

Minireview

Molecular characteristics of the human pandemic influenza A virus (H1N1)

S. PAYUNGPORN¹, N. PANJAWORAYAN², J. MAKKOCH³, Y. POOVORAWAN^{3*}

¹Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; ²Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand; ³Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

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Summary. – The outbreak of the human pandemic influenza A (H1N1) has caused a considerable public concern. The aim of this review was to improve our understanding of this novel virus by analyzing the relationships between its molecular characteristics and pathogenic properties. Results of this analysis indicate that the human pandemic influenza A (H1N1) virus is a new re-assorted virus, which combines genetic materials from the avian flu (H1N1) virus, classical swine flu (H1N1) virus, human flu (H3N2) virus, and Eurasian swine flu (H1N1) virus. Analysis of the sequences for receptor-binding and cleavage sites of hemagglutinin (HA), stalk region of neuraminidase (NA), non-structural protein 1 (NS1), polymerase basic protein 2 (PB2), and polymerase basic protein 1 (PB1) suggested that (i) the human pandemic influenza A (H1N1) virus is a low virulent and low pathogenic virus, (ii) its replication is restricted to the cells of upper respiratory tract, so it does not lead to a systemic infection, (iii) it spreads among humans only, (iv) its replication could be inhibited by oseltamivir, zanamivir, interferon (IFN), and tumor necrosis factor α (TNF- α). A potential application of amantadine might be complicated by the drug-resistant virus strains.

Keywords: molecular characteristics; pandemic; influenza A virus; H1N1

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*Corresponding author. E-mail: Yong.P@chula.ac.th; fax: +66-22564929.

Abbreviations: HA = hemagglutinin; HA₀ = hemagglutinin precursor protein; HPAI = highly pathogenic avian influenza virus; IFN = interferon; LPAI = low pathogenic avian influenza virus; M1 = M1 matrix protein; M2 = M2 ion channel protein; NA = neuraminidase; NEP = nuclear export protein; NP = nucleoprotein; NS = non-structural protein; PA = polymerase acid protein; PB1 = polymerase basic protein 1; PB2 = polymerase basic protein 2; RNP = ribonucleoprotein; SA = sialic acid; TNF- α = tumor necrosis factor α

1. Introduction

The global outbreak of the human pandemic influenza A virus (H1N1) has caused a significant alertness in the general public (Dawood *et al.*, 2009). Analysis of the genetic material allows a prediction of the new influenza virus

characteristics including its capacity to spread the infection, degree of virulence, and drug resistance. This article therefore focuses on the relationship between the molecular characteristics of the human pandemic flu H1N1 virus and its clinical symptoms. It aims at the better understanding of influenza virus in order to alleviate public distress.

2. Virological characteristics of influenza A virus

The most recent human pandemic flu H1N1 virus has been classified as an influenza A virus that displays spherical or filamentous forms of envelope and its size is approximately 80–120 nm in diameter. The genome of influenza A virus contains eight pieces of negative-sense single-stranded RNA, which can be translated into ten viral proteins, namely PB2, PB1, polymerase acid (PA), HA, nucleoprotein (NP), NA, matrix (M1), ion channel protein (M2), NS1, and nuclear export protein (NEP or NS2). Each of these proteins has a specific function and all of them are involved in the viral replication (Nicholson *et al.*, 2003; Ludwig *et al.*, 2003).

HA is the major surface glycoprotein of the virus. It plays two main biological roles: (i) target recognition by serving as a receptor-binding site for the sialic acid (SA) receptors of host cells and, (ii) facilitation of influenza virus entry into the cell cytoplasm through a mechanism known as the receptor-mediated endocytosis. The receptor binding capability of HA is specific and dependent on the type of host SA and its specific linkage to the oligosaccharide of receptor. Therefore, the receptor binding specificity of HA has been considered for determination of the host range.

M2-ion channel is a small transmembrane protein responsible for the pumping protons into viral particles from the endosome. The influx of proton creates an acidic environment inside of the virus and causes viral envelopes to undergo a conformational change. Subsequent uncoating of the virus releases the viral genome and viral proteins into the cytoplasm of infected cells.

