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REVIEW

The threat of avian influenza A (H5N1). Part I: epidemiologic concerns and virulence determinants

Jindrich Cinatl Jr · Martin Michaelis · Hans W. Doerr

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Abstract Among emerging and re-emerging infectious diseases, influenza constitutes one of the major threats to mankind. In this review series epidemiologic, virologic and pathologic concerns raised by infections of humans with avian influenza virus A/H5N1 are discussed. This first part concentrates on epidemiologic concerns and virulence determinants. H5N1 spread over the world and caused a series of fowl pest outbreaks. Significant human-to-human transmissions have not been observed yet. Mutations that make the virus more compatible with human-to-human transmission may occur at any time. Nevertheless, no one can currently predict with certainty whether H5N1 will become a human pandemic virus.

Introduction

In the 1990s, a dogma of influenza epidemiology that had claimed that avian influenza virus cannot be directly transmitted from birds to men was overthrown. In 1995, the influenza virus A/England/268/96 (H7N7), isolated from the eye of a 43-year-old woman with conjunctivitis, was suspected to be directly transmitted from birds [1, 2]. In 1997, a big outbreak of classical fowl pest happened in a chicken mass holding farm of Hong Kong. Molecular analysis revealed avian influenza virus subtype A (H5N1), a high pathogenic avian influenza (HPAI) variant, to spread through live-poultry markets in Hong Kong to humans [3, 4]. Meanwhile, the 1918 pandemic virus is regarded a pure avian virus which adapted to humans [5].

H5N1 infection in Hong Kong caused severe respiratory and partially gastrointestinal disease killing 6 of 18 infected humans who had close contact to the chickens. The fowl pest was eradicated by strict and complete culling of all chickens, including contacts. Patients and contact people were successfully quarantined and no human-to-human transmission of the virus was observed at that time [3, 4]. However, the virus continued to circulate among apparently healthy ducks in the coastal provinces of China.

From 1997 to May 2005, H5N1 viruses were confined to Southeast Asia, but after they had infected wild birds in Qinghai Lake, China, they rapidly spread westward [6, 7]. The continuing evolution of H5N1 viruses and the clusters of human infections in the epicentre raise important questions. Can the source of H5N1 be eliminated? Is the increasing number of clusters of human H5N1 infection an indicator of evolution toward consistent human-to-human transmission? What are the mechanisms underlying H5N1 virulence and severe pathogenicity? How efficient are currently available anti-influenza agents against H5N1 virus? Can effective vaccines for H5N1 be developed?

Epidemiologic concerns

Influenza viruses are divided into three serologically different types (A, B and C) according to the antigenicity of conserved inner virus structures, i.e. the nucleoprotein (NP) and matrix proteins (M1 and M2) of the envelope. While influenza virus types B and C are usually human specific, influenza A virus is preferentially endemic in water birds

J. Cinatl Jr (✉) · M. Michaelis · H. W. Doerr
Institute for Medical Virology,
Hospital of the Johann Wolfgang Goethe University,
Paul-Ehrlich-Str. 40, 60596 Frankfurt/M, Germany
e-mail: cinatl@em.uni-frankfurt.de

H. W. Doerr (✉)
e-mail: h.w.doerr@em.uni-frankfurt.de

such as ducks, geese and shore birds (gulls), which usually do not fall ill from this infection. Depending on the antigenicity of two envelope spikes, which first mediate virus adsorption to target cells in vivo or erythrocytes in vitro (haemagglutinin, HA) and finally the release of viral progeny from the infected cells (neuraminidase, NA), influenza A viruses are divided into 16 HA (H1-H16) and 9 NA (N1-N9) groups resulting in theoretically 16 × 9 serologic subtypes. Up to date, 105 influenza A virus subtypes have been discovered, all endemic in water birds. However, some subtypes have adapted to other birds (chickens) and mammalians (pig, horse, man) in species-specific strains (Fig. 1).

