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REVIEW

H5N1 influenza viruses: outbreaks and biological properties

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All known subtypes of influenza A viruses are maintained in wild waterfowl, the natural reservoir of these viruses. Influenza A viruses are isolated from a variety of animal species with varying morbidity and mortality rates. More importantly, influenza A viruses cause respiratory disease in humans with potentially fatal outcome. Local or global outbreaks in humans are typically characterized by excess hospitalizations and deaths. In 1997, highly pathogenic avian influenza viruses of the H5N1 subtype emerged in Hong Kong that transmitted to humans, resulting in the first documented cases of human death by avian influenza virus infection. A new outbreak started in July 2003 in poultry in Vietnam, Indonesia, and Thailand, and highly pathogenic avian H5N1 influenza viruses have since spread throughout Asia and into Europe and Africa. These viruses continue to infect humans with a high mortality rate and cause worldwide concern of a looming pandemic. Moreover, H5N1 virus outbreaks have had devastating effects on the poultry industries throughout Asia. Since H5N1 virus outbreaks appear to originate from Southern China, we here examine H5N1 influenza viruses in China, with an emphasis on their biological properties.

***Keywords*:** influenza, H5N1, China, human infections, HA, PB2, NS1

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Influenza viruses

Influenza viruses are members of the family *Orthomyxoviridae*, which comprises enveloped viruses with segmented RNA genomes of negative polarity (reviewed in Wright *et al*. [1] and Palese and Shaw [2]). The genome of influenza A viruses is composed of eight RNA segments, each encoding 1-2 proteins. Replication and transcription of the viral RNA (vRNA) require the three polymerase proteins (PB2, PB1, and PA) and the nucleoprotein NP, which are encoded by the three largest segments, and by the fifth segment, respectively. The nuclear export of newly synthesized viral ribonucleoprotein (vRNP) complexes (composed of vRNA and components of the replication complex) is mediated by the M1 and NEP proteins, which are encoded by an unspliced mRNA derived from the seventh and a spliced mRNA derivative of the eighth segment. vRNPs and M1 (the major viral structural protein) are assembled into virions that bud from the cellular membrane. Virions contain two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are encoded by the fourth and sixth segment, respectively. HA executes critical functions in virus binding and internalization, whereas NA promotes the release of viruses from the cell surface. Also embedded in the viral membrane is the M2 ion channel protein (encoded by a spliced mRNA derived from the seventh segment) that mediates functions early and, for certain groups of influenza A viruses, late in infection. The NS1 protein (encoded by an unspliced mRNA derived from the eighth segment) and the PB1-F2 peptide (encoded in the +1 reading frame of the PB1 segment) function as an interferon (IFN) antagonist and a pro-apoptotic factor, respectively.

Based on the antigenicity of the HA and NA surface glycoproteins, influenza A viruses currently form 16 HA and 9 NA subtypes, designated as H1-H16 and N1-N9. Over the past century, only viruses of the H1N1, H2N2, H3N2, and H1N2 subtypes have circulated in humans (reviewed in Wright *et al*. [1]).

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Avian influenza viruses

Influenza A viruses of all known subtypes are maintained in the Orders *Anseriformes* (i.e., ducks, geese, and swan) and *Charadriiformes* (i.e., gulls, terns, surfbirds, and sandpipers) (reviewed in Webster *et al*. [3]). Typically, influenza A viruses are maintained asymptomatically in these hosts, although there have been some recent exceptions with the highly pathogenic H5N1 influenza viruses [4, 5].

Based on their pathogenicity in chickens, avian influenza A viruses can be divided into viruses of high or low pathogenicity (reviewed in Swayne and Suarez [6]). Viruses of low pathogenicity cause mild respiratory disease, a decrease in egg production, and/or depression. By contrast, viruses of high pathogenicity cause significant mortality in chickens. All highly pathogenic avian influenza viruses known to date belong to the H5 or H7 subtype; however, only a small percentage of H5 and H7 viruses are highly pathogenic. Why certain H5 and H7 viruses evolve into pathogenic variants is not known. Viruses of high pathogenicity in chickens are typically benign in ducks and geese, with the exception of some of the recently circulating highly pathogenic avian H5N1 viruses.

