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## **Avian Influenza H5N1 Virus: An Emerging Global Pandemic**

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### **Abstract**

The specter of avian influenza emerging from Asia and spreading all over the globe is causing deeper concern by the day. As we witness the H5N1 virus evolving and becoming increasingly dangerous, a major pandemic may be unavoidable. The bird flu virus has already claimed more than 140 lives worldwide as of August 2006. Should bird flu spark a global pandemic, several hundred million people could die within a matter of weeks, which is many times the number of deaths due to AIDS so far. This pathogen is completely different from seasonal influenza virus, which kills between 1 and 2 million people worldwide in a typical year. In the worst previous pandemic of 1918, more than 20 million humans died of the Spanish flu. The current bird flu virus has emerged from a pool of animals that have previously never infected humans implying that humans do not have antibodies to combat the infection. This virus also causes severe disease and high fatality within a short time span. The only remaining factor to enable the virus to cause a pandemic is if it acquires the capability of swift transmission among humans through coughing, sneezing or just a simple handshake! The evolving bird flu virus has already crossed the species barrier from chickens to other birds and mammals including pigs. Pigs possess flu virus receptors on their respiratory cells which are similar to human receptors. Thus, pigs serve as an excellent mixing vessel for the virus to exchange genes through genetic reassortment to generate an entirely new viral strain that may be capable of efficient human-to-human transmission. This chapter describes the previous pandemics, influenza virus evolution, its molecular biology, replication, zoonosis and pathogenesis. Issues on pandemic preparedness and important strategies to contain or limit the spread of this virus for the present and future are discussed.

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Influenza viruses have been known to cause severe respiratory illness killing 250,000–500,000 people worldwide annually [1]. Ten influenza pandemics

defined by clinical and epidemiological records have occurred in the past three centuries, with an average of 1 every 33 years [2]. Pandemics are different from epidemics, such that they are defined as a global emerging disease caused by a novel virus or its subtype circulating among the human population. Belonging to the *Orthomyxoviridae* family, influenza viruses are classified into three major types A, B, C on the basis of two antigens, i.e. nucleoprotein (NP) and matrix (M1) protein. The avian influenza virus belongs to the type A which is the most virulent, and its further subtyping is based on the distinct glycoprotein antigens haemagglutinin (HA) [H1 to H16] and neuraminidase (NA) [N1 to N9]. Influenza A viruses have been isolated from humans and various animals including horses, pigs, sea mammals (e.g. seals and whales), wild waterfowl (e.g. wild ducks) and poultry [3]. Influenza B and C have been isolated from humans, and are known to cause local epidemics. Past influenza A pandemics that afflicted humans during the 20th century apparently arose from the Eurasian avian lineage of viruses [4].

Currently, H5N1 is considered to pose the greatest potential viral threat to humans. Although efficient human-to-human transmission has not yet been confirmed, it is certainly possible that a different subtype may arise by genetic reassortment of H5N1 with other influenza virus strains to generate the next pandemic strain.

### **Previous Pandemic Outbreaks**

Pandemics are global events which arise abruptly, rapidly cause extensive economic damage, killing millions along its path and subsiding eventually as abruptly as they began. During a pandemic, the causative virus achieves dominance over all other circulating influenza viruses in humans and continues to circulate for decades until replaced by another lethal pandemic strain [5]. Antigenic shift caused by genetic reassortment or antigenic drift may give rise to a pandemic strain. The 20th century has witnessed four human influenza pandemics with intervals of 9–39 years. China is considered the epicenter for pandemic outbreaks of influenza viruses. History indicates that the causative viruses of the influenza pandemics of H2N2 in 1957, H3N2 in 1968, and the re-emergence of H1N1 in 1977 had their origins in China. Recent outbreaks of H5N1 and H9N2 in Hong Kong emphasize the importance of virological surveillance in this region to facilitate early detection of potentially pandemic viruses.

### **The Spanish Influenza**

The H1N1 Spanish influenza (1918–1919) caused the most devastating pandemic witnessed by the human population, exacting a toll of 100 million lives

worldwide [1]. This infection had its origin in Europe, and rapidly progressed to parts of Asia and Africa via ships carrying troops from Europe and America. Evidence to link the origin of the 1918 strain to H1N1 swine influenza virus was not forthcoming until 1997 when Taubenberger et al. [6] succeeded in obtaining genetic information of this pandemic strain from a paraffin-embedded lung tissue sample of an infected individual. In addition to the strong sequence similarity to swine influenza A strain, the 1918 HA gene lacked multiple basic amino acids at the cleavage sites suggesting that pigs may have served as an intermediate host for reassortment from an avian influenza strain. Given the speed at which a new pandemic could spread in the modern world, the emergence of a strain as virulent as that of 1918 would be devastating.