In humans three subtypes have been detected: H1N1, H2N2 and H3N2, which caused pandemics of high morbidity and mortality as the so-called Spanish influenza (H1N1, 1918–1919, about 40 million people killed), Asian influenza (H2N2, 1957, about 2–4 million killed), and Hong Kong influenza (H3N2, 1968, about 1–2 million killed). Newly circulating influenza viruses caused widespread epidemics as they spread rapidly by efficient person-to-person transmission in the human population that lacked antibodies against the new subtype and ultimately replaced the previously circulating strain. The pandemic H1N1 strain of the so-called Russian influenza was essentially identical to those H1N1 strains circulating in the 1950s as shown by oligonucleotide mapping techniques [8]. It is thought likely that the virus was maintained in a freezer until it was somehow reintroduced into the general population. Although the 1977 outbreak was limited (several hundred thousands people killed), because the older population had protective immunity resulting from prior experience with H1N1 strains, this H1N1 virus (and its descendants) has been circulating ever since [9]. At present both H1N1 and H3N2 influenza viruses continue to be present in the human population representing lower pathogenic variants, nevertheless causing over mortality among elderly people in each winter epidemic wave.

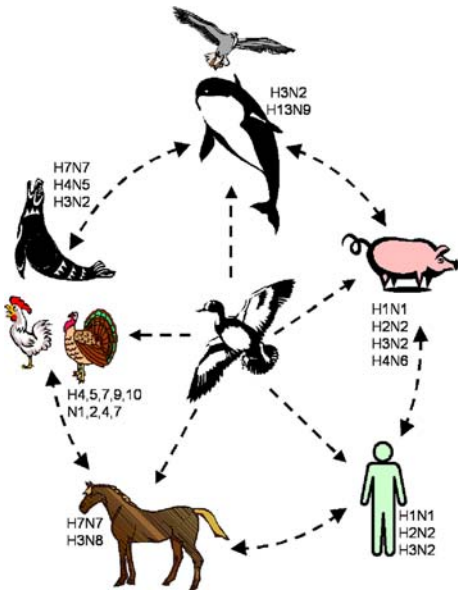

Fig. 1 Dissemination of influenza strains between different species

The emergence of HPAI H5N1 virus which may cause fatal infection in humans has raised great concerns that this virus could start a fourth pandemic [9]. The outbreaks of HPAI in poultry are known since 1959 when the first known HPAI outbreak due to virus of H5 subtype occurred [10]. However, both the increase in HPAI outbreaks and the number of birds affected are alarming since they increase the probability of emerging of strains capable of sustained human-to-human transmission. Table 1 lists outbreaks of classical fowl pest with influenza virus transmission to man. All those transmissions did not spread in significant human-to-human manner. Beside H5N1, the 2003 Dutch outbreak of avian influenza virus A/H7N7 is remarkable, since 89 men had been infected [11]. Of these 89 patients,

Table 1 Fowl pest outbreaks with virus transmissions to humans since 1995

Year	Location	Virus subtype	Human cases (total/fatal)	Clinical manifestation
1995	United Kingdom	H7N7	3/0	Conjunctivitis
1997	Hong Kong	H5N1	18/6	Influenza-like illness, pneumonia
1999	Hong Kong/China	H9N2	2/0	Influenza-like illness
2003	Hong Kong	H5N1	2(imported)/1	Influenza-like illness, pneumonia
2003	The Netherlands	H7N7	89/1	Conjunctivitis, influenza-like illness, pneumonia
2003–2007[a]	Azerbaijan, Cambodia, China, Djibouti, Indonesia, Iraq, Thailand, Turkey, Viet Nam	H5N1	270/164	Influenza-like illness, pneumonia, diarrhea, encephalitis
2004	Canada	H7N3	2/0	Influenza-like illness, pneumonia
2006	United Kingdom	H7N3	1/0	Conjunctivitis