Outbreaks of highly pathogenic avian H5N1 influenza viruses

Since 1997, highly pathogenic avian influenza viruses (HPAI) of the H5N1 subtype have caused numerous outbreaks in poultry in Southeast Asia [7-9] that have led to the death or depopulation of significant numbers of chickens. These outbreaks are accompanied by the occasional transmission of HPAI H5N1 viruses to humans, resulting in a case fatality rate of more than 50%. Since their first recognition, HPAI H5N1 influenza viruses have become endemic in poultry populations in Southeast Asia and have spread to more than 60 countries (Figure 1). The extent of control measures, the number of live poultry markets, and the numbers of poultry backyard flocks may have determined if HPAI H5N1 viruses became endemic or caused only localized outbreaks. Some of these viruses are remarkable in that they also have the ability to kill wild waterfowl [4, 5]. Moreover, HPAI H5N1 influenza viruses have recently become more pathogenic in mammalian species [10, 11]. These viruses thus present an imminent threat to humans, and to the poultry industry and potentially wild birds. A periodically updated timeline of H5N1 outbreaks can be found at http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html. Since Southern China appears to be the epicenter for the emergence and re-emergence of these viruses [7, 9, 12], here we review our current understanding of HPAI H5N1 influenza viruses in China.

HPAI H5N1 influenza viruses in China

Phylogenetic analyses

HPAI H5N1 influenza viruses were first isolated from sick geese in Guangdong Province in China in 1996 [13]. In 1997, these viruses caused outbreaks in chickens in Hong Kong and were transmitted to humans, resulting in six deaths [14-16]. The viruses that caused outbreaks in poultry and transmitted to humans in Hong Kong in 1997 possessed an HA gene that was likely derived from A/goose/Guangdong/1/96 (H5N1; GS/GD/1/96-like virus [13]); the remaining seven viral genes were derived from other avian viruses [17, 18].

Since 1999, HPAI H5N1 influenza viruses have been consistently isolated from apparently healthy ducks in the coastal provinces of Southern China. In early 2004, these H5N1 viruses caused outbreaks in ducks, geese, and chickens in 16 provinces, and more outbreaks were reported in 2005 [19]. Since then, a number of outbreaks have been reported in wild aquatic birds in the Qinghai and Tibet regions (April 2006), in commercial ducks in Guangdong (September 2007), and in poultry in June 2006, and March 2007. More outbreaks in poultry were reported in different Chinese regions in January, February, and March of 2008 (http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html).

After their reappearance in ducks in Southern China in 1999/2000, HPAI H5N1 viruses underwent frequent and extensive reassortment, resulting in a number of different genotypes [7, 10, 12, 20, 21]. One of these genotypes, genotype Z became dominant in 2002 [7, 8]. In late 2005, a new sub-lineage was detected in poultry in Southern China ('Fujian-like' viruses) [9]. These viruses predominate in Southern China and caused outbreaks in Hong Kong, Laos, Malaysia, and Thailand.

The HA genes of most HPAI H5N1 viruses belong to the GS/GD/1/96-lineage, and all HPAI H5N1 HAs possess a series of basic amino acids at the cleavage site (-RRKKR-) that is characteristic of HPAI viruses. Although the HPAI H5 HA genes are represented in different groups in the phylogenic tree, they are considered as one lineage [7, 10].

Based on their HA sequences, HPAI H5N1 viruses are now divided into 10 clades (http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html). Major groups are clade 0 (early progenitor viruses from Hong Kong and China, 1996-2002), clade 2.1 (avian and human Indonesian isolates, 2003-2007), clade 2.2