NP encapsulates the viral RNA genome forming a ribonucleoprotein (RNP). NP also helps to stabilize the viral genome and regulates the synthesis and replicative transcription.

Polymerase complex consists of three different proteins, namely PB2, PB1, and PA. These proteins are involved in RNA transcription and replication. NEP or NS2 plays an important role in the export of nascent RNP from nucleus to the cytoplasm. M1 is the major protein component of the virion. It helps to assemble the viral proteins and drives viral budding.

NA or sialidase is a crucial enzyme for the virus survival and pathogenicity. It cleaves the cellular SA residues that link the newly formed virions to the host cell membrane. This

cleavage releases new virions and allows them to infect the adjacent cells freely.

NS1 is not a part of the viral particles and has been found only in the infected cells. Its most important role is to regulate a viral gene expression such as RNA splicing and translation. Moreover, it plays a significant role in the suppressing of host anti-viral response.

3. Subtypes of influenza A virus and mutation

At present, the influenza A virus can be categorized into different subtypes characterized by the type of HA and NA surface glycoproteins. HA includes 16 different types (H1-H16), whereas NA has been reported to include nine types (N1-N9) (Fouchier *et al.*, 2005). In order to distinguish subtype of the influenza A virus, the types of HA and NA have to be specified. For example, influenza A virus may display the subtype H1N1, H3N2, or H5N1. Each subtype of the influenza A virus exhibits different host preference (Nicholson *et al.*, 2003). Generally, all subtypes of the influenza A viruses (H1-H16 and N1-N9) can infect aquatic birds, while only six subtypes have been documented to infect humans (H1N1, H2N2, H3N2, H5N1, H7N7, and H9N2). Subtypes H1N1 and H3N2 are seasonal human influenza viruses that infect humans annually. On the other hand, the subtypes H5N1, H7N7, and H9N2 have evolved from the avian flu viruses and can potentially infect humans. The likelihood of this cross-infection, however, is very low. As for the swine influenza virus, four subtypes H1N1, H1N2, H3N2, and H4N6 have been reported and only some of them can infect the humans. Particularly, swine H1N1 and H3N2 viruses are significantly different from the human seasonal H1N1 and H3N2 viruses, respectively.

The host specificity of influenza A virus has changed markedly over time due to the genetic mutations that have led to a rapid evolution of the virus. Two major processes can cause mutations in the influenza A virus – antigenic drift and antigenic shift (Suarez *et al.*, 2000).

Antigenic drift presents a process, when minor mutations accumulate and modify the properties of proteins. A lack of proofreading mechanism within the viral RNA polymerase causes frequent mutations in the influenza A genome. Mutations occurring in the essential proteins such as HA or NA can generate a new virus strain resistant to its host's immune system as exemplified by the human seasonal flu viruses. In addition, the antigenic drift enables influenza virus to cross the species barrier as in the case of avian flu viruses (from birds to the humans).

Antigenic shift is a process occurring by a genetic reassortment of at least two different influenza viruses that infect the same cell. This process gives rise to a re-assorted virus that contains a mixture of the genetic material of two original

virus strains. The re-assorted virus with the resulting major antigenic alteration can be especially virulent, because it is not restrained by host immunity to previously prevalent strains of the virus. Notably, the influenza A virus mutated by the antigenic shift have been reported to spread wider than those mutated by antigenic drift.

4. Genetic evolution of the human pandemic influenza A virus (H1N1)

In April 2009, the flu outbreak caused by a novel influenza virus was first reported in Mexico. Bioinformatic analysis of its viral genome indicated that the virus is a new re-assorted virus containing combined genetic materials from human, avian, and swine influenza A viruses (Garten *et al.*, 2009). Phylogenetic analysis indicated that the first genetic reassortment among the classical swine H1N1 virus, contemporary human H3N2 virus, and avian H1N1 virus took place in North America in 1998. This genetic reassortment generated a triple re-assorted H3N2 virus that subsequently recombined with the swine H1N1 virus and resulted in two different re-assorted viruses known as the triple re-assorted H1N1 and the triple re-assorted H1N2. These two re-assorted viruses were later found to infect pigs in North America as well as in Asia generating a new type of the re-assorted virus. The genes of its genome have caused the recent outbreak of the human pandemic influenza A virus (H1N1).

