LPAI, such as H9N2 (Figure 1C–D) and H5N8 (Figure 1E), also replicated productively, but with a lower viral yield. Surprisingly, the replication of human influenza H3N2 viruses (Figure 1B) across the last three decades was greatly inhibited.

Discussion

There are clear differences in viral tropism of human seasonal and avian influenza viruses for replication in the human bronchus and lung. HPAI H5N1 virus can infect and productively replicate in the lower lung, which may

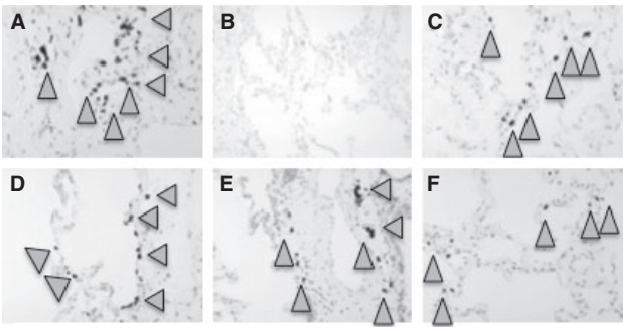

Figure 1. Immunohistochemistry staining of influenza NP protein in lung *ex vivo* culture at 48 hours post infection with influenza A viruses (arrow head).

account for the severity of human H5N1 disease, but not in the conducting airways. Surprisingly, there are marked differences in the replication competence of seasonal influenza viruses in *ex vivo* lung tissues, with influenza H1N1 viruses being able to replicate efficiently while H3N2 viruses do not. This may be related to the more strict Siaα2-6Gal binding preference of H3N2 viruses. On the other hand, the efficient replication of influenza H1N1 viruses in the alveolar spaces indicates factors other than tissues tropism alone play a role in the differences in disease severity between human seasonal H1N1 and avian H5N1 virus infections.

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Influenza C virus NS1 protein up-regulates viral mRNA splicing

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Keywords Influenza C virus, NS1 protein, splicing, viral mRNA.

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Abstract

Pre-mRNAs of the influenza A virus M and NS genes are poorly spliced in virus-infected cells. By contrast, in influenza C virus-infected cells, the predominant transcript from the M gene is spliced mRNA. The present study was performed to investigate the mechanism by which influenza C virus M gene-specific mRNA (M mRNA) is readily spliced. Ribonuclease protection assays showed that the splicing of M mRNA in infected cells was much higher than that in M gene-transfected cells, suggesting that viral protein(s) other than M gene-translational products facilitates the splicing of viral mRNAs. The unspliced and spliced mRNAs of the influenza C virus NS gene encode two nonstructural (NS) proteins, NS1 (C/NS1) and NS2 (C/NS2), respectively. The introduction of translational premature termination into the NS gene, which blocked the synthesis of C/NS1 and C/NS2 proteins, drastically reduced the splicing of NS mRNA, raising the possibility that C/NS1 or C/NS2 enhances the splicing of viral mRNAs. The splicing of influenza C virus M mRNA was increased by co-expression of C/NS1, whereas it was reduced by co-expression of influenza A virus NS1 protein (A/NS1). The splicing of influenza A virus M mRNA was also increased by co-expression of C/NS1, whereas it was inhibited by that of A/NS1. These results suggest that

influenza C virus NS1, but not A/NS1, can up-regulate the splicing of viral mRNAs.

Introduction

Pre-mRNAs of the influenza A virus M and NS genes are poorly spliced in virus-infected cells.[1,2] The inefficient splicing of viral pre-mRNAs can be understood partly by the fact that influenza A virus NS1 protein is associated with spliceosomes and inhibits pre-mRNA splicing.[3,4] *cis*-acting sequences in the NS1 transcript also negatively regulate splicing.[5] By contrast, in influenza C virus-infected cells, the predominant transcript from the M gene is spliced mRNA.[6] The present study was performed to investigate the mechanism by which influenza C virus M gene-specific mRNA (M mRNA) is readily spliced.[7]

Materials and methods

Viruses and cells

The Yamagata/1/88 strain of influenza C virus was grown in the amniotic cavity of 9-day-old embryonated hen's eggs. COS-1 and 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

RNA purification and ribonuclease protection assay

Subconfluent monolayers of COS-1 cells were transfected with pME18S containing influenza C virus M gene cDNA using the lipofectamine procedure and then incubated at 37°C. Total RNA was extracted from both the transfected cells and cells infected with C/Yamagata/1/88 virus using the RNeasy Mini Kit (QIAGEN). Ribonuclease protection assay was performed using a Ribonuclease Protection Assay kit RPA III (Ambion).[6] Briefly, a [^{33}P]-labeled influenza C virus RNA 6-specific RNA probe (vRNA sense) was synthesized by *in vitro* transcription and hybridized with the total RNA at 42°C overnight. Hybrids were digested with RNase A (0·08 U) and RNase T1 (3 U) at 37°C for 30 minutes and then analyzed on a 4% polyacrylamide gel containing 4 m urea.

Indirect immunofluorescent staining

HMV-II cells infected with C/Yamagata/1/88 and COS-1 cells transfected with pME18S expressing influenza C virus NS1 were fixed with carbon tetrachloride at various times after infection and transfection, respectively. The cells were then stained by an indirect method using anti-GST/NS1 serum as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Seikagaku Kogyo) as the secondary antibody.

Results

The splicing efficiency of influenza C virus M gene-specific mRNA (M mRNA) in infected cells was higher than that in M gene-transfected cells

The ratio of M1 encoded by a spliced M mRNA to CM2 encoded by an unspliced M mRNA in influenza C virus-infected cells was about 10 times larger than that in M gene-transfected cells. Ribonuclease protection assays showed that the splicing of M mRNA in infected cells was much higher than that in M gene-transfected cells (Figure 1). These data suggest that viral protein(s) other than M gene-translational products facilitates viral mRNA splicing.

The influenza C virus NS gene translational product may up-regulate the splicing of viral mRNAs

The unspliced and spliced mRNAs of the influenza C virus NS gene encode two nonstructural (NS) proteins, NS1 (C/NS1) and NS2 (C/NS2), respectively. The introduction of translational premature termination into the NS gene, which blocked the synthesis of C/NS1 and C/NS2 proteins, drastically reduced the splicing of NS mRNA, suggesting that C/NS1 or C/NS2 enhances viral mRNA splicing. Immunofluorescent staining showed that NS1 localized in the nucleus in the early phase of infection, and was distributed in both the nucleus and cytoplasm in the late phase

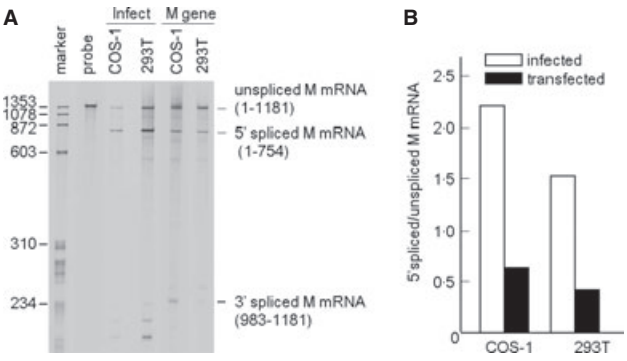

Figure 1. Ribonuclease protection assay of influenza C virus M gene-derived mRNAs in infected and M gene-transfected cells. (A) Total RNA was extracted from COS-1 and 293T cells infected with C/Yamagata/1/88 at an m.o.i. of 10 PFU/cell at 24 hours p.i. (lane Infect) and from those cells transfected with pME18S-CM at 48 hours p.t. (lane M gene) and analyzed by ribonuclease protection assay using a [^{33}P]-labeled influenza C virus RNA segment 6 (M gene)-specific RNA probe (vRNA sense). The protected fragments were separated on a 4% polyacrylamide gel containing 4 m urea. The *Hae*III digest of ψX174 DNA was 5′ end-labeled and used as a size marker (lane marker). The probe lane shows an undigested probe. (B) The ratios of 5′ spliced M mRNA to unspliced M mRNA in infected (open box) and transfected (closed box) of COS-1 and 293T cells were calculated after densitometry of Figure 1A.

of infection, raising the possibility that influenza C virus NS1 protein plays a role in viral mRNA splicing that occurs in the nucleus.

