

## Swine-origin influenza-virus-induced acute lung injury: Novel or classical pathogenesis?

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### Abstract

Influenza viruses are common respiratory pathogens in humans and can cause serious infection that leads to the development of pneumonia. Due to their host-range diversity, genetic and antigenic diversity, and potential to reassort genetically *in vivo*, influenza A viruses are continual sources of novel influenza strains that lead to the emergence of periodic epidemics and outbreaks in humans. Thus, newly emerging viral diseases are always major threats to public health. In March 2009, a novel influenza virus suddenly emerged and caused a worldwide pandemic. The novel pandemic influenza virus was genetically and antigenically distinct from previous seasonal human influenza A/H1N1 viruses; it was identified to have originated from pigs, and further genetic analysis revealed it as a subtype of A/H1N1, thus later called a swine-origin influenza virus A/H1N1. Since the novel virus emerged, epidemiological surveys and research on experimental animal models have been conducted, and characteristics of the novel influenza virus have been determined but the exact mechanisms of pulmonary pathogenesis remain to be elucidated. In this editorial, we summa-

rize and discuss the recent pandemic caused by the novel swine-origin influenza virus A/H1N1 with a focus on the mechanism of pathogenesis to obtain an insight into potential therapeutic strategies.

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**Key words:** Acute lung injury; Influenza virus A/H1N1-pdm; Pandemic; Swine-origin influenza virus

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### INTRODUCTION

Influenza is one of the most important human infectious diseases, which is responsible for 33000 to 51000 annual deaths worldwide<sup>[1]</sup>. Influenza is an acute respiratory disease that is characterized by the sudden onset of high fever, chills, coughing, sore throat, muscle pain, severe headache, prostration, malaise, and inflammation of the upper respiratory tract and trachea, with general discomfort, but it rarely induces severe inflammatory lung diseases, including pneumonic involvement<sup>[2,3]</sup>, due to host innate and acquired immunity<sup>[4]</sup>.

In March and early April 2009, a novel influenza virus emerged in Mexico and the United States and spread rapidly around the world<sup>[5-9]</sup>. On April 15 and 17, two unrelated cases of febrile respiratory illness in children in Southern California were confirmed to be caused by the

novel influenza virus<sup>[9]</sup>. On June 11, the World Health Organization (WHO) declared the global pandemic alert level to phase 6, the pandemic phase. During the early phase of this influenza pandemic, there was a sudden increase in the rate of severe pneumonia and shift in the age distribution of patients toward the aged, which was reminiscent of past pandemics and suggested relative protection of individuals who were exposed to H1N1 strains during the 1918 pandemic. At that time, there were uncertainties about all aspects of the outbreak, including the virulence, transmissibility, pathogenicity, and origin of the virus.

This 2009 pandemic virus originated by reassortment among several influenza A virus strains; sequence analysis of the novel pandemic virus revealed that hemagglutinin (HA), nucleoprotein (NP) and NS gene segments were derived from the classical swine viruses; PB1 gene segment from human seasonal H3N2 influenza viruses; and PB2 and PA gene segments from avian influenza viruses<sup>[9]</sup>. In addition, neuraminidase (NA) and M gene segments originated from the Eurasian swine viruses, which confirmed that the novel swine-derived pandemic influenza virus (A/H1N1pdm) is genetically and antigenically distinct from previously identified human isolates of H1N1 subtype<sup>[9]</sup>.

Another highly pathogenic strain of avian influenza virus of H5N1 subtype also causes severe pneumonia and lymphopenia, which is characterized by high levels of cytokines and chemokines<sup>[10]</sup>. Such deregulated cytokine/chemokine induction, which is called hypercytokinemia and hyperchemokinaemia, might be associated with the level of virus replication; the “cytokine storm” has been thought to be one of the factors that causes acute lung injury induced by H5N1 subtype<sup>[10]</sup>. The A/H1N1pdm virus also causes severe lung injury or other virus-associated diseases in humans, especially children, but the exact mechanisms remain unclear. The cytokine storm induction could be a fascinating explanation for the highly pathogenic potential of the A/H1N1pdm virus. Nevertheless, the A/H1N1pdm virus induces a weak cytokine response<sup>[11]</sup>. Additionally, the deduced amino acid sequence has revealed the absence of markers associated with high pathogenicity seen in avian or mammalian viruses<sup>[12]</sup>. Based on data from current investigations, the 2009 pandemic virus possesses a totally different property that is distinct from other pandemic isolates. In this paper, we summarize and discuss research on the 2009 pandemic caused by the novel influenza virus of A/H1N1 subtype, with particular attention to pathogenesis of influenza-virus-induced diseases.