PB2 and PA genes originated from the 1998 avian H1N1 virus in North America. PB1 gene arose from the human flu H3N2 virus that had evolved in pigs in 1998 as a genetic reassortment from the original 1968 avian flu virus. HA, NP, and NS genes evolved from the classical swine H1N1 virus detected in North America. NA and M genes arose from the Eurasian (Europe and Asia) avian-like swine H1N1 virus.

5. Molecular properties of the human pandemic influenza A virus (H1N1)

Various researchers have studied the molecular biology of the avian H5N1, seasonal human flu H1N1 and classical swine flu H1N1 viruses. The classification of human pandemic flu H1N1 virus as an influenza A virus allows a comparative analysis among this group of viruses to investigate its capacity for the infection, replication, pathogenicity, and drug resistance.

5.1 Receptor binding site of HA

The receptor binding site on HA molecule is crucial for the virus to bind specifically to SA receptor on the cell

surface. This receptor-binding specificity determines the ability of virus to infect its potential host. For example, the receptor binding site on HA of the avian flu H5N1 virus contains amino acids glutamine-serine-glycine (QSG) that specifically bind to SA linked to galactose (Gal) by α -2,3 linkage (SA- α -2,3Gal) on the cells of avian respiratory system. On the other hand, the avian flu H5N1 virus poorly infects humans, because the cell receptors of the human upper respiratory system contain abundant linkage SA- α -2,6Gal. However, the cell receptors of human lower respiratory system contain the linkage SA- α -2,3Gal. Thus, in the case of a very high viral load and a close contact, the avian flu H5N1 virus can infect the human lower respiratory system and cause the illness (Shinya *et al.*, 2006; van Riel *et al.*, 2006).

The receptor-binding site of swine flu virus contains the amino acids sequence QAG that can bind to SA receptors of the swine respiratory system. The receptor-binding site of the seasonal human flu H1N1 virus contains the amino acids QEG that bind specifically to the SA- α -2,6Gal of the human upper respiratory system. Consequently, the seasonal human flu H1N1 virus can infect and cause symptoms in humans.

In order to study the receptor binding specificity of the human pandemic flu H1N1 virus, a comparative analysis was performed using HA molecules of the different H1N1 isolates. The results indicate that the receptor binding site of all isolates of the human pandemic flu H1N1 virus contain the sequence of amino acids QEG as reported for the seasonal human flu H1N1 virus (Fig. 1). Consequently, this finding suggests that the human pandemic flu H1N1 virus has the capacity to infect the human cells of the upper respiratory system.

In addition, a recent study (Recombinomics Commentary; available on line at http://www.recombinomics.com/News/11250902/D225G_Norway_China.html; Kilander *et al.*, 2010; Chen *et al.*, 2010) has indicated that the human pandemic H1N1 virus found in Ukraine, Brazil (Sao Paulo), Hongkong, China (Zhejiang), and Norway contains the D225G mutation (H3 numbering system) at the receptor binding site. The presence of this mutation could facilitate the infection of human lower respiratory system by the binding of virus to SA- α -2,3Gal receptor and to initiate a lung infection (Stevens *et al.*, 2006).

5.2 Cleavage site of HA

The avian flu viruses can be classified into two types according to the viral pathogenicity and cleavage site on the HA molecule. The first type of viruses is called the low pathogenic avian influenza (LPAI) viruses. Its HA precursor protein (HA₀) is cleaved by the enzyme designated as tissue specific trypsin-like protease that is exclusively expressed