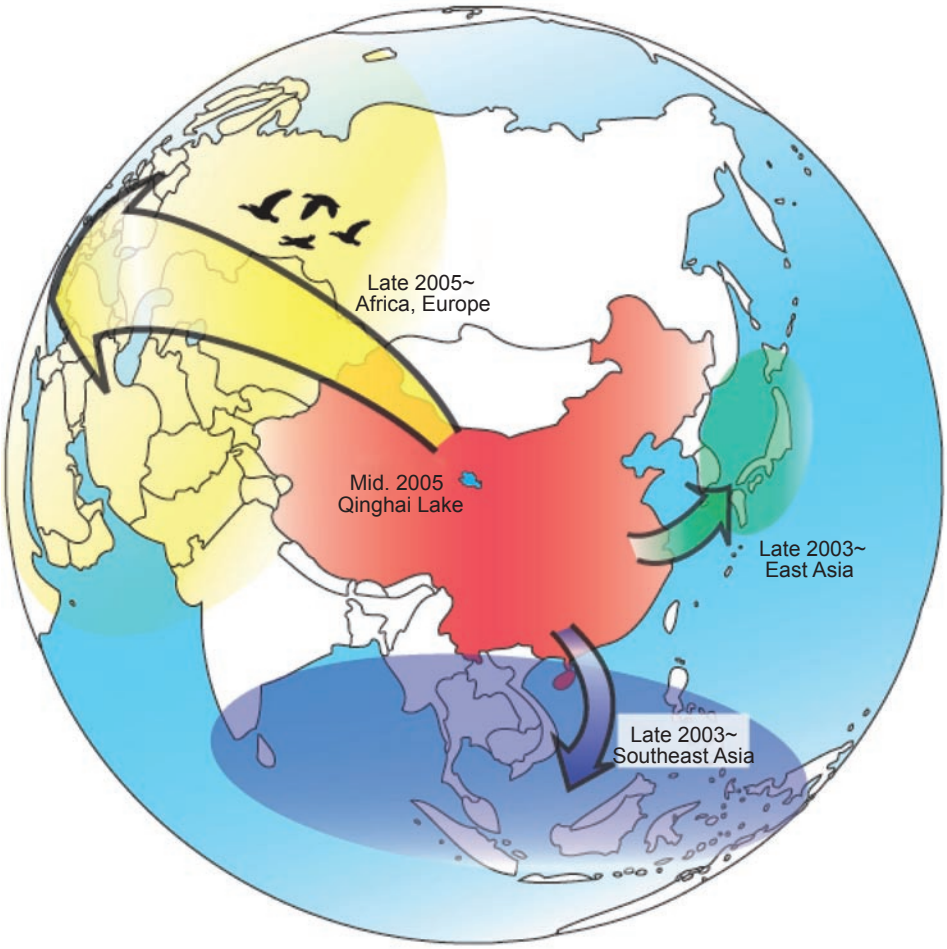

Figure 1 Spread of highly pathogenic avian H5N1 influenza viruses from China. Colors indicate the viruses of genotype V (green), Z (blue), and Qinghai lake genotype Z (yellow). Genotype V viruses have been introduced into East Asia, including South Korea and Japan, since late 2003. At the same time, genotype Z viruses were introduced into Southeast Asian countries, such as Thailand, Vietnam, and Indonesia, from Southern China where they currently prevail. Moreover, H5N1 virus outbreaks among migratory birds at Qinghai Lake in mid-2005 triggered the spread of these viruses into African and European countries.

(2005 progenitors from the Qinghai Lake outbreak and from Mongolia, and avian and human 2005-2007 isolates from Eastern and Western Europe, the Middle East, and Africa), clade 2.3 (avian and human virus isolates from China, Hong Kong, Vietnam, Thailand, Laos, and Malaysia, 2003-2006), clade 2.4 (avian virus isolates predominately from Yunnan and Guangxi Provinces, China, 2002-2005), and clade 2.5 (avian virus isolates from Korea, Japan, and China, 2003/2004, and from Shantou, China, 2006).

The NA genes of HPAI H5N1 isolates are divided into two lineages. One lineage contains the 1997 human and poultry isolates [22], and viruses that were isolated from the eggs of Vietnamese waterfowl in 2005 [23]. The NA genes in this lineage have a 19-amino-acid deletion in the stalk (amino acids 54-72). The second NA lineage can be divided into two sub-lineages, whose NA proteins are GS/GD/1/96-like (i.e., containing a full-length NA stalk), or GS/GD/1/96-derived with a 20-amino acid deletion in the NA stalk (amino acids 49-68). This NA stalk deletion is distinct from, but overlaps with, the 19-amino-acid deletion that characterizes viruses of the first NA lineage.

The phylogenetic trees of the PB2, PB1, PA, and NP genes of Chinese HPAI H5N1 viruses are very similar and can each be divided into two lineages. One lineage contains viruses that were isolated from humans and poultry in Hong Kong in 1997 [22], as well as viruses that were isolated from the eggs of Vietnamese waterfowl in 2005 [23]. The second lineage can be further divided into several sub-lineages. Less than 90% homol-

ogy exists between the two lineages, whereas members of a sub-lineage share 90%-95% homology.

Similar to the polymerase and NP genes, the M genes of the 1997 H5N1 Hong Kong viruses form a separate lineage, whereas the M genes of the remaining Chinese HPAI H5N1 form a second lineage that has evolved into several sub-lineages. The M gene of A/duck/Guangdong/40/00 virus is quite different from that of other Chinese HPAI H5N1 viruses and forms a unique sub-lineage [10].

The NS genes of Chinese HPAI H5N1 duck viruses fall into two alleles, *A* and *B*. The NS genes of GS/GD/1/96 and several HPAI H5N1 duck viruses belong to the *B* allele, whereas the remaining NS genes, including those of the 1997 human Hong Kong viruses, belong to the *A* allele. The *A* allele can be further divided into two branches. The deduced NS1 amino acid sequences of viruses in one branch of the *A* allele revealed a 5-amino-acid deletion at positions 80-84.