## PROPERTIES OF INFLUENZA VIRUSES

Influenza viruses belong to the family *Orthomyxoviridae*, and are enveloped negative-sense strand RNA viruses with segmented genomes that contain seven or eight gene segments<sup>[13,14]</sup>. Each gene segment contains a coding region that encodes one or two viral proteins; three

segments (1, 2 and 3) encode proteins that form the viral polymerase complex: polymerase basic protein 2 (PB2), PB1 and polymerase acidic protein (PA), respectively<sup>[13,14]</sup>. Two segments (4 and 6) encode surface envelope glycoproteins that function as viral antigens, HA and NA, respectively<sup>[13,14]</sup>. Segment 5 encodes NP. Segment 7 encodes two proteins, the matrix protein M1 and M2. The smallest segment 8 encodes two non-structural proteins NS1 and NS2.

Three phylogenetically and antigenically distinct viral subtypes, A, B and C, are circulating globally among human populations, and subtype A influenza viruses have exhibited the greatest genetic diversity, infected the widest range of host species, and caused the vast majority of severe disease in humans<sup>[13,14]</sup>. The influenza A viruses are further subdivided by antigenic characterization of the surface glycoproteins HA and NA; so far, 16 HA subtypes (H1-H16) and nine NA subtypes (N1-N9) are known<sup>[13,14]</sup>.

Influenza viruses accumulate point mutations during replication due to poor fidelity of RNA transcription<sup>[15]</sup>. The continual mutations in the antigenic portion of surface glycoproteins may result in “antigenic drift”; it produces selective advantages for the influenza virus to escape from the immunity of its host<sup>[16]</sup>. Furthermore, the regions that undergo changes in such selective pressure move between antigenic clusters, which correspond to the changes in interaction between virus and host<sup>[17,18]</sup>, which indicates the possibility of natural genome-editing competency of viruses<sup>[19,20]</sup>. Natural selection favors amino acid variants of the HA and NA proteins that allow the virus to evade preexisting immunity, therefore, human immune response to viral infection is not completely cross-protective<sup>[21]</sup>. In addition, the segmented genome of influenza virus facilitates “antigenic shift”, a reassortment between more than one isolate when they co-infect the same host cell; a virus acquires HA of a novel subtype from the other, which generates a novel combination of HA and NA antigens to which the population is immunologically naïve<sup>[14]</sup>.

Receptor preference (usage) of each virus strain can be another factor to determine the cell tropism<sup>[22]</sup>. The binding of influenza viruses to their target cells (basically epithelial cells in upper respiratory tract) is mediated by viral HA, and binding preference to specific receptors is a crucial step in influenza virus infection efficiency<sup>[22]</sup>. The cell receptor for influenza viruses is sialic acid (SiA)<sup>[22]</sup>. The membranes of avian or mammalian cells express slightly different configurations of SiA, which depends on how the galactose residue of SiA is linked to membrane oligosaccharides<sup>[22]</sup>. On avian cells, the galactose residue of SiA is linked *via*  $\alpha$ 2,3 C atoms ( $\alpha$ 2,3 SiA) and on mammalian cells *via*  $\alpha$ 2,6 C atoms ( $\alpha$ 2,6 SiA)<sup>[22]</sup>. During evolution, avian and human influenza viruses have acquired HA that is adapted to these species-specific receptor modifications. In the pig trachea, epithelial cells contain both  $\alpha$ 2,3 SiA and  $\alpha$ 2,6 SiA, which explains why pigs are highly susceptible to both human and avian viruses, and are thought to be a place for avian/human

virus reassortment, which can produce epidemic/pandemic influenza strains<sup>[22]</sup>. Such a continual antigenic drift/antigenic shift, followed by the change in receptor preference, has caused not only regular seasonal epidemics in humans, other mammalian species and birds, but also worldwide influenza pandemics.

## INFLUENZA PANDEMICS: PAST AND PRESENT

We have experienced three influenza pandemics in the 20th century: 1918, 1957 and 1968<sup>[23,24]</sup>. All three have been identified as distinct pandemics by their presumed places of origin, Spain, Asia, and Hong Kong, and by different antigenic subtypes of influenza A virus, H1N1, H2N2 and H3N2, respectively<sup>[23,24]</sup>. Additionally, other epidemics have occurred several times. Here, we look back over the history of influenza pandemics and how they resemble or were different from the 2009 pandemic that was caused by the novel swine-origin influenza virus A/H1N1.

### **Spanish pandemic caused by H1N1 subtype**

The 1918 Spanish pandemic was the most lethal influenza pandemic in history<sup>[25]</sup>. In the spring of 1918, influenza in humans spread rapidly all over the world and was prevalent from Canton, China, to the most northern parts of Manchuria and from Shanghai to Szechuan. In October 1918, a disease diagnosed as influenza appeared in Russian and Chinese pigs in the area that surrounds Harbin; such epidemiological evidence showed the spread of virus from humans to swine. An estimated one third of the world's population (approximately 500 million people) was infected and had clinically apparent illness during the 1918 pandemic. Most communities experienced morbidity of 25%-40%. Children under 15 years of age experienced the highest rates of infection. Although the clinical course was self-limiting, a substantially higher percentage of cases developed severe pneumonic complications. Owing to the lack of virological methods, the 1918 pandemic virus was not isolated during the pandemic. Later, the genomic sequences of the 1918 pandemic virus were determined as an avian-like H1N1 subtype that contains human-like signature amino acids in several proteins<sup>[26]</sup>. After the appearance of a novel H2N2 subtype of influenza pandemic strain in 1957, the 1918 pandemic strain disappeared from human circulation.