	Receptor binding site	Cleavage site
	220 230	320 330 340
	IRPKVRDQEGRMN	TGLRNVPSIQ~~~~SRGLFGAIA
Pandemic human flu H1N1 viruses		
A/Thailand/104/2009 (H1N1)		
A/Arizona/01/2009 (H1N1)		
A/Auckland/1/2009 (H1N1)		
A/Brisbane/17/2009 (H1N1)		
A/California/04/2009 (H1N1)		I
A/Canada-AB/RV1532/2009 (H1N1)		
A/Christchurch/2/2009 (H1N1)		
A/Colorado/03/2009 (H1N1)		
A/Delaware/02/2009 (H1N1)		
A/Denmark/513/2009 (H1N1)		
A/El Salvador/213/2009 (H1N1)		
A/England/195/2009 (H1N1)		
A/Florida/04/2009 (H1N1)		
A/Indiana/09/2009 (H1N1)		
A/Kansas/02/2009 (H1N1)		
A/Korea/01/2009 (H1N1)		
A/Maryland/05/2009 (H1N1)		
A/Massachusetts/06/2009 (H1N1)		
A/Mexico/3955/2009 (H1N1)	G	
A/Michigan/02/2009 (H1N1)		
A/Nebraska/03/2009 (H1N1)		
A/Netherlands/602/2009 (H1N1)		
A/New Hampshire/02/2009 (H1N1)		
A/New Jersey/01/2009 (H1N1)	E	
A/New Mexico/04/2009 (H1N1)		
A/New York/04/2009 (H1N1)	G	
A/North Carolina/05/2009 (H1N1)		
A/Ohio/07/2009 (H1N1)		
A/South Carolina/09/2009 (H1N1)		
A/Tennessee/05/2009 (H1N1)		
A/Texas/04/2009 (H1N1)		
A/Virginia/04/2009 (H1N1)		
A/Washington/09/2009 (H1N1)		
A/Wisconsin/08/2009 (H1N1)		
Seasonal human flu H1N1 viruses		
A/Thailand/CU32/06 (H1N1)	K	I
A/Thailand/CU41/06 (H1N1)	K	IS
A/Thailand/CU44/06 (H1N1)	K	I
Swine flu H1N1 viruses		
A/swine/Chachoengsao/NTAH587/2005 (H1N1)	E	G.A.
A/swine/Chonburi/05CB1/2005 (H1N1)	E	G.A.
A/swine/Chonburi/06CB2/2006 (H1N1)	E	G.A.
Avian flu H5N1 viruses		
A/chicken/Thailand/CU-K2/2004 (H5N1)	T.S..NG.S...E	S.QRERRRKK
A/tiger/Thailand/CU-T7/2004 (H5N1)	T.S..NG.S...E	S.QRERRRKK
A/cat/Thailand/KU-02/04 (H5N1)	T.S..NG.S...E	S.QRERRRKK
A/doq/Thailand-Suphanburi/KU-08/04 (H5N1)	T.S..NG.S...E	S.QRERRRKK
A/Thailand/NK165/2005 (H5N1)	T.S..NG.S...E	S.QRERRRKK

Fig. 1

Multiple alignment of amino acid sequences of the receptor binding and cleavage sites of HA of influenza A viruses

in the respiratory system. Therefore, LPAI virus infects cells in the respiratory system only. In contrast, the second type of viruses called high pathogenic avian influenza (HPAI) viruses contain an insertion of basic amino acids (RRRKK) sequence at the cleavage site of the HA₀ protein that is cleaved by the enzyme furin (ubiquitous protease). This enzyme can be found in every part of the body and in consequence, HPAI virus can infect several cell types and causes a very virulent systemic infection (Horimoto *et al.*, 2005).

Comparative analysis of the HA cleavage site indicates that neither the swine flu H1N1 virus, seasonal human flu H1N1 nor human pandemic flu H1N1 viruses contains the sequence of polybasic amino acids RRRKK (Fig. 1). This outcome suggests that the swine flu H1N1 virus, seasonal human flu H1N1 virus, and human pandemic H1N1 virus are not highly pathogenic viruses and can only infect the cells in the respiratory system.