[a] Up to 29 January 2007

78 presented with conjunctivitis, 5 presented with conjunctivitis and influenza-like illness, 2 presented with influenza-like illness, and 4 did not fit into the case definitions. However, the veterinarian who took care of the affected chicken mass holding farm developed severe pneumonia and died [11]. Serological data indicated that at least 1,000 people contracted H7N7 virus [12]. It was shown that avian H7N7 epidemic extend could be limited by chicken culling, as it was previously achieved by stopping the H5N1 spread in Hong Kong. However, the H5N1 virus re-emerged in south-east Asia in 2005 and produced an avian (semi)pandemic affecting many Asian, European and African countries (Fig. 2). Two isolated incursions of HPAI H5N1 virus into Europe occurred in 2004 and 2005 and are good examples of the influence of humans in the potential spread of avian influenza. The first was detected when eagles smuggled from Thailand and confiscated at Brussels Airport, Belgium, were shown to be infected with H5N1 virus genetically similar to those isolated in Thailand [13]. The second was when investigations of deaths in captive caged birds held in quarantine in England, ostensibly from Taiwan, showed them to be as a result of HPAI H5N1 infection [14].

In all countries where H5N1 infection has spread, the virus sporadically jumped from birds to humans (Fig. 2). Most of the infected individuals presented with lower respiratory tract manifestations early in the course of illness and a progression to respiratory failure has been associated with diffuse, bilateral, ground-glass infiltrates and manifestations of acute respiratory distress syndrome. Multiorgan failure with signs of renal dysfunction and sometimes cardiac compromise has been common. Unlike patients with H7N7 infection patients with H5N1 rarely have conjunctivitis but watery diarrhoea appears frequently and may precede respiratory manifestations up to January 2007 [15]. More than 50% of the patients died (64% in China, 68% in Thailand, 45% in Vietnam and 78% in Indonesia). Similar to Spanish flu 1918, younger people were preferentially affected. It must be considered that avian influenza A/H5N1 is now endemic in poultry houses or even chicken mass holding farms of southern China and south-east Asian countries. In a huge lot of hosts, H5N1 virus has found the basis to produce numerous variants (quasispecies). Some of them have mutated to HPAI [16], which can be retransmitted back to ducks and other wild birds, which have carried the virus throughout the world. Beside bird migration, the trade of contaminated poultry and chicken's food might support the virus spread as well. Continued virus activities in Indonesia were attributed to transmission via poultry movement within the country rather than through repeated introductions by bird migration [17].

In general, transmission of avian influenza viruses from birds to mammals, and especially to humans is a rare event. The risk of avian influenza virus transmission to humans depends on multiple factors including virus dose, exposure material and virus strain. Direct exposure to infected birds poses the greatest exposure risk because high levels of virus are excreted in respiratory secretions and faeces. The main portal of entry is upper respiratory tract and conjunctivae; the latter appears to be an important route for H7N7 and H7N3 infections. Direct introduction to the lower respiratory tract might occur following massive exposure, such as during a culling exercise. Personnel involved in culling operations have occasionally been infected [18]. However,