### **Asian pandemic caused by H2N2 subtype**

After the outbreak of Asian influenza in Southern China in February 1957, it spread to Singapore, Hong Kong and Japan, and eventually even the United States and the United Kingdom in October 1957<sup>[23,24]</sup>. Victims were estimated to be 250 000 people in Hong Kong and 70 000 in the United States. The pandemic virus was caused by a reassortment between avian and human strains; *HA* (H2 subtype) and *NA* (N2 subtype) genes from an avian virus were introduced into a previously circulating human

strain, thus eventually defined as H2N2 subtype<sup>[12]</sup>. Additionally, the Asian pandemic influenza virus possessed a *PB1* gene of avian virus origin<sup>[12]</sup>. The Asian pandemic strain of H2N2 subtype was destined for short survival in the human population and disappeared with the appearance of another novel pandemic strain of H3N2 subtype in 1968.

### **Hong Kong pandemic caused by H3N2 subtype**

A decade after the 1957 pandemic, important differences in the pattern of illness and death were noted as an epidemic progressed throughout Asia<sup>[23,24]</sup>. With the arrival of the virus on the West Coast of the United States, high morbidity and mortality rates were striking, with casualties estimated at 30 000 people<sup>[12]</sup>. The virus was first isolated in Hong Kong in July 1968, and genetic analysis revealed that the 1968 pandemic strain was generated by another reassortment between avian and human strains; *HA* (H3 subtype) genes from an avian virus were introduced into human populations, thus making it a novel virus with H3N2 subtype<sup>[12]</sup>. The Hong Kong pandemic strain also possessed a *PB1* gene of avian virus origin.

### **Another example of an epidemic caused by swine H1N1 subtype virus**

Until now, swine influenza viruses are not known to have caused a global outbreak of influenza in humans, except for one possible example; swine influenza virus was apparently confirmed at an army training base in Fort Dix, NJ, USA in 1976<sup>[27]</sup>. The virus was similar to strains that had been circulating in pigs for several years. Significant human-to-human transmission was observed with up to 230 cases resulting in 12 hospitalizations and one death. It is noteworthy that the first mass vaccination for influenza caused a serious side effect of Guillain-Barré syndrome, an acute inflammatory demyelinating disease that results in paralysis and subsequent death<sup>[27]</sup>.

### **2009 pandemic caused by a novel swine-origin influenza virus A/H1N1**

As described above, human-to-human transmission of swine influenza viruses has been very limited, until March and early April 2009, when a novel influenza A virus of swine origin emerged in Mexico and the United States and spread rapidly around the world<sup>[5-9]</sup>. In seasonal influenza most deaths occur in persons older than 65 years, however, the 2009 pandemic predominantly affected the young, with a median age between 12 and 22 years, which suggested age-related preexisting immunity<sup>[28,29]</sup>. The crystal structure of the HA from A/H1N1pdm showed that antigenic structure within the Sa antigenic site was very similar to that of human H1N1 viruses that were circulating early in the 20th century<sup>[30]</sup>. This antigenic similarity between the A/H1N1pdm and 1918 pandemic H1N1-like viruses could provide an explanation for the age-related preexisting immunity against the current pandemic influenza viruses in people born early in the 20th century.

Initial genetic characterization of the A/H1N1pdm

outbreak suggested swine as its source, based on the sequence similarity to previously reported swine influenza isolates<sup>[9]</sup>. This 2009 pandemic virus contained a unique gene combination that has not been previously reported among human or swine influenza viruses. Genetic analysis has revealed in detail that the novel virus was generated by triple reassortment; *HA*, *NP* and *NS* gene segments were derived from the classical swine viruses with North America lineage; *PB1* gene segment was from human seasonal H3N2 influenza viruses; and *PB2* and *PA* gene segments were from avian influenza viruses<sup>[9]</sup>. Furthermore, *NA* and *M* gene segments originated from the Eurasian swine virus lineage, which confirms that the A/H1N1pdm is a completely distinct virus from previously identified isolates<sup>[9]</sup>.

The A/H1N1pdm is estimated to have infected over 22 million people in the United States alone<sup>[31]</sup>. Despite rapid spread of the virus, A/H1N1pdm appears to be no more virulent than the seasonal strains of influenza virus, with a case fatality rate of less than 0.5%<sup>[31]</sup>, which suggests that A/H1N1pdm is significantly less virulent than many of the avian influenza viruses, as well as the 1918 pandemic strain with H1N1 subtype that caused the most deadly pandemic in human history.

