Thai Avian Influenza (H5N1) Virus

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This article presents knowledge and some updated information about H5N1 virus including of the avian influenza outbreaks in many countries, epidemiology of avian influenza viruses, general characteristic, mechanism of viral entry to the cell, and process of viral mutation and the laboratory diagnostic of infection. As we know, the infectivity of the virus depends on its surface protein, namely hemagglutinin, specifying to infected species. However, there were many cases, suggesting avian-to-human transmission; as a result, knowledge about its virology, infectivity and transmission are interesting and must be further studied. In human cases, most of them died of respiratory illness, whereas other types of influenza are self-limited; hence body defences reacting to the infection help us understanding why its severity was happened. In 2004, there were several probable human-to-human transmission cases in Thailand and led us to study in genetic characterization of H5N1 viruses, isolated in Thailand in that year, so we will review these topics in order to understanding about differences between H5N1 in Thailand and in other countries. Furthermore, these topics can be used as basic knowledge for further study in its possibility to cause human-to-human transmission in the future, leading to influenza pandemic. Besides that, clinical diagnosis is presented because it helps healthcare workers diagnose there patients who have influenza-like symptoms and give the appropriate treatment in a right time.

Keywords: • Avian-to-human transmission
• Probable human-to-human transmission
• Avian influenza

Influenza viruses are members of orthomyxoviridae family, with segmented genome. There are 3 types of influenza viruses that can infect people: A, B and C. Only A and B can cause widespread outbreak1. Influenza A virus may lead to avian-to-human transmission. This influenza viruses can be classified by surface proteins, hemagglutinin (HA) and neuraminidase (NA), dividing influenza A virus into many subtypes depending on types of these two proteins. There are 15 HA and 9 NA. Aquatic birds have all types of influenza A viruses. It has been believed that only H1, 2, 3 and N1, 2 are circulating in human population.

HA and NA are major proteins, determining whether an influenza virus is type A or B1. Both influenza A and B have 8 segmented RNA, regulating synthesis of 10 proteins, whereas influenza C has only 7. HA helps the virus to penetrate into a host cell through sialic acid receptor1. NA is used for catalyzing the cleavage of glycosidic linkage to sialic acid, leading to releasing of progeny of the virus form infected cell1. Moreover,
non-structural protein 1 (NS1) of the virus has been identified as an immune modulator 1 (Table 1).

From 1997 to the end of February 2005, there were several outbreaks in many country’s poultries, all around the world. It was known that these outbreaks especially in Asia were mostly caused by influenza A virus (H5N1) infection. This virus led to epidemic in poultry and economic loss. These events remind us to create a review article, collecting some updated information for healthcare workers, medical students and anyone who deals with avian influenza outbreaks to plan the policy to get rid of or prevent this virulent virus.

**Epidemiology**

Wild waterfowl are the natural reservoir of all influenza A viruses. Domestic ducks without illness can excrete large quantity of highly pathogenic H5N1 (fig.1) to poultry, resulting in severe illness and death. So, it is difficult to control this reservoir with invisible pathogen.

From early 19th century to February 2005, there are many times of avian influenza A outbreaks with reported human cases. “Spanish flu”, caused by H1N1 virus, was the first reported influenza outbreak in 1918 2. It killed 40-50 millions of people all around the world. Its probable cause was that avian influenza virus in animals had evolved themselves to be strain that could be transmitted among human. In 1957, H2N2 caused “Asian flu” in USA and killed 70,000 people in this country 3. Influenza outbreak was occurred again in USA in 1968 by H3N2 virus and it was called “Hong Kong flu” 4. 34,000 people died of this virus 4. The outbreaks on 1957 and 1968 were believed to be the result of genetic reassortment between human and avian influenza virus, originated in