Outbreak at Qinghai Lake

In late April/early May of 2005, migratory birds (especially bar-headed geese, the first species affected) were found ill or dead in the national reserve area of Qinghai Lake (or Lake Qinghaihu) in Western China, a hatching area for migratory birds during spring and summer.

More than 6 000 migratory birds died in the outbreak and intensive studies soon identified the causative agents as HPAI H5N1 viruses [19, 24-26]. Multiple genotypes were isolated from the infected birds [19], although one particular genotype dominated [19, 24-26].

Wild waterfowl are the known reservoir of all influenza A virus subtypes (with the exception of viruses of the H13 subtype) [3]; these species typically carry influenza A viruses without any symptoms. Infection of wild waterfowl by H5N1 viruses had previously been sporadic and involved limited numbers of birds (for example, see Becker [27]). The outbreak at Qinghai Lake was, therefore, unusual in that reservoir species succumbed to influenza virus infection.

Pathogenicity studies have shown that most of the 2005 Qinghai Lake viruses are fatal to chickens and mice, with the exception of A/bar-headed goose/Qinghai/2/05 [19], the genotype of which differs from that of the other viruses tested. Two 2005 Qinghai Lake viruses (A/bar-headed goose/Qinghai/1/05 and A/great cormorant/Qinghai/3/05) were also tested in rhesus macaques. These viruses caused transient fever [19] but did not kill the infected animals despite possessing lysine at position 627 of the PB2 protein, which confers high virulence to H5N1 viruses in mammalian species [7, 28-34] (for more information on the role of PB2-627, see section PB2).

In 2006, HPAI H5N1 viruses again caused outbreaks in migratory birds at Qinghai Lake. Compared to the 2005 outbreak, fewer birds, over a wider area, were affected. Genetic analysis showed that these viruses clustered with those isolated in 2005 [35].

Outbreaks in pigs

Thus far, HPAI H5N1 influenza viruses have not caused widespread outbreaks in pigs anywhere in the world, including China. Nonetheless, HPAI H5N1 influenza viruses were isolated from pigs in Fujian province in 2001 and 2003 during active surveillance [36, 37]. In particular, two viruses were isolated from the same pig farm in 2001 and 2003. Sequence analysis revealed that the two viruses were closely related to and share close homology with a previously reported duck virus, A/duck/Zhejiang/2/00 [10, 36, 37], suggesting that HPAI H5N1 duck viruses may have been introduced into pigs and silently circulate in these animals. Such a scenario is of concern, since pigs are thought to act as 'mixing vessels' for human and avian influenza virus reassortment [38].

Human H5N1 virus infections in China

The first human cases of H5N1 virus infection were reported in Hong Kong in 1997, when six out of eighteen individuals succumbed to the infection [14, 16, 39]. This incident marked the first reported fatal infections of humans with avian influenza viruses. No further human cases occurred until 2003, when two Hong Kong residents contracted the disease, one of whom died [40]. Another family member also died; however, the cause of death was not determined for this individual. Since 2003, when a new HPAI H5N1 virus outbreak started, more than 391 individuals in 15 countries have been infected, with 247 cases resulting in fatal outcomes (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_12_16/en/index.html) (Figure 2). In Mainland China, no human H5N1 virus infections were reported until 2005 [41]. Since then, 30 cases with 20 deaths have occurred (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_12_16/en/index.html).

Comprehensive genetic and antigenic analysis of 16 viruses isolated from 13 fatal and 3 nonfatal human cases in Mainland China between November 2005 and July 2006 (unpublished data) revealed that these viruses did not contain mutations in their M2 and NA proteins associated with resistance to adamantanes and oseltamivir, respectively, although eight patients were treated with antivirals during the course of their disease [42, 43].