## PATHOGENESIS OF INFLUENZA-INDUCED DISEASES

The typical signs and symptoms of influenza are basically both local (nasal congestion, cough, and sore throat) and systemic (headache, fever, chills, anorexia, and myalgia)<sup>[2,3]</sup>. These signs and symptoms are due both to the damage at the site of virus replication and to the local and systemic release of cytokines/chemokines and other inflammatory products. The symptoms of A/H1N1pdm infection are generally similar to those of seasonal human influenza virus infection<sup>[32]</sup>. However, the A/H1N1pdm can further cause severe pulmonary disease, particularly acute respiratory distress syndrome (ARDS), which is the most severe form of acute lung injury<sup>[33]</sup>. There have been several published studies of the ARDS; in the case of Mexico, from March 24 to April 24, 2009, a total of 18 cases of pneumonia and confirmed A/H1N1pdm infection were identified among 98 patients hospitalized for acute respiratory illness<sup>[34]</sup>. More than half of the 18 patients were between 13 and 47 years of age<sup>[34]</sup>. All patients showed not only typical symptoms, but also increased serum lactate dehydrogenase levels and bilateral patchy pneumonia. As seen in ARDS induced by avian influenza virus H5N1, dysregulated cytokine/chemokine production could be involved in ARDS induced by the A/H1N1pdm virus; however, A/H1N1pdm induced weak cytokine responses in *in vitro* assays<sup>[11]</sup>. In the acute stage, multifocal destruction and desquamation of the pseudostratified columnar epithelium of the trachea and bronchi were remarkable characteristics, and only a basal layer of the epithelium remained. Later stages showed mononuclear inflammatory cell infiltration into the bronchial walls<sup>[35]</sup>. The lung pathology of deceased patients infected with

the A/H1N1pdm virus at autopsy has shown primarily diffuse alveolar damage, with extensive hyaline membrane formation and intra-alveolar edema<sup>[36,37]</sup>. Necrotizing bronchitis and bronchiolitis were also observed<sup>[37]</sup>. Immunohistochemical staining has demonstrated viral antigen expression in alveolar macrophages but less in type I and II pneumocytes<sup>[35]</sup>. Such changes in the A/H1N1pdm infection resemble those caused by the 1918 pandemic strain<sup>[38]</sup>, thus, the A/H1N1pdm virus probably possesses unidentified advantages to cause such pulmonary disorders in human lungs.

Although pulmonary disorders have been well studied, another important complication of influenza virus infection is central nervous system dysfunction, including influenza-associated acute encephalopathy (IAAE)<sup>[3]</sup>. This is an uncommon neurological syndrome of children that typically presents during the early phase of influenza virus infection<sup>[39]</sup>. Influenza virus can be occasionally detected in the cerebrospinal fluid (CSF) of patients with IAAE. Some neurotropic influenza viruses have indeed been reported to be involved in neuropathogenesis in mice<sup>[40,41]</sup>. However, the frequent failure to detect the viral antigens in CSF does not support this hypothesis; an alternative idea for the neuropathogenesis is hypercytokinemia, which does not require virus infection outside of the lungs. One study has described acute encephalopathy caused by A/H1N1pdm virus infection, without detecting the viral RNA in CSF by reverse transcription polymerase chain reaction<sup>[42]</sup>. The exact incidence of neurological complications caused by A/H1N1pdm virus has not been determined, and the exact mechanisms of IAAE induced by A/H1N1pdm infection remain controversial.

## INFLUENZA PATHOGENESIS IN EXPERIMENTAL ANIMAL MODELS

Animal models, particularly mice, have proven to be a useful tool for the study of influenza viruses, due to its utility in measuring infectivity and pathogenesis, and subsequent application for the evaluation of vaccines and antiviral compounds<sup>[43]</sup>. Mice are not a natural host for influenza viruses, therefore, adaptation of human strains to murine lung is generally required. During passage, variant virus strains acquire mutations that affect function, which are crucial determinants of virulence in mice<sup>[44]</sup>. Although human influenza viruses can efficiently replicate in mice only after they are adapted to grow in these animals, A/H1N1pdm virus replicates efficiently in mouse lungs without prior host adaptation, which suggests that this virus is more pathogenic in mammalian models than are seasonal H1N1 influenza viruses<sup>[45-49]</sup>. The ability of A/California/04/2009 (CA04) virus, a strain of A/H1N1pdm, to replicate in the lungs of mice, ferrets and non-human primates, and to cause appreciable pathology in this organ, might have contributed to viral pneumonia that was characterized by diffuse alveolar damage. There were marked differences in the induction of pro-inflammatory cytokines in the lungs of mice infected with