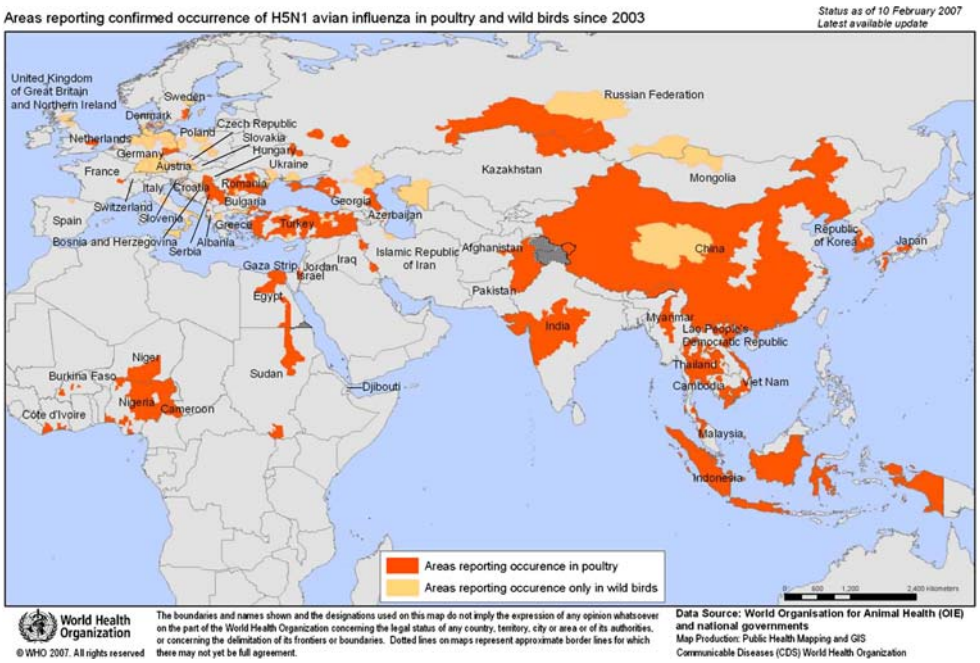

Fig. 2 Areas reporting confirmed occurrence of H5N1 avian influenza in poultry and wild birds since 2003

the importance of this remains uncertain. The risk of human infection, illness and death in the 1997 H5N1 outbreak in Hong Kong was associated with exposure to live poultry 1 week before the person's illness and was not associated with preparing or eating poultry meat [19]. In the recent H5N1 outbreaks, human infections in Thailand, Vietnam and Turkey were associated with direct exposure to infected poultry at the village or smallholdings level [20–22]. Also the prevalence of H5N1 among poultry through Indonesia has inevitably resulted in a rising number of human cases and fatalities [23]. Most Indonesians who have contracted the disease were not commercially poultry farmers; however, they were exposed while doing routine chores. This raised the occasional contact with infected material as an important source of environmental bird-to-human contamination [23]. Recently, avian virus transmission from human-to-birds was suspected in Indonesia where a curious pattern of outbreaks after vaccination teams visited villages occurred. It has been suggested that the teams were likely carrying the virus on contaminated clothing and vehicles and thus infected the birds they vaccinated, which died before they developed immunity [24].

To date, human-to-human transmission of avian influenza viruses has occurred sporadically with very low efficiency and occurred mainly in several household clusters. In the 1997 Hong Kong outbreak, one household contact of an H5N1-infected patient without any history of exposure to poultry and 3.7% of the health-care workers who had looked after the patients were subsequently found to be seropositive [18, 25]. In the recent Southeast Asian H5N1 outbreak, the possibility of human-to-human outbreak has been suggested, although serologic surveys have not found evidence of asymptomatic infections among the contacts. However, more sensitive detection methods may reveal greater numbers of persons with asymptomatic infections. Examination of contacts of patients by reverse transcriptase-polymerase chain reaction (RT-PCR) assay has led to the detection of mild cases, more infections in elderly adults, and an increased number and duration of clusters in families in northern Vietnam. It should be noted that results of enzyme-linked immunosorbent assay and rapid influenza test in all patients in H5N1 outbreak in eastern Turkey in 2005/2006 were negative, and the diagnosis was made by means of PCR [22]. In 2005 infected humans in Indonesia limited person-to-person H5N1 transmission could not be excluded in two clusters among patients who had no known contact with poultry or other animals [23]. The cluster of cases of the deadly H5N1 strain, which occurred early in 2006 in Kubu Sembelang, in North Sumatra was the most likely caused by human-to-human transmission [26]. Interestingly, one-third of Indonesian's human cases have come in clusters of blood relatives. In some clusters, relatives by marriage had similar exposures but did not contract the disease. These findings suggest an involvement of genetic susceptibility in virus transmission. There is also evidence for H7N7 infection that person-to-person transmission has occurred in 2003 in the Netherlands [27]. Documented H7N7 infections developed in three household contacts of the cases presented as conjunctivitis (in two cases) and as influenza-like illness (in two cases). Taken together the observations dealing with changes in epidemiology of avian influenza viruses suggest a growing potential of circulating virus for severe global epidemic.