Influenza C virus NS1 protein may up-regulate the splicing of viral mRNAs

The splicing of influenza C virus M mRNA was increased by co-expression of C/NS1, whereas it was reduced by co-expression of influenza A virus NS1 protein (A/NS1) (Figure 2A). The splicing of influenza A virus M mRNA was also increased by co-expression of C/NS1, though it was inhibited by that of A/NS1 (Figure 2B). These results suggest that influenza C virus NS1, but not A/NS1, can up-regulate the splicing of viral mRNAs.[7]

Discussion

In influenza A virus-infected cells, splicing is controlled so that the steady-state amount of spliced mRNAs is only 5–10% of that of unspliced mRNAs.[1,2] The mechanisms by which influenza A virus NS pre-mRNAs are poorly spliced have been investigated and the following confirmed. Influenza A virus NS1 protein associates with spliceosomes and inhibits pre-mRNA splicing.[3,4] Two *cis*-acting sequences in the NS1 transcript (positions 153–465 in the intron and positions 775–860 in the 3′ exon region) inhibit splicing.[5]

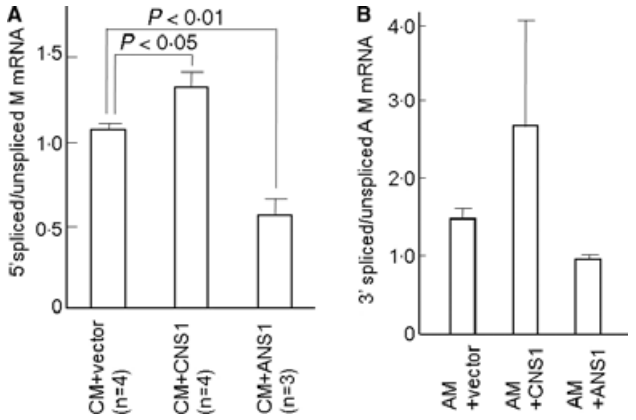

Figure 2. Ribonuclease protection assay of M mRNAs in influenza C and A virus M gene-transfected cells co-expressing influenza C virus NS1 protein. (A) The ratios of 5′ spliced to unspliced influenza C virus M mRNA in COS-1 cells co-transfected with pME18S-CM and the pME18S vector as a control (lane CM + vector), or pME18S-CM and pME18S-CNS1 (lane CM + CNS1) in four independent ribonuclease protection assay experiments and co-transfected with pME18S-CM and pME18S-ANS1 (lane CM + ANS1) in three independent experiments were compared. (B) pME18S-AM (0·33 μg), which can express the M gene-derived mRNA of influenza A virus PR/8/34 strain, was introduced into COS-1 cells with either 0·67 μg pME18S alone (lane AM + vector), 0·67 μg pME18S-CNS1 (lane AM + CNS1), or 0·67 μg pME18S-ANS1, which can express NS1 protein of influenza A/PR/8/34 (lane AM + ANS1). The splicing ratios of 3′ spliced to unspliced AM mRNAs in the cells co-transfected with each construct in the two experiments were compared.

By contrast, influenza C virus M gene-specific mRNA (M mRNA) is efficiently spliced in influenza C virus-infected cells.[6] In this study, we examined the mechanism by which influenza C virus M mRNA is efficiently spliced and the regulatory mechanism of the splicing of NS gene-specific mRNA (NS mRNA).

The introduction of a translational pre-mature termination into the influenza C virus NS gene, thereby blocking the synthesis of influenza C virus NS1 (C/NS1) and NS2 (C/NS2) proteins, drastically reduced the splicing rate of NS mRNA. We further examined whether C/NS1 potentially facilitates viral mRNA splicing. The splicing rate of M mRNA of influenza C virus was increased by co-expression with C/NS1, whereas it was reduced by co-expression with influenza A virus NS1 protein (A/NS1) (Figure 2A). The splicing of influenza A virus M gene-specific mRNA was also increased by co-expression with C/NS1, though it was inhibited by co-expression with A/NS1 (Figure 2B). These results suggest that influenza C virus NS1 can facilitate viral mRNA splicing, but in no way inhibit it, which is in striking contrast to the inhibitory effect of influenza A virus NS1 on pre-mRNA splicing.[3,4]

The mechanism for splicing enhancement by C/NS1 also remains to be determined. We speculate that C/NS1 may interact with some host proteins involved in splicing, thereby leading to an up-regulation in splicing, or that C/NS1 may bind to pre-mRNA, increasing its accessibility to the spliceosome.

The spliced mRNA of the influenza C virus M gene encodes the M1 protein, which plays an important role in virus formation and determines virion morphology.[8,9] Therefore, it is speculated that the mechanism for efficient splicing of M mRNA, which provides the M1 protein necessary for virus assembly in a redundant amount, has been maintained in the influenza C virus. By contrast, unspliced mRNA from the influenza C virus M gene encodes the CM2 ion channel, which is permeable to chloride ions,[10] and also has pH-modulating activity.[11] Although the role of the influenza C virus CM2 ion channel in virus replication remains to be determined, it is conceivable that the over-expression of the CM2 protein has a deleterious effect on virus replication since the fact that a high level of influenza A virus M2 protein expression inhibits the rate of intracellular transport of the influenza A virus HA protein and other integral membrane glycoproteins has been demonstrated.[12] If this is the case, efficient splicing of M mRNA may control the amount of CM2 synthesized to optimize virus replication. Therefore, we speculate that efficient splicing of M mRNA leads to a high level of M1 expression and the reduced expression of CM2, thereby creating conditions that are optimal for virus replication.

In this study, we provided evidence that C/NS1 facilitates the splicing of M mRNA. Furthermore, C/NS1 may regulate the splicing efficiency of its own NS mRNA during infection, controlling the amount of C/NS1 and C/NS2 proteins in infected cells. C/NS2 plays an important role in the nuclear export of vRNP, and is also associated with vRNP in the later stages of infection in virus-infected cells and is incorporated into virions,[13] suggesting that C/NS2 is involved, not only in the sorting of vRNP into the assembly site, but also in virus assembly. Therefore, it is likely that there is a mechanism by which an appropriate amount of C/NS2 is provided during infection to accomplish these functions. In conclusion, C/NS1, which enhances the splicing of viral mRNA, may regulate both the expression level of M gene-derived M1 and CM2 proteins, and that of NS gene-derived NS1 and NS2 proteins, thereby leading to optimal virus replication.

Acknowledgements

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Receptor specificity and replication in human airway cultures of H1N1pdm viruses with HA mutations D222G and Q223R

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Keywords Cell tropism, ciliated cells, egg-adaptation, H1N1/09 pandemic, sialic acid.