CA04 virus compared with a seasonal influenza strain of H1N1 subtype, A/Kawasaki/UTK-4/09. Infection with CA04 virus led to strong induction of both interferon (IFN)- $\gamma$ , interleukin (IL)-4, IL-5 and IL-10 in the lungs of mice<sup>[45]</sup>. In addition, markedly higher levels of monocyte chemoattractant protein-1, macrophage inflammatory protein-1 $\alpha$ , IL-6 and IL-18 were observed in the lungs of macaques infected with CA04 virus<sup>[45]</sup>. The enhanced viral replication and lung pathology observed in CA04-infected mice were not directly linked to dysregulated IL-17 production<sup>[45]</sup>. Similar titers were detected in nasal turbinates of mice infected with A/H1N1pdm or seasonal influenza virus; however, A/H1N1pdm replicated more efficiently in the lungs of infected animals, which could account for the prominent bronchitis and alveolitis with viral antigen expression. Besides lungs, A/H1N1pdm virus was undetectable in the spleen, kidneys, brain, liver and colon, which indicates that A/H1N1pdm virus does not spread to extrapulmonary organs in mice<sup>[45]</sup>.

Ferrets have also been widely accepted as a suitable laboratory animal model for influenza virus research, particularly on pathogenicity and transmissibility<sup>[43]</sup>. Morbidity and lung viral titers are higher in ferrets infected with A/H1N1pdm virus as compared with those infected with the seasonal influenza H1N1 virus<sup>[46,47]</sup>. Histopathologically, infected ferrets show multifocal necrotizing rhinitis, tracheitis, bronchitis, and bronchiolitis, and viral expression in the nasal cavity, trachea, bronchus and bronchioles, but rarely in the alveoli<sup>[47]</sup>. Moreover, antigen is detected in the intestinal tract<sup>[46]</sup>, consistent with gastrointestinal involvement among some human A/H1N1pdm cases<sup>[7]</sup>.

The novel A/H1N1pdm virus exhibits distinct properties from other seasonal or pandemic strains reported previously. Therefore, which factors could determine its pathogenicity in humans?

## DETERMINANTS OF INFLUENZA PATHOGENICITY

Based on genetic composition analysis, the A/H1N1pdm virus appears to have been generated from swine/human/avian triple reassortment: *HA*, *NP* and *NS* gene segments were derived from the classical swine viruses with North America lineage; *NA* and *M* gene segments were from the Eurasian swine virus lineage; *PB1* gene segment was from human seasonal H3N2 influenza viruses; and *PB2* and *PA* gene segments were from avian influenza viruses<sup>[9]</sup>. The A/H1N1pdm virus unexpectedly does not possess markers associated with high pathogenicity.

### PB2

PB2 is a viral RNA polymerase that forms a complex with other viral polymerases PA and PB1<sup>[50]</sup>. The amino acid at position 627 of the PB2 protein was first described as a host range determinant, on the basis of *in vitro* studies<sup>[51]</sup>. The respective amino acid change has been shown to determine the pathogenicity of H5N1 influenza viruses in mice<sup>[52]</sup>. Viruses with Lys at this position were pathogenic

in mice, whereas those with Glu are non-pathogenic<sup>[52]</sup>. Notably, almost all human influenza viruses possess Lys, whereas most avian viruses possess Glu at position 627<sup>[52]</sup>. Lys at position 627 of PB2 is currently recognized as a determinant of viral pathogenicity in several mammalian species<sup>[12]</sup>. Furthermore, Asn at position 701 of PB2 has also been recognized as a determinant of virulence; a role that is probably related to its facilitation of binding of PB2 to importin in mammalian cells<sup>[53]</sup>. The A/H1N1pdm virus possesses low-pathogenic-type amino acids at positions 627 and 701, which are Glu and Asp, respectively<sup>[12]</sup>.

### PB1-F2

The *PB1* gene of human influenza A viruses encodes a second protein, PB1-F2, that is expressed from the +1 reading frame<sup>[54]</sup>. The smallest protein PB1-F2 induces apoptosis, probably by interacting with two mitochondrial proteins, enhances inflammation in mice, and increases the frequency and severity of secondary bacterial infections, which suggests one molecular marker of pathogenicity<sup>[55]</sup>. The PB1-F2 contributes to viral pathogenesis *in vivo* preventing efficient viral clearance, although it does not have any effects on viral replication *in vitro*<sup>[56]</sup>. In fact, it is consistently present in viruses known to be of increased virulence in humans, including the viruses that caused the 1918, 1957 and 1968 pandemics<sup>[56]</sup>. The length of PB1-F2 of swine influenza viruses differs depending on their origin; classical swine viruses possess truncated PB1-F2 proteins of 8-11 amino acids, whereas Eurasian avian-like swine viruses possess full-length PB1-F2 proteins that consist of 87-89 amino acids<sup>[12]</sup>. The A/H1N1pdm virus encodes a truncated PB1-F2 protein of 11 amino acids, possibly with less biological function.