Virulence determinants

Influenza viruses harbour a RNA genome, which is transcribed by its own polymerase. RNA transcription is associated with many point mutations persistently producing many changes in several proteins including the surface protein HA and NA, the polymerase complex [polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA)] and the non-structural proteins (NS) [28, 29]. The mutations in surface proteins result in antigen drift which helps the virus to escape the immunity of its host. Since the influenza virus genome is segmented into eight parts, two or more different virus variants infecting the same cell can produce progeny virus with a mixed genome, which supports the variability of viral structures. It may result in an antigenic shift, if two different subtypes of influenza A virus reassort their genomic segments. The emergence of H2N2 and H3N2 in mankind has been traced to such genomic reassortment [9, 30]. The 1957 Asian influenza virus acquired avian PB1, HA and NA genes, and the 1968 Hong Kong influenza virus acquired avian HA and PB1 genes.

The outcome of influenza virus infection is influenced by both host and virus. If the host has had prior exposure to a related strain, the effects of a highly pathogenic strain will be weakened. In an immunologically naïve host, virulence is mostly determined by the virus. Even in that situation, virulence is a complex phenomenon. Many viral genes can contribute to pathogenicity. Although in most instances virulence is a multigenic trait, a single gene (or a mutation in a single gene) can also markedly effect virulence. Factors that contribute to virulence and enable adaptation of avian influenza in mammalian cells depend on all steps of virus replication and have been intensively studied. Strains of the H5N1 influenza A virus that are virulent in mammals (including humans), have alterations in the sequences of any of three viral proteins including the major surface protein of the virus HA, the viral polymerase complex (including the PB1, PB2 and PA proteins) and the non-structural protein NS1 (Table 2).

Table 2 Virulence determinants of H5N1 virus in humans

Gene	Protein	Function	Change in H5N1
HA	Surface glycoprotein	Binding to receptor [*N*-acetylic neuraminic acid (SiA)]	No mammalian SiA-adapted variant isolated up to date
		cleaved by extracellular trypsin-like proteases in HA1 and HA2 for release of viral RNA	Multibasic cleavage site recognized by multiple cellular proteases
NS1	Nonstructural protein	Inhibits host interferon response in a species-specific manner	Single mutation increases NS1-induced interferon inhibition in mammals NS1 binds to cellular regulatory proteins and disturbs their function
PB2, PB1, PA	Members of polymerase complex	Copies genomic RNAs into viral mRNAs and catalyses replication of genomic RNAs	Adaptation to forms suited for human-to-human transmission likely?
PB1-F2	Encoded by an alternative reading frame in PB1	Induces apoptosis in macrophages thereby down regulating host immune response	Involved in adaptation of the H5N1 virus to humans?
M2	Ion channel	Involved in hydrogen transport during release of viral RNA	Involved in adaptation of the H5N1 virus to humans?

The most crucial and first step of influenza virus infection is an adsorption of the infectious agent to the target cell. This is mediated by a special membrane receptor, which reacts with a defined viral envelope structure. The cell receptor for influenza viruses is membrane-based sialic acid (SiA = *N*-acetylic neuraminic acid). Similar to a key in a lock, this SiA fits into a pocket-like structure at the top of the viral HA protein, which serves as anti-receptor. The membrane of avian or mammalian cells expresses slightly different configurations of SiA, depending on how the galactose residue of SiA is linked to membrane oligosaccarides. On avian cells, the galactose of SiA is linked via α 2, 3 C atoms (α2,3 SiA), on mammalian cells via α 2, 6 C atoms (α2,6 SiA). During evolution avian and human influenza viruses acquired haemagglutinins adapted to those species-specific receptor modifications [31, 32]. In the pig trachea, epithelial cells contain both α2,3-linked SiA (α2,3 SiA) and α2,6-linked SiA (α2,6 SiA) [33], explaining why pigs are highly susceptible to both human and avian viruses and are thought by some to be a "mixing vessel" for avian and human viruses, reassortment of which might give rise to pandemic strain. However, there is no evidence that 1957 Asian and 1968 Hong Kong pandemic viruses were generated in pigs. The 1918 pandemic virus is now regarded as a pure avian virus which adapted to humans [5].