### **The 1957 and 1968 Influenza Pandemics**

The 1957 Asian influenza pandemic resulted from the emergence of a reassortant influenza virus in which both the HA and NA segments had been replaced by gene segments closely related to avian strains [7–9]. The 1957 pandemic strain also acquired a novel N2 subtype replacing N1 of the previous strain. The sequence of the new NA was very closely related to the avian N2 sequences, with only six amino acids differing from the conserved avian sequences [9]. The 1968 pandemic strain H3N2 that circulated in Hong Kong appeared to have had an avian origin as well [10, 11]. This pandemic strain emerged with an avian-derived H3 segment, while retaining the N2 segment derived in 1957. More recently, it was demonstrated that the PB1 gene segment was also replaced in both 1957 and 1968 pandemic strains by a segment of avian derivation. Since PA, PB2, NP, M and NS were preserved from the H1N1 strains of 1918, the hypothesis for the generation of novel pandemic strains became accepted. As in 1918, the epidemic spread resulted in case morbidity, but the mortality rates were much lower. The world was better prepared to cope with the situation in view of the availability of antimicrobial agents and vaccines, and hence the casualties were less compared to the previous pandemic.

### **Influenza A H5N1 Emergence in Hong Kong, 1997**

The first cases of the outbreak appeared in May and November to December 1997 in Hong Kong, where 18 individuals were reported to be infected with H5N1 [12]. The human influenza virus isolates acquired all eight gene segments from Eurasian avian sources, with a preference for binding to the cell surface Neu5Gc 2–3 sialic acid receptor, a feature typical of avian influenza viruses. Although the outbreak was controlled by the slaughter of poultry stocks (1.5 million), the HA gene continued to circulate in geese in southern China [13].

**Table 1.** Human deaths due to bird flu (from 2003 to 23 August 2006)

Country	Cases	Deaths
Vietnam	93	42
Indonesia	60	46
Thailand	24	16
China	21	14
Egypt	14	6
Turkey	12	4
Azerbaijan	8	5
Cambodia	6	6
Iraq	2	2
Djibouti	1	0
Total	241	141

From 1997 to 2001, various genotypes of HA remained antigenically homogenous, but a significant change was observed in 2002. The H5N1 strain that was pathogenic only in chickens exhibited high pathogenicity in ducks and aquatic birds, thus implying antigenic change. No further human cases of H5N1 influenza were identified until February 2003, when H5N1 reemerged in a family in Hong Kong. The first patient was a 9-year-old boy who recovered from the infection during hospitalization, but his 33-year-old father and 8-year-old sister succumbed to the infection [14]. Genetic analysis revealed that the causative strain was similar to the antigenically distinct strain that was highly pathogenic for ducks and poultry.

Hundreds of millions of poultry animals have been slaughtered due to the unprecedented bird flu epidemic of H5N1 infection in Asia in 2004. The outbreaks were observed in countries such as China, Japan, South Korea, Vietnam, Cambodia, Laos, Thailand, Egypt, Turkey, Iraq, Malaysia, Djibouti and Indonesia. But the virus has taken its greatest toll on human lives in Vietnam, Indonesia, and Thailand followed by China (see table 1 for complete list). The H5N1 strain circulating in Asia is of Z genotype, which is endemic in birds in Southeast Asia and has undergone various antigenic changes and is similar to the strain isolated from Vietnam in 2004 [15]. The intensity of mammalian transmission of H5N1 can be attributed to several factors such as antigenic variation of HA, acquisition of high pathogenicity towards aquatic birds, and the susceptibility to genetic reassortment. These events portend that the potential risk of transmission between humans can eventually lead to the establishment of the H5N1 lineage among humans.

The bird flu virus which was initially considered to be endemic in Asia has now spread to Europe and even Africa. Cases of H5N1 infection have been reported from around the globe and the World Health Organization (WHO) now warns that this expanding geographical presence is of great concern as it

increases the susceptibility of human transmission globally. The outbreaks are attributed to contact and sharing of water sources between domestic poultry and migratory birds known to harbour and excrete the deadly virus.