### NS1

The NS1 protein of some strains of influenza A virus has been shown to be a multifunctional immune modulator; the combination of all these functions makes the NS1 protein a very potent inhibitor of immunity and allows influenza virus to escape efficiently from immune surveillance and to establish infection in the host<sup>[57]</sup>. In particular, NS1 plays a crucial role in innate immunity; NS1 regulates IFN- $\beta$  signaling by affecting transcription factors and IFN- $\beta$ -responsive genes in a strain-dependent manner<sup>[58]</sup>. Furthermore, NS1 interacts with the retinoic-acid-induced gene-I (RIG-I) and inhibits the RIG-I pathway, thus preventing activation of IFN- $\beta$ <sup>[59]</sup>. The NS1 protein also affects virulence *via* regulating the immune response in infected cells; indeed several amino acids in NS1 proteins have been shown to affect virulence<sup>[60]</sup>. For example, Glu at position 92 of NS1 is recognized as a determinant of H5N1 viral pathogenicity<sup>[60]</sup>. The A/H1N1pdm virus possesses the low-pathogenic-type amino acid at position 92, which is Asp<sup>[12]</sup>. In addition, the four C-terminal amino acids of NS1 are thought to be crucial for influenza pathogenesis, by forming a PDZ ligand domain motif<sup>[61]</sup>. Introduction of the PDZ ligand domains of highly

pathogenic H5N1 viruses or the pandemic 1918 virus into an otherwise ordinary human virus confers slightly increased virulence and is not paralleled by increased IFN production<sup>[61]</sup>. The A/H1N1pdm virus lacks the 11 amino acids at the C terminus, including the PDZ domain motif, which suggests lower pathogenicity of A/H1N1pdm. However, restoration of Crk/CrkL binding motif or extension of NS1 to 230 amino acids has no impact on virus replication in human or swine cells, as well as minimal effects on replication, pathogenicity and transmission in animal models<sup>[62]</sup>. This suggests that the A/H1N1pdm virus is fully optimized to replicate efficiently without requiring certain NS1 biological functions.

## HA

HA cleavage is a crucial process for viral infectivity; exposure of the amino terminus of the HA2 domain mediates fusion between the viral envelope and endosomal membrane<sup>[13]</sup>. Efficiency of HA cleavage is determined by the amino acid sequence at the cleavage site<sup>[13]</sup>. Avian viruses with low pathogenicity and non-avian viruses possess a single basic amino acid (Arg) at the cleavage site, which is cleaved by proteases in the respiratory or intestinal systems<sup>[13]</sup>, whereas highly pathogenic influenza viruses of H5 or H7 subtype possess several basic amino acids that are recognized by ubiquitous proteases at the cleavage site<sup>[63]</sup>. Increased pathogenicity of avian influenza viruses has been linked to the acquisition of multibasic HA cleavage sites, which indicates the significance of the HA cleavage motif for virulence<sup>[63]</sup>. The A/H1N1pdm virus possesses a single basic amino acid Arg at the cleavage site, as well as other pandemic viruses, including the 1918 Spanish virus H1N1, 1957 Asian virus H2N2, and 1968 Hong Kong virus H3N2<sup>[12]</sup>.

## PREVENTION AND TREATMENT FOR INFLUENZA VIRUS INFECTION

### Vaccines

Vaccination is the preferred strategy for prevention and control of influenza and subsequent inflammation. Most of the currently licensed influenza vaccines are in the form of inactivated antigen preparations. The first of the inactivated vaccine formulations was the whole virion; the experimental use of which dates back to the 1940s<sup>[64]</sup>. The second inactivated formulation was a split virion that was derived by disrupting whole virus particles with detergents. The final formulation was the subunit form, which was prepared by enriching for the viral surface glycoproteins HA and NA following disruption of viral particles<sup>[64]</sup>. Split and subunit vaccines have subsequently proven to be safe and have been delivered to many millions of people for several decades. Seasonal influenza vaccines include human influenza A viruses of the H1N1 and H3N2 subtypes, and an influenza B virus; the 2009-2010 seasonal conventional influenza vaccine was composed from A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2)

and B/Brisbane/60/2008, a reassortant seed virus that possesses the HA and NA segments of the circulating virus<sup>[65]</sup>. These vaccines need to be revised every 1-3 years owing to mutations in the HA and NA proteins of globally circulating influenza viruses. In fact, based on surveillance data of circulating influenza virus strains, the WHO have recommended A/California/7/2009 (H1N1)-like, A/Perth/16/2009 (H3N2)-like, and B/Brisbane/60/2008-like viruses as vaccine strains for the 2010-2011 northern hemisphere season<sup>[66]</sup>. Instead of A/Perth/16/2009 (H3N2)-like virus, A/Wisconsin/15/2009 has been recommended as a vaccine strain for the 2010 southern hemisphere season<sup>[66]</sup>. Remarkably, development of reverse genetics systems<sup>[67]</sup>, which enable one to generate influenza viruses from cloned DNA, made investigation of molecular mechanisms of influenza pathogenesis possible and also efficient vaccine production<sup>[68]</sup>. The WHO summarizes the current status of development and availability of vaccine virus candidates as shown in Table 1<sup>[69]</sup>.