The emergence of pandemic influenza virus from an avian progenitor also appears to involve a switch in preferential binding of the HA protein from α2,3 SiA acid to α2,6 SiA acid. The HA proteins of avian influenza virus species contain Gln226 and Gly228 residues, which form a narrow receptor binding pocket that favours binding of 2,3 SiA [34]. On the other hand, human species usually contain Leu226 and Ser228, which form a broad pocket that prefers α2,6 SiA [35]. High-resolution structures of the reassembled HA of the 1918 virus show that its avian-like Gln226 and Gly228 residues create a narrow avian-like binding pocket that still allows for high-affinity binding of α2,6 SiA [36, 37]. Only a single amino acid change at position 190 (Asp190Glu) in the HA of the 1918 virus switches its receptor binding preference from α2,6 SiA to α2,3 SiA [36–39]. Amino acids at position 190 and 225 in the 1918 virus HA determine not only its receptor binding specificity [39, 40] but also play a role in virus transmission [41]. Two amino acid mutations that cause a switch in receptor binding preference from the human α2,6 SiA to the avian α2,3 SiA (Asp190Glu and Asp225Gly) resulted in a virus incapable of respiratory droplet transmission between ferrets but that maintained its lethality and replication efficiency in the upper respiratory tract. Furthermore, poor transmission of a 1918 virus with dual α2,6 SiA and α2,3 SiA specificity suggests that a predominant human α2,6 SiA binding preference is essential for optimal transmission of this pandemic virus [41]. These findings confirm an essential role of HA receptor for the transmission of influenza viruses among mammals. However, although just a single Glu190Asp mutation in the HA of the H5N1 strain could potentially switch its binding preference to α2,6 SiA, an expected requirement for its evolution into pandemic virus, no avian influenza virus A/H5N1 has been isolated and found to be adapted to mammalian SiA till date.

The second step of influenza viral replication is the release of viral RNA that is complexed with polymerase and NP (ribonucleoprotein RNP) out of the viral particles through membrane fusion [35]. A precursor HA molecule undergoes post-translational cleavage into HA1 and HA2 subunits by host proteases, with the generation of a fusogenic domain at the amino terminus of HA2 that mediated fusion between the viral envelope and endosomal membrane [42]. Proteolytic activation is therefore essential for viral infectivity and dissemination [43]. The HAs of low

pathogenic avian viruses and seasonal human influenza viruses possess a single arginine at the cleavage site, recognized by extracellular trypsin-like proteases [44]. For this reason, the HA of low pathogenic influenza viruses is cleaved in only limited number of organs, resulting in mild or asymptomatic infection. In contrast, highly pathogenic avian viruses possess multiple basic amino acids at the cleavage site that are recognized by ubiquitous, intracellular subtilisin-like proteases that thus trigger systemic infection [45, 46]. A carbohydrate side chain near the cleavage site can affect HA cleavability by interfering with the accessibility of the host proteases to the cleavage site [47]. All avian viruses lethal to humans have a highly cleavable HA and an H5N1 mutant virus whose HA cleavable site had been changed to an avirulent type was attenuated in mice [48]. These findings show that basic residues adjacent to the HA cleavage site are required for the virulence of these viruses. Interestingly, the 1918 virus HA does not have a multibasic cleavage site and possible its own NA protein is involved in cleavage of HA [49]. These findings demonstrate that low pathogenic influenza viruses could potentially increase their virulence through mutations in or reassortment of their NA gene as was shown for neurotropic strain of influenza virus, A/WSN/33 [50]. For WSN virus this NA activity is one of the determinants of the virulence of this virus in mice [51].