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Introduction

Propagation of the human influenza viruses in embryonated hen's eggs always results in a selection of variants with amino acid substitutions in the hemagglutinin (HA) that affect viral receptor-binding characteristics (reviewed[1]). Brookes *et al.*[2] recently studied infection in pigs using the egg-grown virus that contained a mixture of the original A/California/07/09 (H1N1pdm) and its two egg-adaptation mutants with single amino acid substitutions D222G and Q223R (225 and 226 in H3 numbering system). Only the original virus and the variant with 222G were detected in the directly inoculated animals, indicating that the variant with 223R failed to infect. Only the original virus was detected in nasal secretions of contact infected pigs, suggesting that the D222G mutant failed to transmit. In contrast, there was an apparent selection of the D222G mutant in the lower respiratory tract samples from directly inoculated pigs.

The D222G substitution is of a special interest as it can emerge during virus replication in humans and was associated with severe and fatal cases of pandemic influenza in 2009–2010[3–7] and 1918.[8] Here we compared phenotypic properties of the original clinical isolate of H1N1pdm virus A/Hamburg/5/09 and its D222G and D223R mutants to explain observed effects of these mutations on virus replication in swine[2] and to predict their potential effects on virus replication in humans.

Materials and methods

A/Hamburg/5/09 (Ham) was isolated from clinical material by two passages in MDCK cells. The virus was passaged twice in 11-day-old embryonated hen's eggs and plaqued in MDCK cells. The plaques were amplified in MDCK cells and the sequences of the viral HA were determined. The variants with single mutation D222G and Q223R were aliquoted and designated Ham-e and Ham-e1, respectively.

The receptor-binding specificity of the viruses was assessed by assaying their binding to desialylated-resialylated peroxidase-labeled fetuin containing either α2-3-linked sialic acid (2-3-Fet) or α2-6-linked sialic acid (2-6-Fet).[9] In brief, viruses adsorbed in the wells of 96-well EIA micro plates were incubated with serial dilutions of 2-3-Fet or 2-6-Fet, and the amount of bound fetuin probe was quantified by peroxidase activity. The binding data were converted to Scatchard plots (A490/C versus A490), and the association constants of the virus-fetuin complexes were determined from the slopes of these plots.

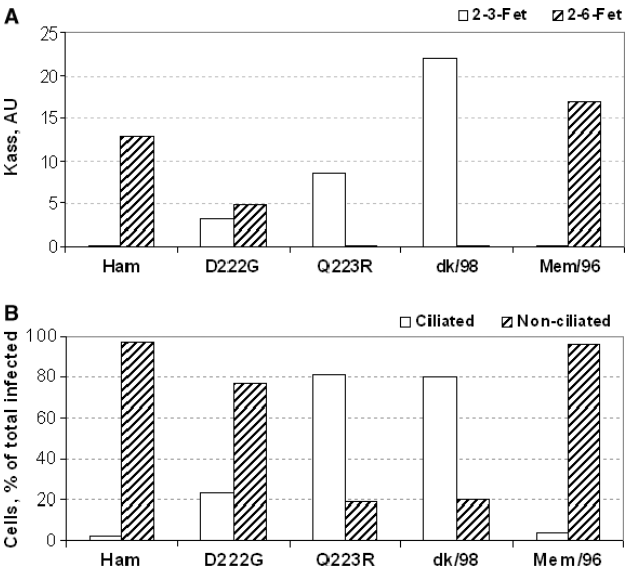

Figure 1. Receptor specificity (A) and cell tropism (B) of Ham, Ham-e (D222G), Ham-e1 (Q223R), and two control H1N1 viruses, A/duck/Alberta/119/98 (dk/98) and A/Memphis/14/96-M (Mem/96). A. Association constants (arbitrary units) of virus binding to 2-6-Fet (open bars) and 2-3-Fet (hatched bars). B. Percentages of infected ciliated cells (open bars) and infected non-ciliated cells (hatched bars) with respect to the total amount of infected cells in HTBE cultures 8 hours post-infection.

Viral cell tropism and replication efficiency in human airway epithelium were studied using fully differentiated cultures of human tracheo-bronchial epithelial cells (HTBE).[10,11] To determine cell tropism, cultures were infected at a moi 1, fixed 8 hours after infection, and double immuno-stained for virus antigen and cilia of ciliated cells. Infected cells were counted under the microscope (100× objective with oil immersion) in the epithelial segment that included 15–30 consecutive microscopic fields containing between 5% and 20% ciliated cells relative to the total number of superficial cells. Percentages of infected ciliated cells and infected non-ciliated cells relative to the total number of infected cells were calculated. Ten segments per culture were analyzed and the results were averaged. To compare growth kinetics of Ham and Ham-e, replicate HTBE cultures were infected with 200 plaque-forming units of the viruses followed by incubation at 35°C under air-liquid interface conditions. At 24, 48, and 72 hours post-infection, we added DMEM to the apical compartments of the cultures and incubated for 30 minutes at 35°C. The apical washes were harvested, stored at −80°C, and analyzed simultaneously for the presence of infectious virus by titration in MDCK cells as described previously.[11]

Results

The non-egg-adapted H1N1pdm virus Ham, similarly to the seasonal human virus A/Memphis/14/96 (H1N1), bound to 2-6-Fet (Figure 1A) and did not show any significant binding to 2-3-Fet. This result contrasted with the binding of H1N1pdm viruses to several 2-3-specific probes in carbohydrate microarray analysis.[12] Reduced avidity of virus interactions with soluble glycoprotein in solution as compared to its binding to the probe clustered on the microarray surface could account for these differences in the assay results. The D222G mutant Ham-e differed from the parent virus by its ability to bind to 3-Fet and by its reduced binding to 6-Fet. The Q223R mutant only bound to 2-3-Fet, although less strongly than did the avian virus A/Duck/Alberta/119/98 (H1N1).

The viral cell tropism in HTBE cultures (Figure 1B) correlated with receptor specificity. Ham and Mem/96 showed a typical human-virus-like tropism[10,11] with preferential infection of non-ciliated cells (<5% of infected cells were ciliated). The mutant with 223R and control duck virus displayed a typical avian-virus-like tropism (preferential infection of ciliated cells). The D222G mutant displayed a cell tropism that was intermediate between those of human and avian viruses; in particular, this mutant infected significantly higher proportion of ciliated cells than Ham and Mem/96.

Observed alteration of receptor specificity and cell tropism (Figure 1) suggested that egg-derived mutations can affect replication of the H1N1pdm virus in human airway epithelium. To test this, we first compared the capacity of the viruses to initiate infection in HTBE cultures. Replicate cultures were infected with identical doses of the viruses, fixed 8 hours post-infection, and immuno-stained for viral antigen. Under these conditions, Ham and Ham-e infected comparable numbers of cells, whereas Ham-e1 infected at least 10 times less cells (data not shown). This result indicated that the mutation Q223R markedly impaired the ability of Ham-e1 to infect human airway epithelial cultures. We next compared two other viruses Ham and Ham-e for their multi-cycle replication in HTBE cultures and found that the original virus reached threefold higher peak titers 48 hours post infection than did the D222G mutant (Figure 2).

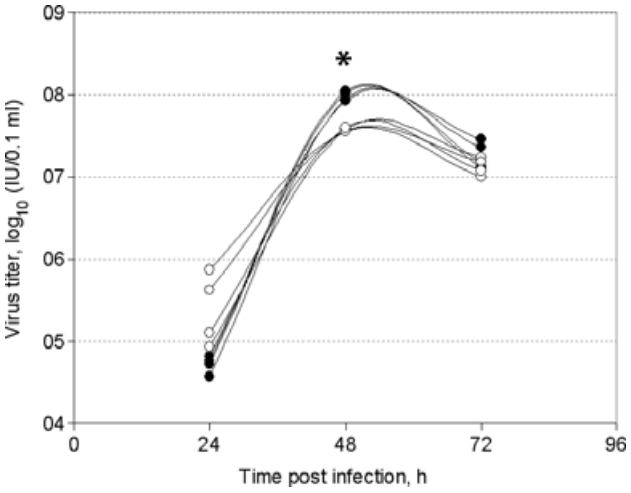

Figure 2. Replication kinetics of Ham (closed circles) and Ham-e (open circles) in HTBE cultures. Data for four individual replicate cultures are shown.*$P < 0{\cdot}0001$.