5.3 Stalk region of NA

Neuraminidase cleaves SA residues to which the newly formed virions are attached and in this way facilitates release of the virus during budding from the host cell membrane. Cleavage of the SA allows the new viral particles to be released and to invade the adjacent cells. Analysis of the NA sequence of avian H5N1 virus has revealed a deletion of 20 amino acids (positions 49–68) within the stalk region (the region between the viral membrane and the globular head). This deletion shortens the NA stalk-motif localized on the viral surface that results into the high pathogenicity and virulence of avian H5N1 virus (Zhou *et al.*, 2009). The swine H1N1 virus, seasonal human H1N1 virus, and human pandemic H1N1 virus do not display the deletion within the stalk region of their NA (Fig. 2). Therefore, the human pandemic H1N1 virus is not a virulent and highly pathogenic virus.

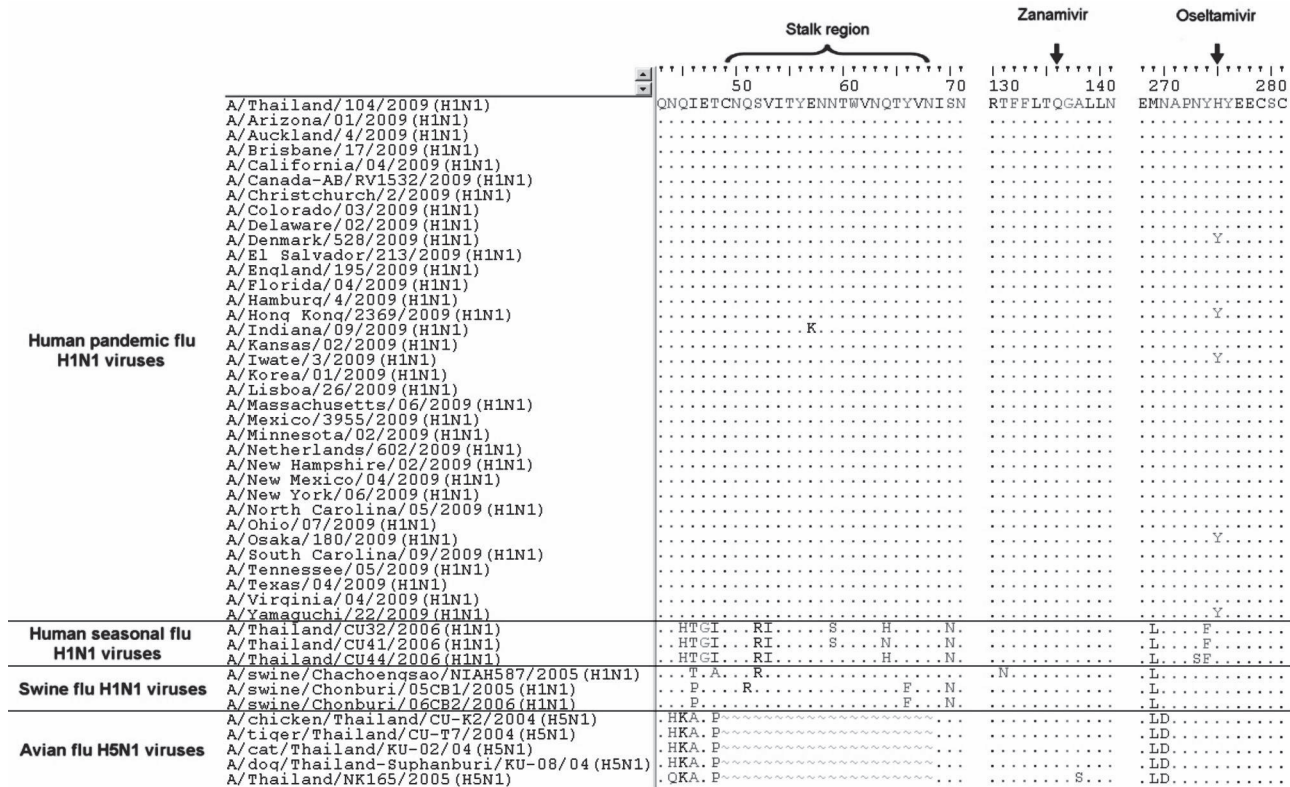


Fig. 2

Multiple alignment of amino acid sequences of the stalk region and zanamivir/oseltamivir resistance regions of NA of influenza A viruses