Live attenuated virus vaccines are administered by intranasal inoculation of replication-competent viruses<sup>[64]</sup>. In ferrets, the effect of the 2008-2009 formulations of commercially available cold-adapted live attenuated (FluMist<sup>®</sup>) or inactivated split (Fluviral<sup>®</sup>) vaccines, and a commercial swine vaccine (FluSure<sup>®</sup>) against A/H1N1pdm has been evaluated<sup>[70]</sup>. The advantage of the live attenuated virus vaccine approach is that both humoral and cellular immune responses can be theoretically elicited, thus it is believed to be superior to inactivated vaccines<sup>[71]</sup>. Cell-mediated immunity would be more preferable as a vaccine, since it targets the conserved viral proteins<sup>[72]</sup>. In fact, in infants and young children, live attenuated virus vaccine provides better protection than inactivated vaccine.

As a result of the inherent variation of the influenza virus HA protein, a number of attempts have been made to design influenza vaccines based on conserved viral epitopes<sup>[72]</sup>. One of the candidates for universal target antigen vaccines is the extracellular domain of the M2 protein, M2e. It consists of 24 amino acids at the N terminus of M2 protein, including the N-terminal epitope SLLTEVET (amino acids positioned at 2-9), which is remarkably conserved among all subtypes of influenza viruses<sup>[72,73]</sup>. Phase I clinical trials have been completed with the M2e approach<sup>[73]</sup>. Besides M2e, a number of other proteins have been targeted, including NP and PB through T-cell-mediated approaches and conserved domains of HA<sup>[72]</sup>. The immunogenicity of this part of the HA protein and its protective potential when used as the immunizing antigen on A/H1N1pdm infection remain to be determined. The development of faster, safer, more cross-reactive and more effective vaccines for the current pandemic and the next is expected.

### Antiviral compounds

There are two approved classes of antiviral compounds used against influenza, including NA inhibitors oseltamivir (sold as Tamiflu<sup>®</sup>) and zanamivir (sold as Relenza<sup>®</sup>), and M2 protein inhibitors amantadine (sold as Symmetrel<sup>®</sup>)

Table 1 Summary of status of development and availability of A/California/7/2009 (H1N1)-like candidate vaccine viruses

Parent virus	Candidate vaccine virus	Type of virus or reassortant	Developing institute	Available from	
A/California/7/2009 (H1N1)	X-179A	Wild type virus		WHO CCs	NIBSC, UK
	IVR-153	Classical	NYMC, USA	CDC, USA	NIBSC, UK
	X-181		CSL, Australia	CSL, Australia	NIBSC, UK
	X-181A		NYMC, USA	NYMC, USA	NIBSC, UK
	NIBRG-121	Reverse genetics	NIBSC, UK	NIBSC, UK	
	NIBRG-121xp NIIDRG-5			NIID, Japan	NIID, Japan
A/California/4/2009 (H1N1)	CBER-RG2	Wild type virus		WHO CCs	
		Reverse genetics	CBER/FDA, USA	CDC, USA	
A/Texas/5/2009 (H1N1)	IDCDC-RG15 IDCDC-RG20	Wild type virus		WHO CCs	
		Reverse genetics	CDC, USA	CDC, USA	
A/England/195/2009 (H1N1)	NIBRG-122	Wild type virus		WHO CCs	NIBSC, UK
		Reverse genetics	NIBSC, UK	NIBSC, UK	
A/Texas/5/2009 (H1N1) and A/New York/18/2009 (H1N1)	IDCDC-RG18	Reverse genetics	CDC, USA	CDC, USA	
A/New York/18/2009 (H1N1)	IDCDC-RG22	Wild type virus		WHO CCs	NIBSC, UK
		Reverse genetics	CDC, USA	CDC, USA	

Adapted from WHO<sup>[65]</sup>. CBER/FDA: Center for Biologics Evaluation and Research/Food and Drug Administration; CDC: Centers for Disease Control and Prevention; NIBSC: National Institute for Biological Standards and Control; NIID: National Institute of Infectious Diseases; NYMC: New York Medical College; WHO CCs: World Health Organization Collaborating Centers on Influenza.