## **H5N1 Virology**

The influenza A virus genome consists of eight segments of negative-sense single-stranded RNA that encode 10 proteins (HA, NA, M1, M2, NP, PB1, PB2, PA, NS1 and NS2). Under electron microscopy, the viruses are pleomorphic and subsequently appear spherical (approximately 120 nm in diameter). Two distinct types of spikes (approximately 16 nm in length), corresponding to the HA and NA molecules, reside on the surface of the virions. The HA spike appears rod-shaped and protrudes from the envelope as a trimer, while the NA spike is a mushroom-shaped tetramer. These two glycoproteins together with the M2 protein are anchored to the lipid bilayer envelope derived from the plasma membrane of host cells by short sequences of hydrophobic amino acids (transmembrane region). Inside the lipid envelope, eight segments of RNA are associated with nucleoprotein (NP) which resembles a helix. This RNA-NP complex contains the three polymerase proteins (PB1, PB2 and PA) [16].

### *Haemagglutinin*

HA is a type I glycoprotein containing an N-terminal ectodomain and a C-terminal anchor which is the major surface antigen of influenza A virus. HA plays a significant role in host cell recognition, attachment and fusion of the viral envelope with the host cell. Encoded by RNA segment 4, HA enables the viral particles to specifically attach to the cell surface receptors containing sialic acid. It is synthesized as a polyprotein precursor (HA0) that is post-translationally cleaved (involving processes such as proteolytic cleavage, glycosylation and fatty acid acylation) into two subunits HA1 and HA2, connected by disulphide linkages. This cleavage is carried out by trypsin-like proteases of the host. After cleavage, HA1 consists of 324 amino acids with variable carbohydrate groups and contains the antigenic determinants, while HA2 has about 222 amino acids with variable carbohydrate and 3 palmitate residues. Due to the error-prone activity of the RNA polymerase, the HA molecule undergoes a high rate of mutation ( $2 \times 10^{-3}$ ) resulting in the current 16 HA sub-types which are serologically distinct or partially cross-reactive. However, the amino acids that constitute the receptor-binding sites, as well as cysteine and proline residues, are highly conserved [17].

### *Neuraminidase*

NA is a type II glycoprotein containing an N-proximal anchor and a C-terminal ectodomain. This second surface antigen of influenza A virus can be

observed as a tetramer unevenly distributed in the lipid bilayer envelope. Encoded by RNA segment 6, the neuraminidase (sialidase) cleaves terminal sialic acid residues from glycoproteins and glycolipids [18] and hence permits virion entry. This enzyme is also required for elution of newly synthesized virions from infected cells, and aids in the movement of the virus through the mucus of the respiratory tract [19, 20], thus behaving as an essential adjuvant for pathogenicity. The amino-terminal hydrophobic sequence of NA directs transport of the virion to the cell membrane, and prevents viral clumping before the next infectious cycle begins. Similar to HA, NA is prone to mutation and its nine subtypes are not cross-reactive [21].

#### *M1 Protein*

Influenza virus RNA segment 7 is bicistronic, encoding both M1 and M2 proteins. The M gene encodes two partly overlapping proteins, i.e. a highly conserved 252-amino acid M1 protein and a 97-amino acid M2 protein [22]. The M1 protein forms an envelope over the nucleocapsid complex. In the infected cell, it is distributed both in the cytoplasm and in the nucleus. It is considered as an important determinant of species specificity, and there is speculation on its role in progeny viral assembly.

#### *M2 Protein*

The bicistronic RNA segment 7 encodes the M2 protein which is derived after splicing from its precursor M1 transcript. The HA and NA glycoprotein spikes together with M2 form tetramers on the infected cell surface forming ionic channels that maintain the pH, preventing the exposure of the viral HA to low intracellular pH to which it is sensitive, thereby facilitating uncoating of the viral nucleoprotein during replication. Despite this preferential association between HA and matrix proteins, the role of the M gene in highly pathogenic influenza virus has not been accorded its due significance.

#### *Nucleoprotein*

The NP is encoded by RNA segment 5, and functions as a structural protein. NP forms a loose association with the RNA of the virion and encapsidates the RNP complex (comprising RNA-NP and the polymerases PB1, PB2 and PA). Besides its structural role, NP is involved in the switching of viral RNA polymerase activity from mRNA synthesis to cRNA synthesis and vRNA synthesis [23, 24]. NP may also play a role in host selection based on its phosphorylation, which is host-dependent.

#### *Polymerases (PB1, PB2 and PA)*

*PB2.* The polymerase protein constitutes an integral part of the RNP complex, and is encoded by RNA segment 1. Its function includes recognition of the

5' mRNA cap of the host for use as viral mRNA transcription primers, and cleavage of the cap structures to generate primers for viral mRNA transcription [23, 24].

*PB1*. Localized in the nuclei of infected cells, PB1 is encoded by RNA segment 2. The basic function of the PB1 protein is in the elongation of viral mRNA primers and vRNA synthesis.