NS1 protein of influenza viruses, encoded by the unspliced mRNA derived from the shortest RNA segment, acts as virulence factor at least in part due to its ability to overcome the IFNα/β response during influenza A infection [52–54]. The basis of the IFNα/β antagonistic properties of the NS1 of influenza A virus relies on its ability to prevent IFNβ synthesis. NS1 prevents the activation of transcription factors such as ATF-2/c-jun, NFκB and IRF-3/5/7, all of which stimulate IFN expression. The NS1 protein also binds and inhibits the function of two cellular proteins that are required for the modification of the 3' ends of cellular mRNA [54]. Consequently, the production of IFNβ mRNA is substantially reduced. Moreover, NS1 binding of dsRNA generated in the cytoplasm during viral infection, results in suppression of 2'5' oligo (A) synthase (OAS)/RNase L pathway which represents important defence mechanism against viral infections [55]. Nevertheless, the effects of NS1 protein on IFN response remain only partially understood [56]. The NS1 gene of the 1918 virus was compared with the NS1 gene of the mouse-adapted H1N1 influenza A virus strain WSN. Replacement of the NS1 gene of WSN virus with that of the 1918 virus resulted in an attenuated virus in mice, but this virus more efficiently inhibited the IFNα/β system in human cells [57, 58]. This probably results from the fact that the viral interferon antagonist activity encoded by the NS gene shows a high degree of species specificity (a human NS gene being more active in human cells than in mouse cells) [57, 58]. An NS1 gene derived from a highly virulent avian H5N1 virus showed similar properties. In this case, a single mutation was shown to alter considerably the phenotype of an influenza virus in a specific host. Pigs infected with H1N1 virus carrying the NS1 gene of H5N1 experienced significantly greater and prolonged viremia, fever and weight loss as compared to animals infected with the control virus [59]. These effects required the presence of glutamic acid at position 92 of the NS1 protein.

In addition to blocking IFN response, the NS1 protein of avian H5N1 viruses may act as a virulence factor by specific binding of cellular proteins and disrupting their functions in regulatory pathways. The carboxyl terminus of the NS1 proteins of the vast majority of avian H5N1 viruses contains a sequence motif, Glu-Ser-Glu-Val (ESEV) [60]. These residues are predicted to mediate binding to proteins bearing a region called a PDZ domain. The multitude of human proteins that contain a PDZ domain function in diverse cellular signalling pathways including those that regulate protein traffic within the cells and those that maintain cell morphology and organization. Another PDZ-binding sequence, Glu-Pro-Glu-Val (EPEV), was identified at the carboxyl terminus of the NS1 proteins of all the virulent H5N1 viruses isolated from humans. In contrast, the carboxyl terminus of the NS1 proteins of low-virulence human influenza A usually contains a different sequence which is not a PDZ binding motif [60]. Functional studies confirmed that the C terminus of NS1 in low-pathogenic human influenza viruses has no interactions with PDZ domains, but the highly pathogenic viruses from 2003, 1997 and 1918 are all able to interact with some PDZ proteins [60]. On the other hand, virulence of H5N1 viruses in ferrets does not depend on carboxyl-terminal ESEV/EPEV sequence in NS1 protein and the highly pathogenic human isolate A/Vietnam/1203/04 encodes for an NS1 protein which is truncated and consequently lacks the suspect ESEV/EPEV motif [56, 61]. Therefore, further experiments are required to examine whether eliminating the carboxyl-terminal ESEV/EPEV sequence of NS1 protein of other H5N1 viruses has any effect on their virulence.

The polymerase complex (including the PB1, PB2 and PA proteins) is also implicated in virulence. The polymerase is associated with each of the viral genomic RNAs in the virus particle. The polymerase copies these genomic RNAs into viral mRNAs and also catalyzes the replication of the genomic RNAs in infected cells. Some mutations that can enhance the activity of polymerase and increase virulence in mice have been found in H5N1 HPAIV strains [62]. Notably, H5N1 viruses that are virulent in mice encode lysine at position 627 in PB2, whereas H5N1 viruses that are not virulent in mice, as well as other avian influenza A virus strains, encode glutamic acid at this position [48]. It is