and rimantadine (sold as Flumadine<sup>®</sup>)<sup>[74]</sup>. The M2 sequence of A/H1N1pdm virus possesses a mutation at position 31 from Ser to Asn, which is known to confer resistance to the M2 proton channel inhibitors such as amantadine and rimantadine<sup>[7]</sup>. NA inhibitors are currently preferred for influenza virus infections since they are less toxic and more effective than the M2 inhibitors. In mice, an A/H1N1pdm strain CA04 was highly susceptible to oseltamivir phosphate, zanamivir, R-125489 [the active form of CS-8958 (laninamivir)], and viral RNA polymerase inhibitor T-705 (favipiravir)<sup>[75,76]</sup>. Peramivir is another NA inhibitor under development for the treatment and prevention of influenza<sup>[77]</sup>. In the initial 13 clinical isolates of the 2009 pandemic reported by the Centers for Disease Control and Prevention, all cases were susceptible to NA inhibitors including peramivir<sup>[7]</sup>. In October 2009, the US Food and Drug Administration granted a license for peramivir for intravenous administration for the treatment of certain adult and pediatric patients with 2009 pandemic influenza. A drug such as amantadine, for which resistance frequently emerges, should be used in combination therapy to decrease the emergence of resistant virus strains. Besides approved anti-influenza virus compounds, ribavirin, IFN or antiviral substances approved for other indications other than influenza viruses, might serve as additional treatment options to inhibit viral replication completely.

### Immunomodulatory function of probiotics

The addition of adjuvant to the vaccine is associated with enhanced immunogenicity and subsequent improved protection against influenza virus infection<sup>[64]</sup>. An alternative method to improve the response to the vaccine would be the consumption of probiotics in food and drinks<sup>[78]</sup>. Probiotics are defined as live microorganisms or micro-

bial food components<sup>[79]</sup>. Oral administration of probiotics has been reported to enhance innate and adaptive immunity in the host<sup>[80]</sup>. Lactobacilli, non-pathogenic Gram-positive inhabitants of normal human intestine, are known for their health-promoting effects such as non-specific enhancement of the immune system, protection against intestinal infection, decrease of serum cholesterol levels, and anti-carcinogenic activities<sup>[81]</sup>. There are several lines of evidence that oral administration of *Lactobacillales* increases resistance against influenza A virus infection<sup>[82-88]</sup>. Oral administration of *Lactobacillus casei* strain Shirota (LcS) to neonatal mice ameliorated influenza virus A/Puerto Rico/8/34 (H1N1) infection, as assessed by survival rate and virus titers in nasal washing. Oral administration of LcS accelerated innate immunity of the respiratory tract, which resulted in protection against influenza virus infection in neonates<sup>[82]</sup>. In addition, *Bifidobacterium breve* YIT4064 and *Lactobacillus fermentum* CECT5716 have been reported to show anti-influenza virus effects by oral administration<sup>[83-85]</sup>. Intranasal as well as oral administration of *Lactobacillus rhamnosus* GG and *Lactobacillus gasseri* TMC0356 also protected mice from influenza virus A/Puerto Rico/8/34 (H1N1) infection<sup>[86,87]</sup>. Very recently, oral administration of heat-killed *Lactobacillus plantarum* L-137 (HK-LP)<sup>[88-92]</sup>, a strain isolated from fermented food, exhibited protection against influenza virus A/FM/1/47 (H1N1) infection in C57BL/6 mice; the survival time was significantly prolonged in mice treated with HK-LP<sup>[88]</sup>. Moreover, the viral titers in the lung were significantly lower in mice treated with HK-LP at the early stage after influenza virus infection, and an appreciable level of IFN- $\beta$  was detected in the serum of mice treated with HK-LP, which suggested that HK-LP, a potent IFN- $\beta$  as well as IL-12 inducer, is useful for



prevention against influenza virus infection<sup>[88]</sup>. Given the promising results in these studies, it will be interesting to investigate the specific effects of the probiotics on the novel A/H1N1pdm infection.

## CONCLUSION

Major influenza pandemics show no predictable periodicity or pattern, and all differ from one another, thus influenza viruses will continue to be a major health threat. The rapid, continuous, and unpredictable nature of influenza A virus evolution makes vaccine strategies and pandemic preparation difficult, although our assumption is that there will be a pandemic every 30-50 years. We experienced the first influenza pandemic of the 21st century, which was caused by the novel A/H1N1pdm virus subtype. In contrast to previous pandemics, virulence and pathogenicity seemed much milder in the current pandemic, and the molecular characteristics of the circulating virus based on the current research support this conclusion. However, there is some considerable concern that co-circulating seasonal influenza A viruses during the winter season, or highly pathogenic avian viruses might lead to the emergence of more virulent reassortant strains, although there have been no reports of emergence of such reassortant viruses since April 2009. With the continuous threat of an influenza pandemic, there is an urgent need to develop safe and effective vaccines and/or antiviral compounds against divergent influenza viruses, particularly the current pandemic strain A/H1N1pdm and the highly pathogenic H5N1 strain.

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