5.4 NS1 protein

Previous studies on NS1 protein have reported that the last four amino acids at C-terminus (positions 227–330) are formed by the ESEV motif (glutamic acid-serine-glutamic acid-valine). The ESEV motif functions as PDZ (postsynaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein) ligand domain for the protein-protein recognition and is involved in the several cell-signaling cascades within abnormal cells. Viruses that contain the ESEV motif are considered as highly pathogenic such as the Spanish flu H1N1 and avian flu H5N1 viruses (Jackson *et al.*, 2008). Analysis of the NS1 has shown that the human pandemic H1N1 virus does not contain the ESEV motif within the NS1, but displays a stop codon at the position 220 instead. This piece of evidence, therefore, indicates that the human pandemic flu H1N1 virus is a low pathogenic virus (Fig. 3).

5.5 PB2 protein

The rate of viral replication is regulated by the PB2 protein. According to the previous research, E627K mutation within PB2 protein can significantly increase the rate of viral replica-

tion and consequently to augment the virulence (Shinya *et al.*, 2004). Alternatively, the amino acids E627 and N701 within PB2 protein have been reported to increase the ability of viral replication and transmission in mammals (Steel *et al.*, 2009).

The result of amino acid analysis of PB2 protein shows that the swine H1N1 and human pandemic H1N1 viruses contain E627. In addition, the swine H1N1 virus displays D701N mutation, whereas the human pandemic H1N1 virus still holds D701. Therefore, the result suggests that the human pandemic flu H1N1 virus has a normal rate of viral replication and transmission in mammalian host (Fig. 4).

5.6 PB1-F2 protein

PB1-F2 protein is found only in some subtypes of influenza A virus. This protein is generated from the PB1 gene by an alternative open reading frame (ORF). It is a small protein composed of 87–90 amino acids. The PB1-F2 protein is involved in apoptosis and causes significant delay in the clearance of influenza virus by the host immune system. Therefore, a virus that produces the active PB1-F2 protein is considered as a highly pathogenic virus (Zamarin *et al.*, 2006), such as the 1918 flu virus dubbed as the Spanish flu

virus (McAuley *et al.*, 2007). Moreover, a single amino acid substitution N66S in the PB1-F2 molecule has been reported as a contributing factor for the high virulence and lethality of the virus (Conenello *et al.*, 2007).

Amino acid analysis of the human pandemic H1N1 virus indicates that it can generate a truncated PB1-F2 protein comprising only 11 amino acids due to an early stop codon at position 12. Therefore, the shortened PB1-F2 protein is not able to induce the apoptosis. This result suggests that the human pandemic flu H1N1 virus is a low pathogenic virus (Fig. 5).

5.7 Resistance to oseltamivir

Oseltamivir or Tamiflu is an anti-viral drug that inhibits the replication of influenza A and B viruses. The drug acts as a substrate analogue that binds specifically to the active site of NA and inhibits a viral spread. Studies of the avian H5N1 virus and seasonal human H1N1 virus have reported that the viruses can resist oseltamivir by H275Y mutation (N1 numbering system). This mutation on NA is frequently found in the seasonal human flu H1N1 virus that has caused infection since last year (Cheng *et al.*, 2009).

Analysis of the NA amino acid sequence has shown that the majority of human pandemic flu H1N1 viruses contain H275. Consequently, most of the viruses have not acquired drug resistance by mutation yet (Fig. 2). However, various strains of the human pandemic flu H1N1 viruses reported from Japan, Hong Kong, and Denmark contain the H275Y mutation in NA molecule and as a result, these viruses could

resist the oseltamivir action (Leung *et al.*, 2009). Therefore, oseltamivir treatment for patients infected with the human pandemic flu H1N1 virus must be reconsidered.

5.8 Resistance to zanamivir

Zanamivir or Relenza is an alternative NA inhibitor that inhibits the viral spread. Previous studies on seasonal human flu H1N1 virus reported that Q136K mutation on the NA could reduce zanamivir susceptibility by approximately 300-fold (Hurt *et al.*, 2009). According to the amino acid sequence alignment of NA (Fig. 2), the amino acid Q136 of human pandemic H1N1 has not undergone a mutation yet, what means that the virus is not resistant to the zanamivir. Therefore, the drug can effectively inhibit the viral replication of human pandemic H1N1 virus and in this way represents an alternative drug for the flu treatment.