Conclusion

The D222G mutation in H1N1pdm virus facilitates virus binding to 2-3-linked receptors and alters viral cell tropism in human airway epithelium. These changes could account for increased replication of the D222G mutant in the lower respiratory tract in humans[5–7] and pigs[2] and correlation of this mutation with severe pulmonary disease.[3–7]

The D222G mutant replicates less efficiently in human airway cultures than the original virus. This finding correlates with an apparent lack of transmission of variants with 222G in humans[3] and pigs.[2]

Egg-derived mutation Q223R abolishes virus binding to 2-6-linked receptors and strongly decreases infection in cultures of human airway epithelium. This result agrees with poor infectivity of the Q223R mutant in pigs[2] and highlights potential pitfalls of using egg-adapted viruses with this mutation for the preparation of live influenza vaccines.

Acknowledgements

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Role of palmitoylation of membrane protein CM2 in influenza C virus replication

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Keywords CM2 protein, influenza C virus, palmitoylation.

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Abstract

CM2 is the second membrane protein of influenza C virus and is posttranslationally modified by phosphorylation, palmitoylation, N-glycosylation, and dimer/tetramer formation. In the present study, we generated rCM2-C65A, a recombinant influenza C virus lacking CM2 palmitoylation site, and examined viral growth and viral protein synthesis in the recombinant-infected cells. The rCM2-C65A virus grew less efficiently than did the wild-type virus. Membrane flotation analysis of the infected cells revealed that less NP was recovered in the plasma membrane fractions of the rCM2-C65A-infected cells than that in the wild-type virus-infected cells, suggesting that palmitoylation of CM2 is involved in the affinity of the ribonucleoprotein complex to the plasma membrane, leading to the efficient generation of infectious viruses.

Introduction

Influenza C virus has seven single-stranded RNA segments of negative polarity, encoding PB2, PB1, P3, haemaggluti-

nin-esterase-fusion (HEF), nucleoprotein (NP), matrix (M1) protein, CM2, and the non-structural proteins NS1 and NS2.[1,2] CM2 is the second membrane protein of the virus and is encoded by RNA segment 6 (M gene).[3–8] It is composed of three distinct domains: a 23-residue N-terminal extracellular domain, a 23-residue transmembrane domain, and a 69-residue cytoplasmic domain.[3,9,10] It is abundantly expressed at the plasma membranes of infected cells and is incorporated in a small amount into virions.[10,11] CM2 forms disulphide-linked dimers and tetramers, and is posttranslationally modified by N-glycosylation, palmitoylation, and phosphorylation.[10–12] Analyses of a number of CM2 mutants revealed the positions of the amino acids involved in the posttranslational modifications.[10,13] Evidence was obtained that the *N*-glycosylation was not required for either the formation of disulfide-linked multimers or transport to the cell surface,[10] and that none of dimer- or tetramer-formation, palmitoylation or phosphorylation was essential to the transport of CM2 to the cell surface.[13]

In the present study, in order to investigate the effect of CM2 palmitoylation on influenza C virus replication, we generated a CM2 palmitoylation-deficient influenza C virus, in which a cysteine at residue 65 of CM2 was mutated to alanine, and examined the viral growth and viral protein synthesis in infected cells.

Materials and methods

Cells and antibodies

293T and HMV-II cells were maintained as described previously.[14,15] LLC-MK_2 cells were maintained at 37°C in minimal essential medium with 5% foetal bovine serum and 5% calf serum. Monoclonal antibodies (mAbs) against the HEF, NP, and M1 proteins of C/Ann Arbor/1/50 (AA/50), and antisera against the AA/50 virion and the CM2 protein were prepared as described previously.[3,16–18]

Plasmid DNAs

The seven Pol I plasmids for the expression of viral RNAs of AA/50, and the nine plasmid DNAs for the expression of the influenza C viral proteins were reported previously.[6,14] Plasmid DNA, pPolI/CM2-Acy(-), in which 995-TGT-997 of the M gene was replaced with 995-GCT-997, was constructed based on pPolI/M.

Generation of recombinant influenza C viruses

To generate a recombinant wild-type (rWT) virus, the above-mentioned 16 plasmids were transfected into 293T cells as described previously.[6] To rescue a mutant virus, rCM2-C65A, a recombinant influenza C virus lacking a CM2 palmitoylation site, the plasmid pPolI/CM2-Acy(-), instead of pPolI/M, was transfected together with the other 15 plasmids. At 90 hours posttransfection (p.t.), the respective culture medium of the transfected-293T cells was inoculated into the amniotic cavity of 9-day-old embryonated chicken eggs, and a stock of the recombinant virus was prepared.

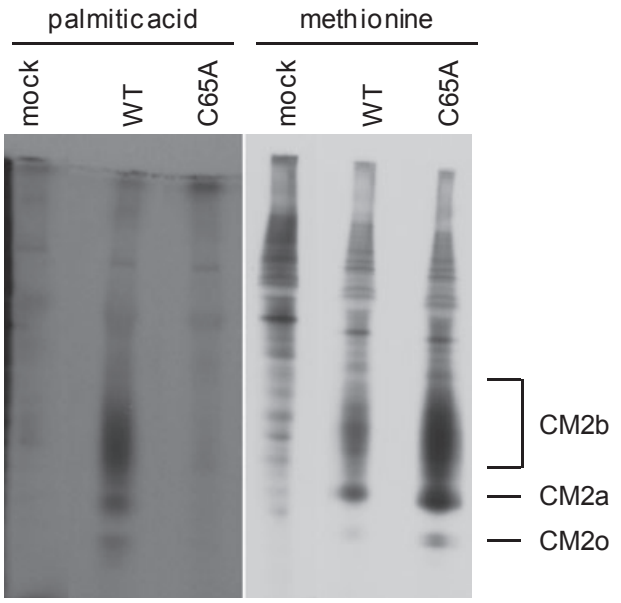

Figure 1. Synthesis of CM2 in recombinant-infected cells. HMV-II cells infected with recombinant wild-type or rCM2-C65A were pulse-labeled with [^{35}S]methionine or [^{3}H]palmitic acid at 48 hours p.i. The lysates of the cells were immunoprecipitated with anti-CM2 serum and analysed by SDS-PAGE.

Determination of infectious titers of viruses

The infectious titres of the stocked recombinant viruses and the supernatants of recombinant-infected HMV-II cells were determined according to the procedure reported previously.[19]

Radioimmunoprecipitation

HMV-II cells infected with recombinants were labeled with [^{35}S]methionine or [^{3}H]palmitic acid. Cells were then disrupted and subjected to immunoprecipitation with the indicated antibodies. The immunoprecipitates obtained were then analysed by SDS-PAGE on 17·5% gels containing 4 m urea, and processed for fluorography.[18] Flotation analysis was performed according to the procedure described previously.[6]

Results

Synthesis of CM2 in rCM2-C65A-infected HMV-II cells

To examine whether the CM2 protein without palmitoylation is synthesized in rCM2-C65A-infected cells, HMV-II cells infected with the recombinants were subjected to

radioimmunoprecipitation. The infected cells were pulse-labeled with [^{35}S]methionine or [^{3}H]palmitic acid for 4 hours at 48 hours postinfection (p.i.), and the lysates of the cells were immunoprecipitated with anti-CM2 serum and analysed by SDS-PAGE. As shown in Figure 1, the CM2 protein was synthesized both in the rWT- and rCM2-C65A-infected cells, but no incorporation of [^{3}H]palmitic acid into the CM2 proteins synthesized in the rCM2-C65A-infected cells was observed, indicating that CM2 in the rCM2-C65A-infected cells was not palmitoylated.