**Table 1:** The relationship between eight segmented RNAs of influenza A, its products and viral proteins functions

<table>
<thead>
<tr>
<th>RNA Segment</th>
<th>Encoded-Proteins</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>NO. 1</td>
<td>PB2</td>
<td>These 3 proteins act as polymerase complex in viral replication.</td>
</tr>
<tr>
<td>NO. 2</td>
<td>PB1</td>
<td>Surface protein receptor.</td>
</tr>
<tr>
<td>NO. 3</td>
<td>PA</td>
<td>An essential molecule of nucleocapsid</td>
</tr>
<tr>
<td>NO. 4</td>
<td>HA</td>
<td>The enzyme that cleaves terminal acetyl neuraminic residues from oligosaccharides, glycoproteins or glycolipids, in the releasing process of viral progeny.</td>
</tr>
<tr>
<td>NO. 5</td>
<td>Nucleoprotein (NP)</td>
<td>Plays a role in viral replication.</td>
</tr>
<tr>
<td>NO. 6</td>
<td>NA</td>
<td>Ion channel in pH regulation</td>
</tr>
<tr>
<td>NO. 7</td>
<td>Matrix protein 1 (M1)</td>
<td>Assists the virus escape from host immunity.</td>
</tr>
<tr>
<td>NO. 8</td>
<td>Matrix protein 2 (M2)</td>
<td>Regulate releasing of vRNP.</td>
</tr>
</tbody>
</table>

These 2 proteins cannot be found in the virus, but can be found in infected cells.
Thai Avian Influenza (H5N1) Virus

Fig.1. The structure of H5N1 avian influenza virus: The virions with the genes encoded in its eight genomic negative-sense RNA segments, with glycoprotein - spikes on lipid envelope.

pigs, intermediated host¹ (fig 2). Another time of influenza outbreaks in USA was 1980, occurred by H7N7 virus. There were 4 patients with purulent conjunctivitis were reported in this event². This virus was

Fig.2. Antigenic shift process: genetic reassortment occurs between human and avian Influenza virus. The accidental recombination of the genetic material between those two viruses were happened in the intermediated host such as pigs.
also the origin of an outbreak in England in 1996 that had a human case\(^6\).

In 1997, H5N1 virus caused a great influenza outbreak in poultry in Hong Kong. 1.5 millions of poultry were culled in order to eradicate the virus\(^6\). Furthermore, there were 18 cases in human and six of them died of acute respiratory distress syndrome (ARDS)\(^7\), 33% mortality rate.

Influenza A H9N2 outbreak in China and Hong Kong was occurred in 1999 with two reported cases of children illness. Both of them were recovered. However, during 1998-1999, there were several additional H9N2 infection cases in China\(^6\). Virginia, 2002, H7N2 virus\(^8\) originated avian influenza outbreak with a human infection through direct contact with poultry. Influenza outbreak was again occurred in England in 2002 by H1N1, believed to be the reassortant between H1N1 and H3N2\(^2\).

2003 February, H5N1 outbreaks were happened in China and Hong Kong. Two patients who had just come back from China developed illness owing to H5N1 infection\(^5\) and one of them died\(^5\). A member of these two patients’ family in China died of respiratory illness without any investigation\(^6,8\). Moreover, in the middle of December, H5N1 outbreak occurred in poultry in Cambodia, Indonesia, Japan, Laos, Korea, Thailand and Vietnam. From that time to May 2004, 22 Vietnamese were infected and 15 of them died (68%).

The outbreak of H7N7 virus was recurrent in the Netherlands in 2003\(^8\). It brought about 89 cases of poultry-to-human transmission\(^8\). Most of them had conjunctivitis or influenza-like-illness; whereas, there was a dead case of ARDS and complications of the infection. Dutch authorities reported three possible instances of transmission from poultry workers to family members\(^8\). Another time of avian influenza in Hong Kong was late 2003, occurred by H5N2. There was a confirm case of a child with mild symptoms. And in the same year, there was an avian influenza outbreak, caused by H7N7 virus, in New York\(^4\). A confirmed patient with severe respiratory symptom, but reversible, was reported in November.

2004: Thailand and Vietnam (H5N1) in the early of year 2004, it was another time of H5N1 outbreaks in many countries in Asia. By the end of March, 12 Thai were infected by H5N1, 8 of them died of ARDS and multiple organ dysfunction syndrome (MODS)\(^5\). In addition, 23 Vietnamese was affected too\(^7\), 15 of them died (65.22%). In June, new lethal outbreak of H5N1 occurred again in poultry in Cambodia, China, Indonesia, Malaysia, Thailand and Vietnam\(^7\). In August, human cases of H5N1 infection in Thailand and Vietnam were happened again and in September, a report from Thailand suggested that human-to-human transmission is probable\(^7\). During the beginning of 2004 to October in the same year Thailand had 16 cases of H5N1 infection in human, 11 cases died\(^8\). From last year to Jan 21 2005, there had been 52 human cases of H5N1 in Vietnam and Thailand resulting in 37 deaths\(^7\). It has been reported in canada that there was an eye infection due to H7N3 in poultry workers\(^6\).