5.9 Resistance to amantadine

Amantadine is an anti-viral drug designed to inhibit the function of M2 protein that pumps protons into viral particles. A large number of protons inside the cells create an acidic environment that induces uncoating of the viral particles. This process is essential in the early steps of viral replication, so amantadine can inhibit the influenza virus replication. Previous studies have shown that the influenza A virus can escape the effect of drug by mutation of several amino acids on the M2 protein, such as those in positions 26, 27, 30 or 31 (Furuse *et al.*, 2009). The most significant

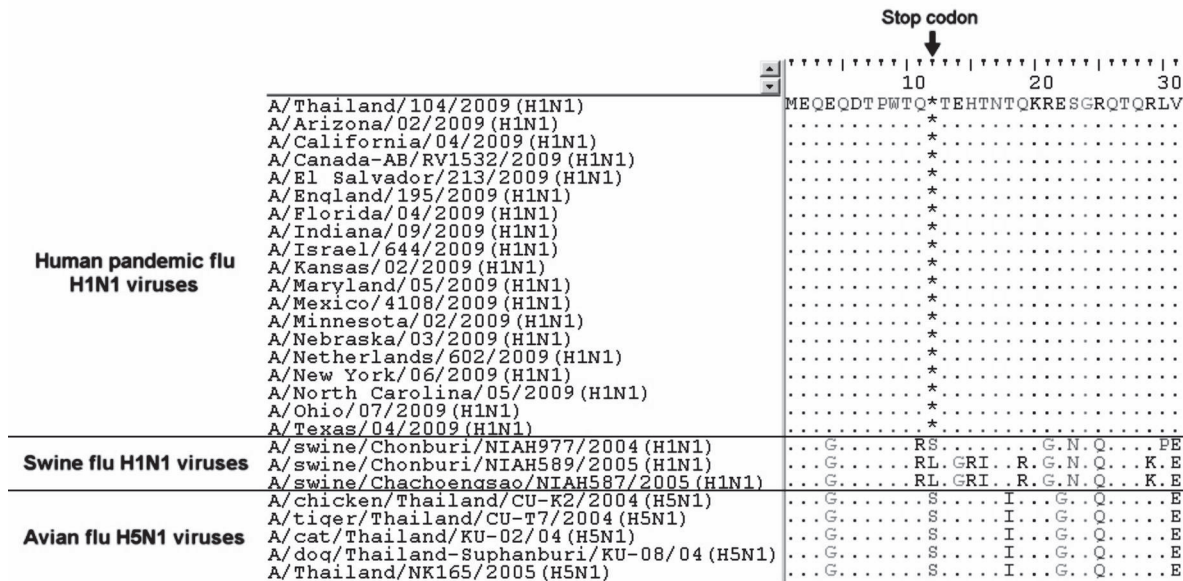


Fig. 5

Multiple alignment of amino acid sequences of an alternative ORF region of PB1 (PB1-F2) of influenza A viruses