Growth kinetics of the recombinants in HMV-II cells

The rWT or rCM2-C65A viruses were infected to HMV-II cells at an m.o.i. of 5 and incubated at 33°C for up to 96 hours. The infectious titres (p.f.u./ml) of rWT were approximately 5- to 10-fold higher than those of rCM2-C65A at 24–96 hours p.i. (data not shown), indicating that rWT grew more efficiently than did rCM2-C65A. Thus palmitoylation of CM2 appears to have some effect on the generation of infectious virions in cultured cells.

Viral proteins synthesized in HMV-II cells

To investigate the reason(s) for the difference in growth kinetics between the two recombinants, we analysed viral proteins synthesized in the infected HMV-II cells. Pulse-chase experiments of HMV-II cells revealed no significant differences in the synthesis and maturation of the HEF, NP, M1, and CM2 proteins between the rWT- and rCM2-C65A-infected cells (data not shown). The infected cells pulse-labeled and chased were respectively immunoprecipitated with anti-CM2 serum in the presence of 50 mm iodoacetamide and analysed by SDS-PAGE in non-reducing condition. In both populations of infected cells, several bands corresponding to CM2a-monomer, -dimer, and -tetramer, as well as CM2b-dimer and -tetramer were detected (data not shown). These results demonstrate an absence of any significant differences between palmitoylation-deficient CM2 and authentic CM2 in terms of conformational maturation and transport in infected cells.

Membrane flotation analysis of virus-infected cells

Membrane flotation analysis revealed that no significant differences in the kinetics of the HEF, M1, and CM2 proteins were observed between rWT- and rCM2-C65A-infected cells (data not shown). In contrast, a slight difference in NP kinetics was observed. The pulse-labeled NP proteins were recovered in the bottom fractions in both rWT- and rCM2-C65A-infected cells. In the chase experiment, the amount of membrane-associated NP proteins in fractions 3 and 4 was 30 % of the total NP in the rWT-infected cells, which was higher than that (19%) in the rCM2-C65A-infected cells (data not shown). This finding may suggest that the affinity of the NP protein, presumably representing the viral ribonucleoprotein (vRNP) complex, to the plasma membrane in the rCM2-C65A-infected cells is lower than that in rWT-infected cells, leading to the less efficient generation of infectious virions.

Discussion

Since CM2 is structurally similar to M2, an influenza A virus membrane protein known to be involved in infectious virus production,[20–24] it is possible that the cytoplasmic tail of CM2 participates in the genome packaging through interaction with vRNP. In the present study, we showed that the affinity of NP to the plasma membrane of rCM2-C65A-infected cells was slightly lower than that to the plasma membrane of rWT-infected cells. This observation may suggest that palmitoylation of CM2 is involved in the viral ribonucleoprotein (vRNP) incorporation, leading to efficient infectious virion generation. We hypothesize that palmitoylation contributes to proper regional structure formation in the CM2 cytoplasmic tail, which is competent to recruit vRNP efficiently into virions. Alternatively, the CM2 cytoplasmic tail without palmitoylation is not likely to reach the proper conformation, resulting in reduced interaction with vRNP and less efficient generation of infectious progeny virions. The questions of if and how the M1 protein is involved in the interaction between the CM2 cytoplasmic tail and NP remains to be clarified.

We showed that CM2 synthesized in rCM2-C65A-infected cells was oligomerized and transported to the cell surface. This finding is consistent with the previous observation that palmitoylation is not required for the transport of CM2 to the cell surface in CM2-expressing COS-1 cells.[13] However, the use of reverse-genetics system has enabled us to conclude that the palmitoylation of CM2 is required for efficient infectious virus production. This suggests that the significance of the other posttranslational modifications of CM2 during virus replication can be clarified using recombinant viruses lacking the respective modification sites.

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The regional distribution of different types of influenza receptors in cultured human alveolar epithelial cells and correlation with *in vitro* infection

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Keywords Maackia amurensis agglutinin, *Sambucus nigra* agglutnin, sialic acid receptors, type II pneumocytes, type-I-like pneumocytes.

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Background

Sialic acid (Sia) linked glycoproteins are the classical influenza receptors for influenza virus haemagglutinin to bind. The distribution of Sia on cell surfaces is one of the determinants of host tropism, and understanding its expression on human cells and tissues is important for understanding influenza pathogenesis. Previous research has shown the differences in apical versus basolateral infection and release of different influenza virus from polarized epithelial cells[1] and correlated this with sialic acid distribution in the human respiratory tract. Moreover, mass spectrometric analysis was recently employed to elucidate the glycans present in the tissue in a higher resolution in human lung.[2] The objective of this study was to examine in detail the distribution of these Sia-linked glycans at the cellular level by the use of confocal microscopy.

Materials and methods

Human primary type I-like and type II pneumocytes were isolated from human non-tumor lung tissue by tissue fragmentation, percoll density gradient centrifugation, and magnetic cell sorting.[1] The cells were seeded on coverslips and maintained in small airway growth medium. When confluence was reached, cell monolayers were fixed with 4% paraformaldehyde. We used the plant lectins, *Sambucus nigra* glutinin (SNA) from Roche which binds to Siaα2-6Gal, *Maackia amurensis* agglutinin (MAA)I and MAAII from Vector Lab, which bind the Siaα2-3Gal linked glycans using Vector Red as fluorescent chromogen. The cells were counter-stained with DAPI or with FITC-conjugated antibody against endoplasmic recticulum (protein disulfide-isomerase, PDI). The cells were imaged with multi-photon excitation laser scanning microscopy using Zeiss 510 LSM. The optical cross-section pictures were reconstructed by Zeiss LSM510 META.

Results

We found that there was more binding of MAAI and MA-AII to type II pneumocytes than type I-like pneumocytes and more overall binding of these lectins than binding of SNA (Figure 1). In keeping with results from other polarized cells there was more binding to the apical than basolateral aspect, thus, explaining the previously published data on apical versus basolateral infection.[1] As sialic acid has been implicated in the targeting of proteins to the surface, the relative lack of sialic acid on the basolateral aspect can explain why there is little seasonal influenza virus dissemination to the systemic circulation in human infections. Furthermore, though there was little binding of SNA to the

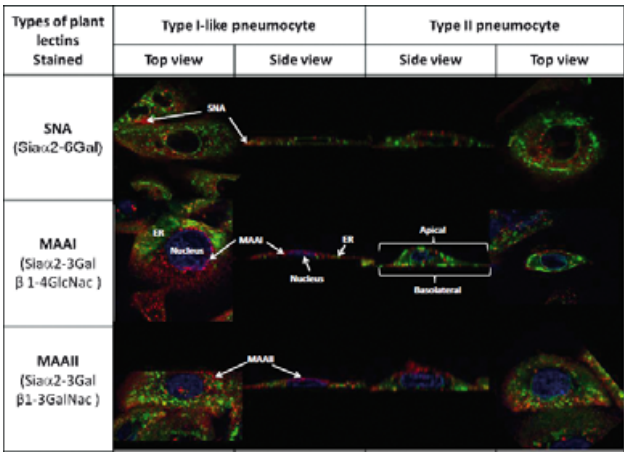

Figure 1. Primary human type I-like and type II pneumocytes stained with lecins (red), pdi (green), and DAPI (blue) and imaged captured with confocal microscope.

apical or basolateral aspects of the pneumocytes, the experimental findings of infection by influenza H3N2 virus that has a strict Siaα2-6Gal tropism[3] suggests that there are Siaα2-6Gal glycans present, which are not readily bound by the lectin SNA.