For this year 2005, influenza A H5N1 viral infection has been still an outbreak in three countries: Thailand, Vietnam, and Cambodia (9). The recent incidences in Thailand are reported H5N1 infection cases in Poultry(9). Sick, died with unknown cause and culled poultry are delivered for detection of H5N1 infection. There are not any reported human cases.

WHO reported that during January 28 to February 2, 2005 there were 37 confirmed cases and 29 of them are dead cases\(^9\). From the middle of December 2004, there are 10 confirmed cases with 90% mortality rate. Right now, April 2005, a dead confirmed case was reported from Cambodia\(^9\).
As we know, viral infection requires binding to host cell surface receptor and fusion of the virus and host cell membrane, following by endocytosis and the fusion of the virus and endosomal membrane. The protein which is responsible for binding and fusion of this virus to a host cell is HA, which is the primary target of neutralizing antibody. HA consists of homotrimers which each monomer is presented as a precursor (HA0), cleaved into HA1 and HA2 on infectious cells. HA2 is often referred to as ‘fusion peptide’, because it has hydrophobic domain at its N-terminus, essential for membrane fusion.

**Binding to host cell receptor**

Influenza A binds to oligosaccharide, containing N-acetyl neuraminic acid (sialic acid), attached to cell surface glycoprotein or glycolipid. This event can be inhibited by specific antiviral sera, enzyme, involved

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**Fig.3.** The arrangement of (HA) and (NA) molecules on the phospholipid L bilayer membrane of H5N1 avian influenza virus envelope. The HA, a rod-shaped glycoprotein with triangular cross-section, has importance functions in the attachment and entry of virus to the host cells and in determining virulence. The role of NA molecules involves in the releasing of virus progenies from the infected cells by excising neuraminic (sialic acid) from the host cell receptor protein.
in viral activities or by exogenous bacterial NA. From studies, amino acids on the receptor-binding site of HA are conserved; on the contrary, other amino acids around this site are usually changed by antigenic drift due to viral evolution.

In addition, HA molecule is consisting of 550 amino acid residues\(^1\), the area which most effectively binds to sialic acid receptor may be called ‘binding pocket’\(^10\). There are H-bonds, linked to amino acid residues at position 98, 153, 183 and 195 of HA, leading to the integrity of the binding site. If there are substitutions of Tyr 98, Trp 153, His 183, and Tyr 195 individually by Ala, the capacity of binding to the cell is decreased\(^10\). Additionally, substitutions of Ser 136 and a Leu to Ala at residue 194 decrease binding capability more than the former event. In the case that mutation in receptor binding site of HA lowers the capability of binding to a host cell; the decreased capacity can be compensated by high density of receptors on the host cell surface. The attachment to a host cell requires cooperation of multiple receptors interaction and balance between receptor and binding affinity. HA in each strain of virus has different binding property, depending on types of sialyloligosaccharides linkage. HA of human influenza virus binds preferentially to \(\alpha-2, 6\) linkage of sialyloligosaccharides whereas HA of avian influenza binds to \(\alpha-2, 3\) linkage\(^10,11\). The residues at position 222 and 226 of H5N1 hemagglutinin are most frequently implicated to involve in this process\(^10\). HA of strain for example H3N2, favouring \(\alpha-2, 3\) linkage possesses glutamine at 226 and glycine at 228\(^10\). On the contrary, binding to \(\alpha-2, 6\) linkage strains have leucine and serine in these positions respectively\(^10\).

\(\alpha-2,6\) oligosaccharides are found in pig’s trachea\(^10\). Nevertheless, pig can be co-infected by both human and avian influenza. This suggested that pig may play a role as the origin of influenza pandemic because it acts as ‘a mixing vessel’ to initiate a new reassortment of avian and human influenza. The result of this phenomenon is a new subtype of influenza, leading to avian-to-human transmission. Interestingly, in 1997 Honk Kong H5N1 outbreak, it was reported that it was directly transmitted from poultry to man.

Modulation of receptor affinity

Glycosylation and sialylation site, closed to RBS (receptor binding site) are major factors in releasing of H5N1 from an old cell to infect others\(^12\). Binding of glycans to SA reduces affinity of SA to RBS. Length of NA stalk is another modulator of H5N1 releasing\(^12\).