*PA*. The PA polymerase is encoded by RNA segment 3. Also localized in the infected cell nucleus, not much is known about its significance but a possible role as a helix-unwinding protein is suspected.

#### *Nonstructural Proteins NS1 and NS2*

NS1 and NS2 are encoded by RNA segment 8. NS2 is derived from precursor NS1 by splicing. In the infected cell, NS1 is localized in the nucleus and NS2 in the cytoplasm. NS2 protein is now known to exist in virions [25, 26], and is thought to play a role in the export of RNP from the nucleus [26, 27] through interaction with M1 protein. Based on the evidence that the NS2 protein contains a nuclear export signal and facilitates vRNP export, O'Neill et al. [28] have proposed to rename this protein as NEP (viral nuclear export protein). Subsequent studies also confirmed that this protein is essential for vRNP nuclear export [29].

## **Influenza Virus and Its Replication**

Despite their common avian lineage, influenza A viruses exhibit a pattern in host range and replication. This is attributed to the receptor specificity of HA molecules, and how they respond to the presence or absence of certain sialic acid-galactose linkages in the host.

Sialic acid is a nine-carbon, acidic amino sugar (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid), and is attached to the outermost ends of N-glycan, O-glycans and glycosphingolipids. The 5-carbon position situated at the terminal region of N-glycans, O-glycans and sphingolipids on modification gives an N-acetyl group yielding N-acetylneuraminic acid (Neu5Ac), and on hydroxylation the 5-N-acetyl group gives 5-N-glycolylneuraminic acid (Neu5Gc). The sialic acids can undergo further structural diversities, including substitution of hydroxyl groups with acetyl, methyl, phosphate or sulphate groups, and also by the different  $\alpha$  linkages from the 2 carbon to the underlying sugar chain to give SA2-3 Gal  $\beta$ - (2-3) and SA2-6 Gal  $\beta$ - (2-6). These factors are attributed to the diversity of sialic acid sugar chain receptors among animal species. About 40 different sialic acid receptors have been reported in nature [30, 31].

Krug et al. [32] conducted extensive studies on the replication and expression of influenza viruses. The virion particle infects the target host cell as the HA molecule recognizes and attaches to the specific terminal sialic acid on the cell

membrane surface. This is followed by endocytosis of the virus. In the acidic environment of the endosome, the virus undergoes a conformational change in the HA, the amino terminus of HA2 is inserted into the membrane of the endosome, fuses the envelope with the endosomal membrane, and the nucleocapsid is released into the cytoplasm of the cell. In the nucleus, the polymerase complex initiates primary transcription to produce necessary proteins for replication. The primary transcription involves what is known as 'cap snatching' [33]. The PB2 polymerase cuts the 5' methylguanosine cap along with thirteen nucleotides of the host RNA which serves as a primer for the transcription of the protein PB1, viral transcriptase followed by the translation of viral proteins, NP and NS1. During the early stage of infection, the host RNA transcription mechanism is inhibited. As the concentration of NP accumulates in the nucleus, a shift in mRNA synthesis to cRNA (positive sense) and vRNA synthesis is observed. The vRNA serves as template for secondary transcription of viral mRNA which in turn results in the synthesis of M1, HA and NA proteins. Accumulation of M1 protein in the nucleus signals the translocation of NP from the nucleus to the cytoplasm. Simultaneously, the HA and NA proteins undergo post-translation modifications (glycosylation, polymerization and acylation), and migrate to the plasma membrane along with the M2 proteins. As the nucleocapsid begins to take form, it gets encased within the shell of M1, initiating the budding process. Finally, the NA cleaves the sialic acid receptors and facilitates the elution of the progeny virions from the host cell.

An infectious virion is formed only when the precursor unit of the HA molecule (HA0) is cleaved to produce HA1 and HA2. As the cleaved unit is susceptible to low pH, in birds the cleavage of HA in avian influenza viruses occurs intracellularly, whereas in mammals the HA is cleaved by extracellular proteases of the respiratory tract [34].

## **Zoonosis**

Although certain influenza virus subtypes (e.g. H9) cause mild disease in poultry, the H5 and H7 subtypes are known to cause widespread outbreaks inflicting massive fatalities among domestic poultry. Among animals, wild waterfowl represent the major reservoir for influenza viruses. All influenza viruses in other animal species are believed to be derived from these birds. Migratory birds are considered as carriers, merely serve to carry the virus over great distances, and shed large quantities of virus in their faeces but remain healthy. The excreted virus can survive for several days and withstand low temperatures. Some strains remain infectious for up to 207 days at 17°C, and they remain infectious for longer periods at 4°C. Hence, the virus is acquired by