Discussion

The *in vitro* model of primary human type I-like and type II pneumocytes system formed a polarized epithelium that has a similar lectin distribution to human alveoli *in vivo* which demonstrated that it is a physiologically relevant model to study the tropism and pathogenesis of influenza A virus.

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Differential onset of apoptosis in avian influenza H5N1 and seasonal H1N1 virus infected human bronchial and alveolar epithelial cells: an *in vitro* and *ex vivo* study

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Keywords Alveolar epithelial cells, apoptosis, avian influenza H5N1 virus, bronchial epithelial cells, *ex vivo*, *in vitro*, pathogenesis.

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Introduction

Human disease caused by highly pathogenic avian influenza (HPAI) H5N1 virus is associated with fulminant viral pneumonia and mortality rates in excess of 60%.[1] Cytokine dysregulation is thought to contribute to its pathogenesis.[2,3] We previously found delayed onset of apoptosis in H5N1 infected human macrophages and, therefore, a longer survival time of the target cells for prolonged virus replication and cytokine and chemokine secretion, which may contribute to the pathogenesis of H5N1 disease in humans.[4] As bronchial and alveolar epithelial cells are target cells of influenza virus because of their proximal physiological location and interaction with macrophages, we further investigated if the differential onset of apoptosis could be found in influenza H5N1 and seasonal influenza H1N1 infected human respiratory epithelia. We dissected the apoptotic pathways triggered by influenza virus infection.

Materials and methods

Seasonal influenza H1N1 virus (A/HK/54/98), a low pathogenic avian influenza H9N2 lineage isolated from poultry (A/Quail/HK/G1/97), and two virus isolates of HPAI A subtype (A/HK/483/97 and A/VN/1203/04) were included. Primary human bronchial and alveolar epithelial cells were infected with influenza viruses at MOI of 2 and the cell monolayer was collected at 24, 30, and 48 hours post infection for TUNEL assay, and supernatant were collected for LDH assay. Fragments of human lung tissues were cut into multiple 2–3 mm fragments within 2 hours of collection and infected with influenza A viruses at a titer of 10^6 $TCID_{50}$/ml. These tissues fragments were infected for 1 hours and incubated for 48 hours at 37°C. One part of the infected tissue was fixed in formalin and processed for immunohistochemistry for influenza antigen, and the other part was homogenized and underwent RNA extraction. Apoptosis cDNA superarray platform (SABioscience) was employed to conduct apoptosis pathway analysis.

Results

In bronchial epithelial cells, seasonal influenza H1N1 virus induced a high percentage of apoptotic cells by TUNEL assay at 24, 30, and 48 hours post infection with a peak of

Figure 1. Differential time of onset of apoptosis in primary human bronchial epithelial cells infected by different influenza viruses. Bronchial epithelial cells were infected with H1N1 (A/HK/54/98), H9N2 (A/Quail/HK/G1/97), H5N1 (A/HK/483/97 and A/VN/1203/04) virus at an M.O.I. of 2 or treated with mock reagents. The percentage of apoptotic cells were determined by TUNEL assay at 24, 30, and 48 hours post infection. The chart showed the mean and the standard deviation pooled from two independent experiments. Asterisk indicates significant difference with $p < 0{\cdot}001$.

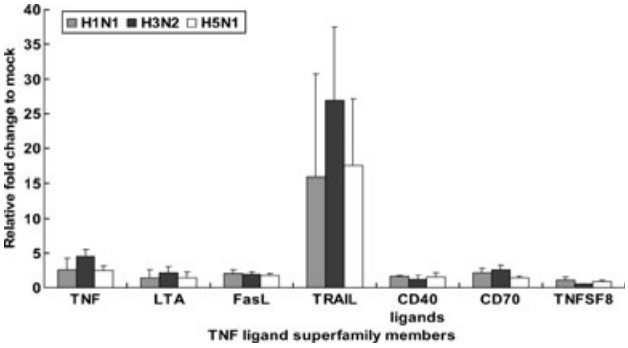

Figure 2. Transcription of Tumor Necrosis Factor (TNF) ligand in lung infected by different influenza viruses. *Ex vivo* lung tissues were infected with H1N1 (A/HK/54/98), H3N2 (A/HK/1174/99), and H5N1 (A/Vietnam/3046/04) virus with virus titer at 1×10^6 $TCID_{50}$/ml or treated with mock reagents. Total RNA was extracted for first-strand cDNA synthesis. The gene expression of key apoptotic genes were profiled by RT-PCR-based RT2 Profiler Apoptosis PCR Arrays (Superarrays Bioscience). The chart showed the mean and the standard deviation pooled from three independent experiments.

approximately 50% apoptotic cells at 30 hours maintained through 48 hours post infection. The percentage of apoptotic cells induced by influenza H9N2 and H5N1 viruses remained low (below 10%) at 24 and 30 hours post infection with a minor increase at 48 hours post infection (up to approximately 20%) (Figure 1). A similar observation of delayed onset of apoptosis was found in influenza H5N1 and H9N2 infected alveolar epithelial cells. Besides, cDNA array data of *ex vivo* infected human lung showed that both influenza H5N1 and H1N1 virus induced TRAIL expression compared with mock-infected tissue (approximately 15 folds) at 48 hours post infection, but influenza H3N2 virus infected lung induced significantly more TRAIL (27 folds compared to mock infected cells), albeit with a limited viral replication (Figure 2). Influenza H3N2 virus infected lung also elicited more TNF-alpha and FasR transcription than either H5N1 or H1N1. These observations can account for the greater apoptotic response in influenza H3N2 virus infected lung. As little impact on the expression of intrinsic pathway components was observed, it seems that the apoptotic response to influenza virus infection in lung was mainly through the extrinsic pathways. No significant changes in the expression of anti-apoptotic protein gene was found, except for a moderate induction of BIRC3 by influenza H3N2 virus, which may act to modulate the apoptotic response.

Discussion

The delayed onset of apoptosis by HPAI H5N1 and low pathogenic avian influenza H9N2 virus infected respiratory epithelial cells may be a mechanism for the influenza viruses to have more prolonged replication within the human respiratory tract, and this may contribute to the pathogenesis of human disease.

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Identification of dual receptor-binding specific strains of human H5N1 viruses in China

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Keywords China, human H5N1 virus, receptor-binding specificity.

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Abstract

Both the α2, 6 linkage and its topology on target cells were critical for human adaptation of influenza A viruses. The binding preference of avian flu virus H5N1 HA to the α2, 3-linked sialylated glycans is considered the major factor that limited its efficient infection in human. Currently, the switch in binding-specificity of human H5N1 viruses from α2, 3 to α2, 6-glycans did naturally occur, and limited human-to-human transmission was found. To monitor their potential adaptation in the human population, receptor-binding specificity surveillance was made in China. Here, the binding specificity of 32 human H5N1 virus strains isolated from 2003 to 2009 was demonstrated. Dual binding preference to α2, 3 and α2, 6-glycans were found in A/Guangdong/1/06 and A/Guangxi/1/08. Furthermore, both of them showed a high affinity to the long-branched α2, 6-glycans, which predominate on the upper respiratory epithelial in human. Our data suggests that the existence of H5N1 virus with binding specificity to humans should be of concern.