The HA genes of 15 human isolates from Southern China belong to sub-clade 2.3.4, they are closely related to each other and to H5N1 poultry viruses isolated in the

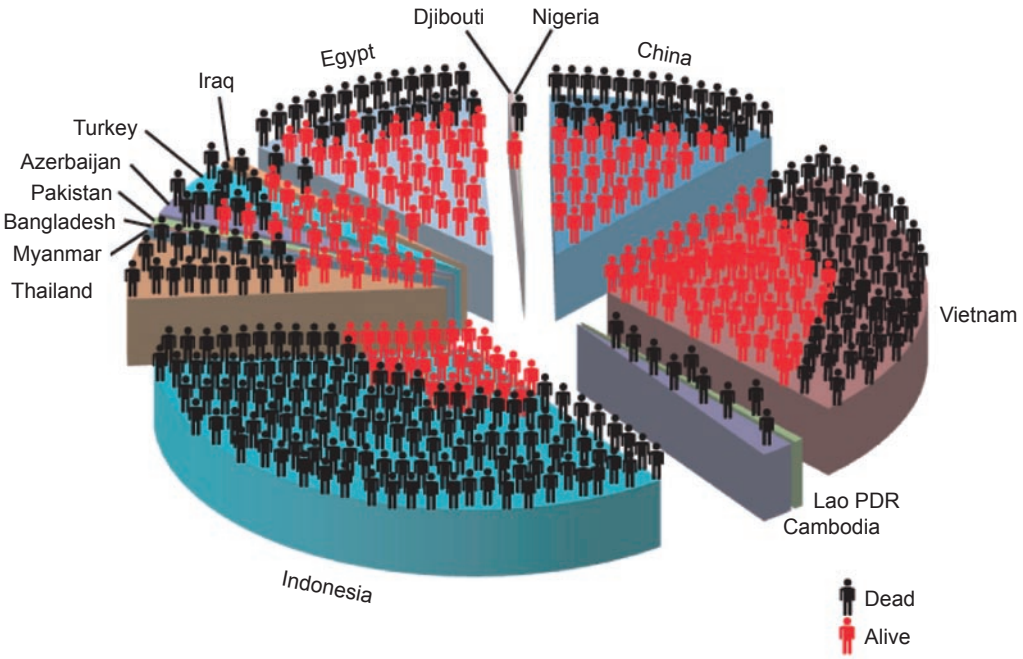

Figure 2 Confirmed human cases of H5N1 virus infection from 1997-2008 (reported to WHO). Colors indicate whether the infected individual survived (red) or succumbed to the infection (black).

same geographic location at the same time, suggesting infected domestic poultry as the source of the human infections [42, 43]. By contrast, the HA gene of one human isolate from the Xinjiang Autonomous Region in Northern China is more closely related to Qinghai Lake-like viruses. The amino acid sequences at the cleavage site of the HA proteins of these 16 human viruses differ between isolates from Southern and Northern China; however, they all contain multiple basic amino acids at the HA cleavage site, a known determinant of high pathogenicity for avian influenza viruses (see section HA).

Antigenic studies revealed that the human virus isolates from Southern China are antigenically closely related to each other (unpublished data). They are, however, antigenically distinguishable from H5N1 virus isolates from Indonesia, Vietnam, and Turkey (unpublished data). Hence, the currently WHO-recommended pandemic vaccine strain, A/Vietnam/1203/04, may provide only limited protection against H5N1 viruses currently circulating in Southern China.

Clinical data are now available for 26 confirmed human H5N1 cases in China that occurred between October 2005 and April 2008; 17 of these individuals died [44]. Most patients presented with fever and cough, but rapidly developed lower respiratory disease. Leukopenia, lymphopenia, moderate thrombocytopenia, and liver and renal function impairment were common during hospitalization. All fatal cases had multi-organ failure and a high frequency of acute respiratory distress syndrome. Treatment with antivirals resulted in more survivors, that is, 8/12 patients who received antivirals survived compared to only 1 survivor of 14 patients who did not receive antivirals [44].

The current HPAI H5N1 viruses do not spread efficiently among humans. Nonetheless, possible cases of human-to-human transmission have been described [45-47]. One such case occurred in December 2007 in Jiangsu Province, China: a 24-year-old man died of H5N1 virus infection, while his 52-year-old father became ill but recovered after early antiviral treatment and treatment with post-vaccination plasma [42]. The H5N1 viruses isolated from these two individuals were identical except for a non-synonymous nucleotide substitution in the NS gene that resulted in a glutamic acid to glycine substitution at amino acid position 82 of the NS2 protein [42].

Dissemination of HPAI H5N1 viruses

The mechanisms of HPAI H5N1 virus spread are intensely debated, and migratory birds, as well as the poultry and wild bird trades have been discussed as potential factors. Outbreaks in areas that are not part of known flyways may suggest (illegal) poultry trade as a source of HPAI H5N1 virus outbreaks [48]. The phylogenetic

relationships of HPAI H5N1 viruses from Southeast Asia also suggest the poultry trade as a likely factor for H5N1 virus introduction into Vietnam [49]. On the other hand, the spread of HPAI H5N1 into Europe correlates with migratory bird movements [50]. Most likely, both migratory birds and infected poultry have contributed to HPAI H5N1 virus outbreaks [8, 25].