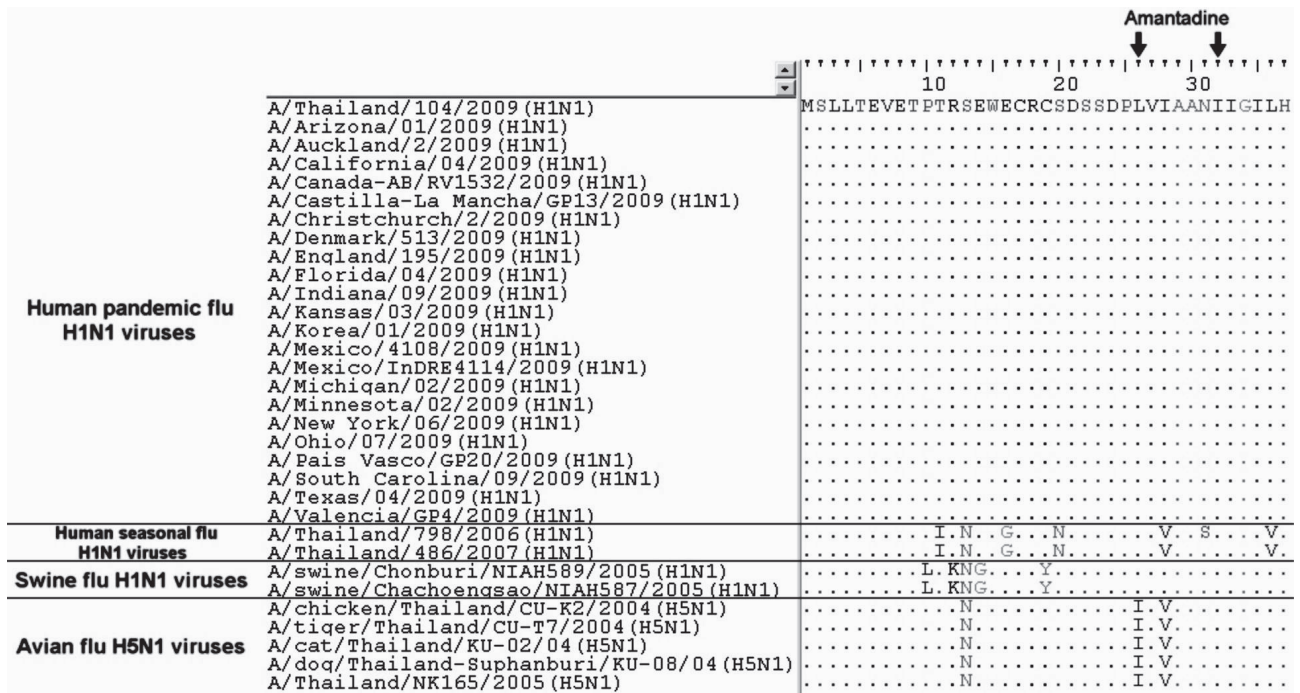


Fig. 6

Multiple alignment of amino acid sequences of the amantadine resistance region of M2 of influenza A viruses

mutation that could lead to the drug resistance is S31N mutation (Cheng *et al.*, 2009).

Bioinformatic analysis of the M2 protein showed that most of the current influenza A viruses including the human pandemic flu H1N1 contain the S31N mutation (Fig. 6). Consequently, amantadine cannot be used for the treatment of patients, who are infected with the human pandemic flu H1N1 virus.

5.10 Resistance to IFN and TNF- α

NS1 protein is expressed in the infected cells and involved in the suppression of host immunity. Within the host cells, IFN and TNF- α are cellular anti-viral molecules. However, the influenza viruses are able to resist the effect of IFN and TNF- α by the D92E mutation within NS1 protein (Seo *et al.*, 2004).

Analysis of the NS1 amino acid sequence has indicated that the human pandemic H1N1 virus contains non-mutated D92. This finding suggests that IFN and TNF- α can be alternatively used to suppress the viral replication of human pandemic H1N1 virus (Fig. 3).

6. Conclusions

Analysis based on the evolutionary research suggests that the human pandemic flu H1N1 virus is a re-assorted virus,

which is composed from various genetic materials of the North American avian flu H1N1 virus (PB2 and PA), human flu H3N2 virus (PB1), North American classical swine flu H1N1 virus (HA, NP, and NS), and Eurasian avian-like swine flu H1N1 virus (NA and M). Amino acid sequence analysis of the HA binding site suggests that the human pandemic flu H1N1 virus can easily cause the infection in humans. Further sequence analysis of the stalk region of NA, PB2, PB1-F2 proteins, specifically the ESEV motif at C-terminus of the NS1 suggests that the human pandemic flu H1N1 virus is a low pathogenic virus. According to the amino acid sequence analysis of NA, M2, and NS1, the human pandemic flu H1N1 virus can be treated with zanamivir, IFN, and TNF- α , but could not be treated with amantadine. In addition, oseltamivir treatment should also be considered with caution, since some strains of the human pandemic flu H1N1 virus have developed the drug resistance.

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