Introduction

Via envelope glycoprotein hemagglutinin (HA), influenza viruses bind to cell-surface glycosylated oligosaccharides terminated by sialic acids (SA) where their linkage is cell- and species-specific. Differential receptor binding preference is a host barrier for influenza virus transmission. Although most H5N1 viruses have low affinity to Neu5Acα2, 6Gal (human-type) receptor, recent findings suggested that the adaptation of H5N1 virus to human by mutations in the receptor-binding site (RBS) do indeed happen and resulted in enhanced affinity to human-type receptor.[1–3] In contrast to its putative precursor, A/Gs/GD/1/96, diverse genotypes were presented in currently circulating H5N1 virus, accelerating evolution and widespread occurrence.[4,5] To date, 10 distinct phylogenetic clades (0–9) were identified based on H5N1 HA, and the confirmed human infections were caused by clade 0, 1, 2·1, 2·2, 2·3, and 7.[6] In China, human H5N1 disease was mostly caused by clade2·3·4, which was identified in 28 isolates from 39 confirmed patients from 17 provinces since 2003. Clade 7 and clade 2·2 are responsible for the case in 2003 and 2006, respectively. Two current cases of 2009 and 2010 were due to clade 2·3·2. Now information on receptor property has been documented in some H5N1 viruses of clade 1, 2·1, and 2·2.[1–3,7] Little is known about H5N1 virus of clade 2·3·4, particularly from human.

Recently, α2, 3-specific sialidase-treated red blood cell (RBC) agglutination assay was developed and used for receptor specificity screening of H5N1 virus.[3,8] The α2, 6 or α2, 3-binding preference can be distinguished by the change of hemagglutination titer reacted with RBCs and enzymatic RBCs. Since fine receptor specificity existed in H5N1 viruses,[9,10] the glycan array including sulfated-, fucosylated-, linear sialosides, di-sialosides, or direct binding assay with synthetic polyacrylamide (PAA)-based sialylglycopolymers was also recommended for the receptor-specificity surveillance on H5N1 viruses. Furthermore, the long-branched α2, 6 sialylated glycans were currently identified to predominate on the upper respiratory epithelial in human and the recognition of this topology, 6′SLN-LN is the key determinant for the human-adaptation of influenza A virus.[11]

Here, we analyzed the receptor-binding specificity of human H5N1 viruses isolated in China from 2003 to 2009.

Materials and methods

Sampling, virus isolation

Since 2003, a total of 39 H5N1 infection cases were confirmed in China from 17 provinces. The pharyngeal swabs and lower airway aspirations from the patients were collected within 12 days after disease onset, maintained in viral-transport medium, and tested within 24 hours.

Table 1. Hemagglutination titers of influenza viruses with CRBC and sialidase-treated CRBC

Virus and subtype	Passage history from initial isolate	Genetic clade	1%CRBC (HAU/50 μl)	1% sialidase-treated CRBC(HAU/50 μl)
A/Brisbane/59/2007(H1N1)	Chicken egg *4		512	512
A/AH/1/05 (H5N1)	Chicken egg*2, MDCK*2	2·3·4	64	neg
A/AH/2/05(H5N1)	Chicken Egg *1, MDCK*2	2·3·4	32	neg
A/JX/1/05(H5N1)	Chicken Egg *4	2·3·4	256	neg
A/GX/01/05(H5N1)	Chicken Egg *4, MDCK*1	2·3·4	16	neg
A/FJ/1/05(H5N1)	Chicken Egg *4	2·3·4	512	neg
A/AH/1/06(H5N1)	Chicken Egg *4	2·3·4	64	neg
A/HN/1/06(H5N1)	Chicken Egg *4, MDCK*1	2·3·4	8	neg
A/SH/1/06(H5N1)	Chicken Egg *4, MDCK*1	2·3·4	8	neg
A/ZJ/1/06(H5N1)	Chicken Egg *4	2·3·4	256	neg
A/SC/1/06(H5N1)	Chicken Egg *4	2·3·4	64	neg
A/SC/2/06(H5N1)	Chicken Egg *4	2·3·4	64	neg
A/GD/1/06(H5N1)	Chicken Egg *3	2·3·4	32	16
A/SC/3/06(H5N1)	Chicken Egg *3	2·3·4	16	neg
A/GD/2/06(H5N1)	Chicken Egg *3	2·3·4	1024	neg A/XJ
1/06(H5N1)	Chicken Egg *3	2·2	256	neg
A/HB/1/06 (M2, A31S)(H5N1)	Chicken Egg *4, MDCK*2	2·3·4	64	neg
A/HB/1/06 (M2, A31T)(H5N1)	Chicken Egg *4, MDCK*2	2·3·4	32	neg
A/HB/1/06 (M2, S31N)(H5N1)	Chicken Egg *4, MDCK*2	2·3·4	16	neg
A/BJ/1/03(H5N1)	Chicken Egg *7	7	256	neg
A/FJ/1/07(H5N1)	Chicken Egg *3	2·3·4	512	neg
A/AH/1/07(H5N1)	Chicken Egg *3	2·3·4	256	neg
A/JS/2/07(H5N1)	Chicken Egg *2	2·3·4	256	neg
A/HN/1/08(H5N1)	Chicken Egg *2, MDCK*1	2·3·4	128	neg
A/GX/1/08 (H5N1)	Chicken Egg *1, MDCK*1	2·3·4	64	4
A/GD/1/08(H5N1)	Chicken Egg *2	2·3·4	256	neg
A/BJ/01/09(H5N1)	Chicken Egg *1, MDCK*1	2·3·4	64	neg
A/HN/1/09(H5N1)	Chicken Egg *2, MDCK*1	2·3·4	64	neg
A/SD/1/09(H5N1)	Chicken Egg *1, MDCK*1	2·3·4	64	neg
A/XJ/1/09(H5N1)	Chicken Egg *3, MDCK*1	2·3·4	64	neg
A/GZ/1/09(H5N1)	Chicken Egg *3, MDCK*1	2·3·4	64	neg
A/GX/1/09(H5N1)	Chicken Egg *2, MDCK*1	2·3·2	128	neg
A/HN/2/09 (H5N1)	Chicken Egg *1, MDCK*1	2·3·4	32	neg

Hemagglutination (HA) assay

10% CRBC suspension was treated by 625 mU α2, 3-specific sialidase at 37°C for 15 minutes. Complete elimination of α2, 3-receptor on sialidase-treated CRBCs was confirmed by receptor staining and flow cytometry. HA assay of live viruses with 1% CRBC or 1% sialidase-treated CRBC were performed in BSL-3 facility.

Direct binding assay with synthetic sialylglycopolymers

Synthetic 3′SLN-PAA-Biotin(PA191), 6′SLN-PAA-Biotin(PA190), 6′SLN-LN-PAA-Biotin(PA343) was provided by The Scripps Research Institute (TSRI). As described elsewhere with some modifications,[3] generally, serial dilutions of sialyglycopolymers were coated in 96-well-flat–bottom polystyrene plates, and 32 HAU live virus/well were added. Alternatively, the plates were precoated with 5 μg/ml sialylglycopolymers, and then 8, 16, 32, 64, 128 HAU live virus/well influenza viruses were added. Rabbit antisera against A/AH/1/05 diluted in PBS containing 1% BSA was added into the wells. Bound antibody was detected by use of HRP-conjugated anti-rabbit IgG antibody and tetramethylbenzidine substrate solution. Each sample was determined in duplicates and the absorbance read at 450 nm.