HPAI H5N1 virus dissemination through migratory birds assumes long-distance travel of (subclinically) infected birds, yet some studies suggest that migration of infected birds is limited [51]. An alternative scenario is that infected migratory birds may carry the virus over only short distances, before passing it on to other migratory birds, for example in densely populated resting areas. Such "bird shuttles" would allow virus dissemination over long distances in relatively short periods of time. In fact, some HPAI H5N1 viruses isolated in 2006 at Qinghai Lake were closely related to an Croatian virus isolated in 2005 (A/Cygnus olor/Croatia/1/05) [49], suggesting that HPAI H5N1 viruses may have traveled from and to Asia by hitchhiking on migratory 'bird shuttles' [49]. This mechanism of virus dissemination may have allowed the spread of these viruses along the Eurasian migratory-bird flyways and may aid in their dissemination into other areas via other migratory-bird flyways. Migratory birds may thus play an important role in the dissemination of HPAI H5N1 viruses around the world, providing a strong rationale for surveillance of appropriate bird species.

Molecular determinants of H5N1 virulence, pathogenicity, and host range restriction

Three influenza virus proteins, PB2, HA, and NS1, are recognized as major determinants of virulence, pathogenicity, and/or host range restriction. Recently, PB1-F2 has emerged as another determinant of pathogenicity. However, other viral proteins including the remaining components of the replication machinery also seem to contribute to viral pathogenicity (reviewed in Neumann and Kawaoka [52] and Neumann *et al*. [53]).

HA

The HA protein mediates virus attachment and the fusion of the viral and endosomal membranes (reviewed in Wright *et al*. [1]). It is synthesized as a single polypeptide chain (HA0), which is posttranslationally cleaved by cellular proteases. HA cleavage is critical for influenza virulence, since it exposes the hydrophobic N-terminus of HA2, which mediates fusion of the viral and endosomal membranes. A clear link exists between HA cleavability and virulence (reviewed in Horimoto and Kawaoka [54], Klenk and Rott [55], and Garten and Klenk [56]) in that the HA cleavage site of highly pathogenic H5 and H7 viruses is composed of multiple basic amino acid residues that are recognized by ubiquitous proteases, such as furin and PC6, resulting in systemic infection. By contrast, the HAs of nonpathogenic avian viruses lack this series of basic residues at the cleavage site and thus are cleaved by proteases in a limited number of organs, restricting their replication to such organs. Not surprisingly, the HA proteins of all HPAI H5N1 influenza viruses contain multiple basic amino acids at their cleavage site.

Influenza virus *host range restriction* is partly determined by the receptor-binding specificity of the HA protein (reviewed in Wright *et al*. [1], Neumann and Kawaoka [52]). In general, avian and equine viruses bind preferentially to sialic acid that is linked to galactose by an α2,3-linkage (SAα2,3Gal), whereas human influenza viruses have higher binding affinity to SAα2,6Gal. This difference in binding affinity is mirrored by a predominance of SAα2,3Gal on the epithelial cells of duck intestine (the major replication site of avian influenza viruses), but of SAα2,6Gal on the epithelial cells of human trachea. Most of the HPAI H5N1 viruses isolated from humans, however, have avian-type receptor specificity [57], demonstrating the ability of avian influenza viruses to infect humans [57]. In fact, avian-type receptors have recently been identified on the ciliated cells of *in vitro* differentiated human epithelial cells from tracheal/bronchial tissues [58], on the epithelial cells of the lower human respiratory tract [59, 60], and to some extent also on the epithelial cells of the upper human respiratory tract [61]. These findings offer an explanation for human infections with avian influenza viruses. Nonetheless, it is believed that avian influenza viruses will have to acquire human-type receptor-binding specificity for sustained replication and transmission in humans. This assumption is supported by the finding that the earliest human isolates of the 1918, 1957, and 1968 pandemics preferentially recognized SAα2,6Gal [62]. Human H5N1 viruses isolated in 1997 bound to avian-type receptors [57, 63]. However, two H5N1 viruses isolated from infected individuals in Hong Kong, in 2003, showed reduced binding to avian-type, and low but detectable binding to human-type receptors [63]. A single amino acid change at position 227 was responsible for this change in receptor-binding specificity. Analysis of 21 recent human H5N1 virus isolates identified three viruses with increased affinity for SAα2,6Gal [64]. Two independent amino acid changes (Asn182-to-Lys and Gln192-to-Arg, equivalent to positions 186 and 196, respectively, in H3 numbering) were responsible for the altered receptor-binding properties [64]. These mutations had not previously been

associated with receptor-binding specificity [64]. Amino acid changes at position 186 or 196 were also detected in H5N1 viruses isolated from infected individuals in Azerbaijan and Iraq. Together, these data suggest that HPAI H5N1 can acquire mutations in humans that likely reflect adaptation to the new host and may support virus replication and spread.