Membrane fusion

After binding to cell surface receptor, the virus must be endocytosed by fusion of its particle and host cell membrane. Fusion occurs in endosomal compartment. From this phenomenon, the virus can send its nucleocapsid into cytoplasm and then to nucleus in order to initiate RNA transcription and translation. Dissociation of viral nucleocapsid depends on acidification, regulated by cellular proton pumps and viral M2 protein, as an ion channel for itself. The reduction of endosomal pH triggers conformational change in HA2 structure, leading to membrane fusion. The rate of fusion is influenced by surface HA2 density and target lipid composition\(^10\).

There are some theoretical models for membrane fusion. One of these is an interesting hypothesis, called ‘stalk-pore hypothesis’ (figure 4).

Ability of H5N1 replication

PB2, PB1, PA, NP, M and NS proteins are involved
in host-species-specific lineages especially amino acid 627 in PB2 protein. Differences in these proteins affect duration and virulence of infection. (fig.5)

**Body Defense Over H5N1 Infection.**

When infection occurs, 2 systems of immune response: humoral immune response (HIR) and cell-mediated immune response (CMIR) are involved in this event. For the first system, Antibodies, reacting to HA reduce viral infectivity, while those reacting to NA; inhibit viral spreading from cell to cell. It is found that only additional glycosylation site on in HA lowers neutralizing activity of antibodies. In the CMIR, cytotoxic T-cells react to protein PA, NP, M and PB_2 of the virus. In addition, there is an innate immunity, also responses to virus through mediators-TNF-α and IFN. All influenza A susceptible species have IFN-inducible Mx protein, possessing anti-influenza property.

Double stranded RNAs (dsRNA), generated during replication, and are translated into proteins that induce immune responses. In viral evolution, NS1 binds with dsRNA in order to inhibit immune-inducing protein.
synthesis. So, NS1 helps virus evade from our immune.

H5N1 virus induces apoptosis (program cell death) of the host cell. Although the mechanism is unclear, the induction of apoptosis is occurred by transforming growth factor β (TGF-β), activated by NA which is not the only one activator of this apoptotic inducing factor. Moreover, it was found that PB1-F2 could induce apoptosis too.

1997 H5N1 was a great inducer of TNF-α, IFN-β and other inflammatory cytokines in human cultured macrophage, but in contrast, it resisted to these cytokines. A high potency of inflammatory cytokines induction, apoptotic induction, and the resistance to cytokines of the virus cause severe symptoms.

**H5N1 infection in other species**

H5N1 can be directly transmitted to mammals without mutation or recombination. Furthermore, in cases of avian-to-human transmission, it provides gradual adaptation of its receptor specificity from α-2.3 to α-2.6 (human) receptor without reassortment.

**Genetic characters of Thailand H5N1**

The complete genome of Thailand H5N1 was identified from 3 human samples, HA and NA genes from 2 humans and a sample from chicken. All of them have multiple basic amino acids in the cleavage site of HA gene. Their amino acids in receptor binding site were similar to those from Hong Kong 1997 H5N1. There is an interesting finding that this H5N1 has been still amantadine resistant, caused by M2 gene mutation. Besides that, it has amino acids, more specific to chicken than Hong Kong 1997 strain, indication that Thai strain has higher potential in avian transmission.

Avian-to-human transmission was firstly occurred in Hong Kong in 1997 by H5N1 virus. This virus is believed to be the reassortant that obtained HA gene from A/goose/Guangdong/1/96, NA from A/teal/Hong Kong/W132/27 and internal gene from A/quail/Hong Kong/G1/97 or A/teal/Hong Kong/W312/97.

In 2001, H5N1 was classified into 5 strains: A-E. But, after year 2002, those strains disappeared. However, there were new 8 strains: V, W, X1, X2, and X3, Y, Z and Z+.

H5N1 viruses, causing outbreaks during 2003-2004 had the same origin, by the way, their antigens differed from 1997 and early 2003 ones.

**Laboratory investigation of Thai H5N1 genome**

1. **Isolation and identification**

   Isolated viruses were identified by influenza A-specific monoclonal antibody staining on cultured tissue. The tissue was then stained with the second antibody, conjugated goat anti-mouse IgG, under indirect immunofluorescent assay. HA typing was done by RNA extraction, whose products would be amplified by reverse transcription PCR (RT-PCR). Nucleotide was used to identify types of NA genes.