Results

Human avian influenza cases (H5N1) in China from 2003 to 2009

A total of 31 H5N1 virus strains were obtained from 2003 to 2009. The name and passage history of influenza viruses used in the study are listed in Table 1. As the same sequences of eight RNA segments were detected in A/JS/1/07 and A/JS/2/07, only A/JS/2/07 was tested here. Three amantadine-resistant variants with M2 mutation of

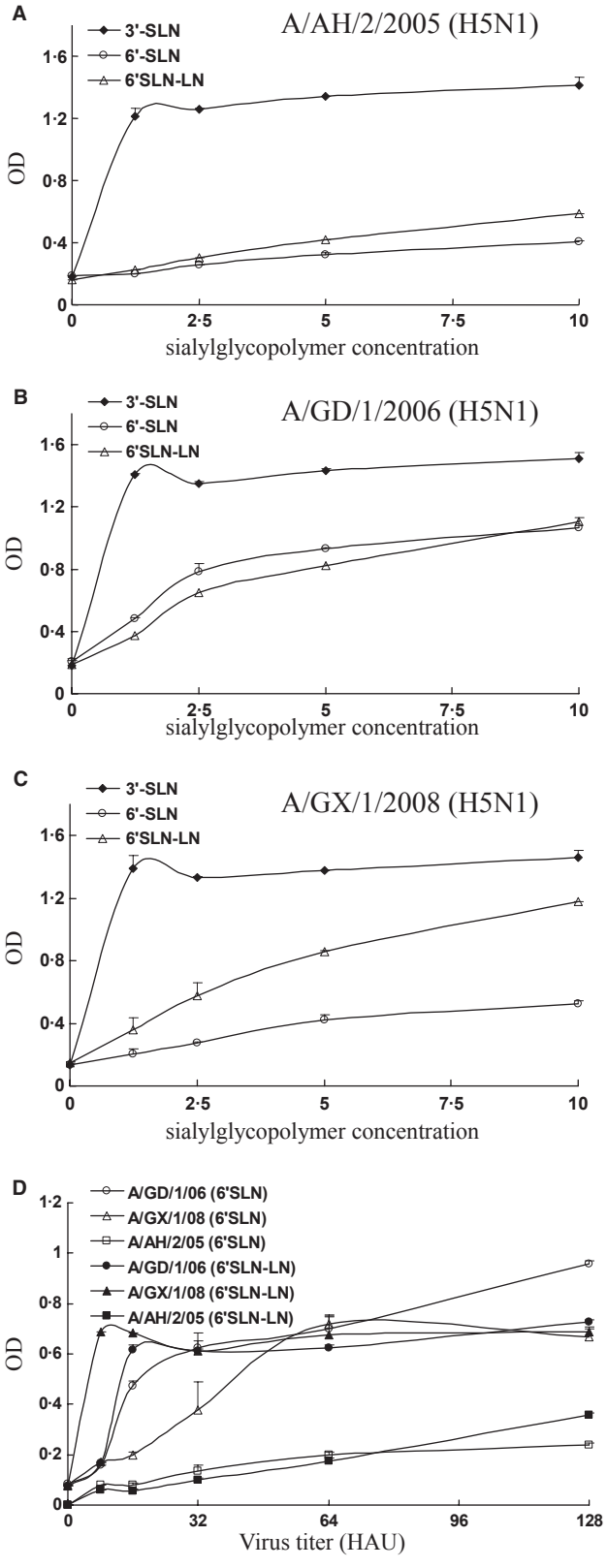

Figure 1. Direct binding assay using sialyglycopolymers. The dose-dependent direct binding tests on the three viruses (A) A/AH/2/05(H5N1) (B) A/GD/1/06(H5N1) (C) A/GX/1/08 (H5N1) were performed in the same set of experiment. (D) Binding of 6′SLN and 6′SLN-LN to A/GD/1/06, A/GX/1/08 and A/AH/2/05. The data represent mean ± SEM from duplicate wells in one representative test from two sets of independent experiments.

A30S, A30T, and S31N, respectively, were cloned from the A/HB/1/06 isolate.

Screening of receptor-binding preference by HA assay

Representative results from three sets of independent experiments are shown in Table 1. Complete HA with sialidase-treated CRBCs, which were only with α2,6-receptors, was detected in human influenza virus (A/Brisbane/59/2007, H1N1) and two human H5N1 virus strains, A/GD/1/06 and A/GX/1/08.

Identification of subtle receptor specificity by direct binding assay

High binding of α2,3 oligosaccharides to H5N1 viruses was detected (Figure 1A–C). And enhanced α2, 6-binding preference was also detected in A/GD/1/06 and A/GX/1/08. The α2, 6-binding was dose dependent for sialyglycopolymers and virus titer. Notably, as compared with A/GD/1/06 of both short- and long-α2, 6 recognition, A/GX/1/08 prefers to bind to long-α2, six oligosaccharides at low viral titer (Figure 1B,C). However, both of them showed strong affinity to short- and long-α2, 6 oligosaccharides at high viral loads (Figure 1D).

Discussion

Sialoside-, galactoside-, mannoside- and sulfo-OS-binding are the four types of carbohydrate-binding properties of influenza virus.[12] Binding of influenza virus to the α2, 3-or α2, 6-linked sialylated glycans on cell surface is important for host range restriction, and the preference to α2, 3 of H5N1 virus limited its efficient infection in human. Here, dual receptor-binding preferences were detected in A/GD/1/06 and A/GX/1/08, which are of clade 2·3·4. Although there is no direct evidence supporting the occurrence of human-to-human transmission in these infection events or the association between viral virulence and receptor-binding switching, viral systemic disseminations are found in the both fatal cases (data not shown). Furthermore, with the introduction of clade 2·3·4 into the adjacent countries of China,[4] the finding of H5N1 virus with 2-6-binding in human should be of concern.

Though H5N1 virus with human-type receptor-binding was isolated from one patient treated by oseltamivir and

those viruses were with HA and/or NA substitutions,[13] whether the substitutions responsible for receptor specificity switching is pre-existed or selected in human host remains unknown. Our finding that three mutant viruses bearing M2 mutations of A30S, A30T, and S31N cloned from one isolate A/HB/1/06 suggested it is likely that the resistant viruses emerged in the host environment. No variation was found in their HA and NA sequence, and all of them show high affinity to α2-3-binding. Our data suggest that the binding-specificity was not affected by the mutations on viral envelope protein M2.

With the adaptation from wild aquatic birds to domestic poultry or even in human host environment, influenza virus may possess broader carbohydrate-binding spectrum or topology conformation.[11,14] We demonstrated differential α2, 6-binding property of two human H5N1 viruses, A/GD/1/06 and A/GX/1/08. Though minor effect of short- α2, 6-binding was detected in viruses A/GX/1/08 at low virus titer, both were of high affinity to long- α2, 6 glycans, even at the low titer which are rich on apical side of human upper respiratory epithelia.[11] Notably, no evident binding preference switching was detected in the viruses isolated from the sporadic human infection cases at the early of 2009 in China (Table 1). However, higher affinity to the long- α2, 6 glycans was observed in BJ/1/09, GZ/1/09, and XJ/1/09 (data not shown). The discrepancy from the findings obtained by sialidase-treated CRBC maybe associated with a limited abundance of N-linked α2-6 with long branches on CRBC, as demonstrated in a recent study.[11] Therefore, glycan dose-dependent binding assay is valuable and should be applied in flu surveillance. The underlying cause of the tendency is unknown, and further research on receptor-binding specificity of H5N1 viruses is required.

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