PB2

The PB2 protein is an essential component of the viral replication complex, together with the PB1, PA, and NP proteins. In 1993, a report described the importance of a 'human-like' amino acid at position 627 of the PB2 protein, which was required for the virus to form plaques in Madin-Darby canine kidney cells [65]; however, the full implications of this finding were not appreciated at the time. In 2001, PB2-627 was recognized as a major determinant of influenza virus pathogenicity when Hatta *et al*. [28] identified this residue as the critical determinant of HPAI H5N1 influenza virus pathogenicity in mice. HPAI H5N1 influenza viruses of high pathogenicity in mice contained Lys at this position [28], which is conserved in human influenza viruses. By contrast, H5N1 influenza viruses of low pathogenicity in mice possessed Glu at this position [28], which is typically found in avian influenza viruses. The recently emerged Qinghai Lake-lineage of HPAI H5N1 viruses is a notable exception in that isolates from wild birds contain the 'human-like' amino acid at PB2-627 [19], raising concerns that viruses of this lineage may cause a human pandemic. Lys at PB2-627 has now been found in a substantial number of H5N1 viruses isolated from infected humans [7, 29-31], in H5N1 viruses isolated from tigers in Thailand (in 2004 and 2006) [32], and in an H7N7 virus isolated from a Dutch veterinarian who died of pneumonia during an outbreak of H7N7 influenza viruses in poultry in 2003 [33]. Viruses isolated from nonfatal human cases during this outbreak all possessed PB2-627Glu [33]. Moreover, an equine H7N7 influenza virus acquired the PB2-627 Glu-to-Lys mutation during replication in mice [34]. Collectively, these data suggest that PB2-627Lys is selected during replication in mammals.

Several studies have assessed the mechanism by which the amino acid at PB2-627 determines pathogenicity and found that it affects both host range and the replicative ability of the virus [66-69]. In murine, but not in avian cells, PB2 possessing 627Lys confers more efficient replication compared to the presence of Glu at this position [67]. By contrast, there were no differences in tissue tropism between viruses encoding PB2-627Lys or Glu [67]. Lys at PB2-627 thus likely supports efficient virus replication in mammalian species, which explains its selection in these hosts.

Chen *et al*. [10] performed a detailed analysis in chicken and mouse models of HPAI H5N1 viruses isolated from ducks in Southern China from 1999 to 2002. The duck viruses were all highly pathogenic for chickens based on the criteria of the Office International Des E'pizooties, although some viruses did not uniformly kill chickens when inoculated intranasally. When tested in mice, the viruses formed four groups based on their replicative ability and lethality. A temporal pattern became evident with progressively increasing pathogenicity of these isolates in the mammalian model. Li *et al*. [70] compared two of these duck viruses that share high homology but display disparate pathogenicity and found that the amino acid at position 701 of PB2 played a key role in pathogenicity: a single Asp-to-Asn substitution at position 701 of PB2 conferred lethality in mice, while the inverse change attenuated the virus in mice. A similar pattern was found for an H7N7 virus, in which Asn at position 701 of PB2 conferred higher replicative ability in mice relative to a virus possessing Asp at this position [71].

Thus, although PB2-627 has emerged as a critical determinant of H5N1 virus pathogenicity, the amino acid at position 701 of this protein also contributes to viral pathogenicity. Moreover, two recent studies demonstrated a role for the polymerase complex in viral pathogenicity [71, 72], although the gene products and particular amino acids that are critical for these effects have yet to be identified.

NS1

The NS1 protein (encoded by the NS gene) is an IFN antagonist that executes its function through a multi-pronged approach (reviewed in Krug *et al*. [73], Garcia-Sastre *et al*.[74], and Garcia-Sastre [75]). It interferes with IFN-β production, the activation of IFN-β-stimulated genes, the expression of protein kinase R (PKR; gene name: EIF2AK2; a central player in innate immune responses), and the double-strand RNA-dependent activation of 2′-5′ oligoadenylate synthetase (OAS, gene name: OAS1), which in turn activates RNase L (gene name: RNASEL), another key player in the innate immune response. Moreover, NS1 interacts with RIG-I (gene name: DDX58) [76-78], which may account for the observed block in activation of several transcription factors [79-81], and suppression of the induction of RNA interference [82, 83].