2. **Amantadine in the anti-influenza virus agent**

   The mean OD (optical density) between amantadine sensitive virus and infected tissue culture cells without amantadine was compared.

3. **RNA extraction**

4. **Gene amplification**

   RT-PCR was used to amplify influenza genes because they are segmented RNA. As a consequence, this process changed viral RNA to DNA on order to initiate gene amplification. In this study, one-step RNA PCR kit was used to amplify PB1, PB2, PA and HA genes, while Qiagen One-step RT-PCR kit was for NP, NA, M and NS genes.

5. **Nucleotide sequencing**
Thai Avian Influenza (H5N1) Virus

PCR product from the process above was electrophoresed in 1% agarose gel. Pieces of gel containing bands of size-expected DNA were then purified by Qiagen Gel Extraction kit. Purified DNA was used as the target of direct nucleotide sequencing which its result was then analyzed by ABI PRISM version 310 or 377 DNA sequencer.

According to Puthavathana, et al. From Siriraj Hospital, a few specific characteristics of the avian flu virus such as the shortening of NA molecule (missing of 20 amino acids) and the additional glycosylation site in HA molecule (at 156 aminoacid residue) were detected. Accounting for the fact that the shortening of NA molecule decreased the spreading of viral progeny, the real H5N1 outbreak in Thailand was greatly spread, suggesting that it might be an extraglycosylation of 156 residues in HA can compensate the low infectivity of virus owing to its shorten NA.

The recently reports were indicated that the amantadine and rimantadine resistant viruses were found. These can be explained that was amino acid substitutions in one of these 4 positions in M2 protein: 26 (Leu → Phe), 27 (Val → Ala or Thr), 30 (Ala → Thr or Val) and 31 (Ser → Asn or Arg). Asn was found in position 31 in Thailand avian influenza.

An interesting fact was isolated viruses from both humans and chicken had 100% identity of amino acid residues in their receptor binding site. This confirmed that human samples were infected by direct avian-to-human transmission.

From the study, 32 amino acid residues in M1, M2, PA and NP protein play a role as host-specific residues but 31 from 32 of these residues in Thai avian influenza viruses were specific to avian species.

Possibility of avian influenza virus H5N1 transmitted from human to human

As mentioned above, avian influenza virus evolves to be more efficient strain for transmission from human to human. The major mechanisms are changes of HA and NA structure. The changes occur in two ways, antigenic drift (fig.5), occurred by point mutation and antigenic shift (fig.6, 7, 8, 9) which is the result of genetic reassortment of two difference subtypes of influenza virus. Human-to-human transmission can be
Fig. 6. Antigenic drift

Fig. 7. Antigenic shift process.

Fig. 8. Relationship between influenza epidemics and antigenic changes
Thai Avian Influenza (H5N1) Virus

Fig. 9. Influenza subtype and genetic variation

explained by three hypotheses.

1. Pigs act as a mixing vessel to initiate genetic reassortment because they can be infected by both avian and human influenza. Their genetic materials are exchanged during viral replication; leading to generation of new subtype of influenza that is can be transmitted from human to human.

2. Human is infected by both human and avian influenza, as a result, the reassortant; causing human-to-human influenza outbreak is occurred.

3. Human is directly infected by avian influenza virus. The viruses have evolved to be human-to-human transmittable strain.

Conclusion

This review focused on genetic characters of avian influenza (H5N1) virus, new emerging infection that was lethal outbreak since 2004 in Thailand. These outbreaks of avian influenza in Thailand we concerned that H5N1 become progressively more pathogenic in poultry, expanding the mammalian host range and highly lethal in humans Thus, the good surveillance system and laboratories for both human and birds testing are essential. Early detection and rapid response to the disease should be made in order to reduce the outbreaks in Asia region. However, there are several strategies to minimize the risk of virus transmission and spread the disease including segregation in farm settings of chickens, ducks, and other animals such as pigs and a reduction in contact between animals and humans or even though humans to humans. Even the bird flu vaccines in animals and next step in human is the best hope of prevention, Thailand has to learn more about the results of experimental bird flu vaccine tests in animals before take the next step forward to human and more concern with safety of vaccine for Thai people as well.

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