thought that this change from glutamic acid to lysine represents an adaptation of H5N1 viruses for efficient replication in mammalian cells [61]. H5N1 viruses recovered from the brains of dead mice infected with A/chicken/Yamaguchi/7/2004 isolate from Japan had substituted glutamic acid to lysine at position 627 of PB2 after a single passage in mice [62]. The mouse lethality of the recovered virus had increased by approximately 5×10^4 times over that of the original virus. However, molecular analysis of virus isolates from 12 H5N1-infected individuals who were admitted to referral hospitals in Ho Chi Minh City during 2004 and 2005 did not reveal unique amino acid changes when performed pairwise comparison of all gene segments of viruses isolated from eight fatal and four surviving cases [63]. No viruses contained glutamic acid at position 92 of the NS1 protein, which is associated with adaptation and virulence in mammals [59], but all contained the PDZ-domain ligand ESEV [60]. The substitution of glutamic acid to lysine at position 627 of PB2 was present in five of eight isolates from fatal cases and in three of four isolates from patients who survived. There was no association between the presence of lysine at position 627 of PB2 and the clinical outcome. Three of four viruses without this substitution, but none of the viruses containing lysine at position 627, contained the substitution of aspartic acid to asparagine at position 701 in PB2 that has been associated with adaptation of H7N7 viruses to mammalian cells [64]. This suggests that the substitution of aspartic acid to asparagine at position 701 may compensate for the absence of lysine at position 627 in conferring enhanced viral polymerase activity and virulence in the mammalian host [63]. Other amino acids residues in the viral polymerase complex implicated in mammalian adaptation, including proline at position 13 in PB1 protein and arginine at position 615 in PA protein, were present in all viruses. These clinical observations are in agreement with experimental study using reverse genetic to generate reassortants combining genes of lethal A/Vietnam/1203/04 (VN1203), a fatal human case isolate, and nonlethal A/chicken/Vietnam/C58/04 (CH58). The results demonstrated that exchanging HA and NA genes did not alter pathogenicity but substituting CH58 polymerase genes completely attenuated VN1203 virulence and reduced viral polymerase activity [65].

These findings demonstrate that changes in only the polymerase genes of an H5N1 influenza virus are sufficient to dramatically alter its pathogenic potential. Even changes in single segments PB2 and/or PB1 attenuated the human virus isolate's pathogenicity. Notably, the 1957 and 1968 influenza pandemics were caused by viruses in which the PB1 was replaced by avian PB1 [66]. Use of prototypic pandemic virus 1918, reconstructed by reverse genetic [49] demonstrated that all eight virus segments originated from an avian host [5]. Therefore, three pandemic viruses had avian-like PB1 genes. This stresses the potential importance of PB1 for the high replication efficiency that was suggested by the experiments using VN1203 [65]. The sequences of the polymerase proteins of the 1918 virus and subsequent human viruses differ by only ten amino acids from the avian influenza virus consensus sequence [5]. Noteworthy, the human forms of seven of the ten polymerase residues have already been observed individually in currently circulating H5N1 influenza viruses recovered from birds and humans. It is conceivable that the polymerase genes of an avian H5N1 virus that is currently circulating could potentially mutate upon selective pressure in infected humans to the forms that are better suited for efficient human-to-human transmission [67].

Molecular analysis of H5N1 avian and human virus isolates in Indonesia and Vietnam shows that only the M2 and PB1-F2 genes were under positive selection pressure, suggesting that these genes might be involved in adaptation of the H5N1 virus to new hosts following interspecies transmission [17]. The M2 ion channel, which is involved in hydrogen transport, may be under positive selection as the viruses repeatedly adapt between aquatic and terrestrial hosts that have different pH and cellular environments [68]. The PB1-F2 protein encoded by an alternate reading frame in the PB1 gene segment of influenza A virus has been shown to enhance viral virulence in a mouse model [69]. Interestingly this alternate reading frame is found in the pandemic strains of 1968, 1957, and the infamous 1918 strain [69]. The PB1-F2 protein contributes to virulence by inducing apoptosis in macrophages resulting in down regulation of the host immune response to infection [70]. It is worthy to further investigate whether novel variants of PB1-F2 developed upon a positive selection pressure during ongoing evolution of H5N1 viruses might influence their virulence.

In summary, the current H5N1 virus is apparently not well "fitted" to replication in humans. However, the number of infection in humans continues to increase and the virus changes its virulence and epidemiology. Considering the overall risk for a pandemic H5N1 strain, the relevant figure is not the risk that a single infection will lead to a pandemic, but the probability that any of the human or animal cases occurring over that period will give rise to a strain capable of sustained human-to-human transmission [71]. Mutations that make the virus more compatible with human-to-human transmission may occur at any time. Nevertheless, no one can currently predict with certainty whether H5N1 will become a human pandemic virus [72].

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