The NS genes of 1997 and 2003 HPAI H5N1 influenza viruses are of different origin – yet both induce high levels of cytokines/chemokines and confer resistance to the antiviral effects of IFN [40, 84-87]. For example,

the NS gene derived from a 1997 H5N1 virus conferred higher pathogenicity in pigs than an NS gene derived from a non-H5N1 control virus [85, 86]. This difference was linked to a single amino acid change at position 92 of NS1 (Asp-to-Glu) [85, 86]. Likewise, the NS gene of 2003 H5N1 viruses induced high cytokine levels in cell culture and in patients [40, 87]. In another study, Jiao *et al*. [88] demonstrated that the amino acid at position 42 of the NS1 protein affected the pathogenicity of H5N1 duck viruses in mice, and that the respective amino acid change affected host cell IFN induction.

Another study found that amino acid 149 of NS1 affects pathogenicity [89]. Two avian H5N1 viruses, A/goose/Guangdong/1/96 (GS/GD/1/96) and A/goose/Guangdong/2/96 (GS/GD/2/96) differed in their ability to infect and kill chickens [89]. GS/GD/1/96 was highly pathogenic for chickens, whereas GS/GD/2/96 virus did not replicate in this animal model [89]. Both viruses bear multiple basic amino acids at their HA cleavage site, indicating that this known marker of virulence did not account for the observed difference in virulence. Reverse genetics studies demonstrated that the NS gene affected the pathogenicity of these two viruses in chickens, and that a single amino acid change at position 149 of the NS1 protein was critical for this effect [89].

Recently, the four C-terminal amino acids of NS1 have emerged as another virulence determination factor [90, 91]. Large-scale sequence analysis of avian influenza viruses identified a PDZ ligand domain of the X-S/T-X-V type at the C-terminus of NS1 [90], and experimental testing demonstrated that the PDZ ligand domains of HPAI H5N1 viruses and the pandemic 1918 virus indeed conferred increased virulence in mice [91]. The increase in pathogenicity was not caused by altered IFN production, suggesting that NS1 also affects viral pathogenicity through some other means.

In addition to single amino acid changes, deletions in the NS1 gene may affect viral pathogenicity. Since 2000, an increasing number of HPAI H5N1 viruses have been shown to possess a 15-nucleotide deletion at nucleotide positions 263-277 of the NS gene (amino acid positions 80-84 of NS1). This deletion seems to increase pathogenicity in chickens and mice [92]. Two H5N1 viruses isolated from pigs in Fujian Province in 2001 and 2003 differ in their lethality in chickens [37]. Both viruses carry the deletion at position 263-277 of the NS gene. An additional 15-nucleotide deletion (nucleotide position 612-626 of the NS gene, resulting in amino acid deletions in both the NS1 and NS2 proteins) was found for the low pathogenic virus. This deletion attenuated recombinant viruses in chickens and interfered with the viruses' ability to antagonize IFN induction [37]. The mechanisms by which these deletions in NS1 affect pathogenicity are not yet understood.

PB1-F2

The most recently discovered influenza virus protein, PB1-F2, is expressed from the +1 reading frame of the PB1 gene of most, but not all, influenza A viruses [93]. It localizes to mitochondria and induces apoptosis, likely through interaction with two mitochondrial proteins [94]. A recent study found that Ser, but not Asn, at position 66 of PB1-F2 conferred high pathogenicity to an H5N1 virus in mice [95]. Interestingly, the 1918 virus that killed an estimated number of 40-50 million people also possessed Ser at position 66 of its PB1-F2 protein [95]. Replacement of this amino acid with Asn attenuated the 1918 virus [95], identifying PB1-F2 protein as an important determinant of pathogenicity. A recent study also demonstrated that PB1-F2 interacts with PB1 and affects the nucleocytoplasmic distribution of this protein [96], thereby probably also affecting virulence.

Conclusions

High-pathogenicity influenza viruses of the H5N1 subtype are now endemic in poultry populations in Southeast Asia. These viruses will continue to circulate in avian species and to occasionally transmit to humans. It is therefore of the utmost importance to understand the genetic determinants of pathogenicity, the factors that facilitate transmission to and/or replication in humans, and the molecular changes that may allow H5N1 viruses to transmit among humans. Much has been learned since these viruses first appeared in 1997; yet many key questions await answers. The combination of reverse genetics and bioinformatics approaches for the 'mining' of the thousands of influenza virus sequences that have become available over the past decade may bring us closer to answering these critical questions in influenza virus research.

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