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Review

Avian influenza A (H5N1)

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Contents

Abstract

Since their reemergence in 2003, highly pathogenic avian influenza A (H5N1) viruses have reached endemic levels among poultry in several southeast Asian countries and have caused a still increasing number of more than 100 reported human infections with high mortality. These developments have ignited global fears of an imminent influenza pandemic. The current knowledge of the virology, clinical spectrum, diagnosis and treatment of human influenza H5N1 virus infections is reviewed herein.

Keywords: Avian; Influenza; H5N1; Human; Review

1. Introduction

Periodically, completely novel antigenic subtypes of influenza viruses have been introduced in the human population, causing large-scale global outbreaks with high death tolls. The most devastating influenza pandemic in modern recorded history, known as the “Spanish flu”, occurred in 1918–1919, killing up to 100 million people worldwide. Other less destructive pandemics during the previous century occurred in 1957 and 1968. Avian influenza A viruses are key to the emergence of human influenza pandemics.

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The virus strains implicated in the 20th century's influenza pandemics originated directly from avian influenza viruses, either through genetic reassortment between human and avian influenza strains (1957 and 1968) or possibly through adaptation of purely avian strains to humans (1918). It was long thought that the restricted host range of avian influenza viruses precluded direct transmission to humans, and that the emergence of pandemic strains required genetic reassortment between avian and human strains. However, occurrences of direct bird-to-human transmission of avian influenza viruses have increasingly been reported in recent years, culminating in the ongoing outbreak of influenza A (H5N1) among poultry in several Asian countries with associated human infections. These unprecedented developments have resulted in increasing global concerns about the pandemic potential of these viruses.

2. Virology

Influenza viruses are pleomorphic, enveloped RNA viruses belonging to the family of Orthomyxoviridae. Protruding from the lipid envelope are two distinct glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). HA attaches to cell surface sialic acid receptors, thereby facilitating entry of the virus into host cells. Since it is the most important antigenic determinant to which neutralizing antibodies are directed, HA represents a crucial component of current vaccines. NA is the second major antigenic determinant for neutralizing antibodies. By catalyzing the cleavage of glycosidic linkages to sialic acid on host cell and virion surfaces, this glycoprotein prevents aggregation of virions thus facilitating the release of progeny virus from infected cells. Inhibition of this important function represents the most effective antiviral treatment strategy to date. A third membrane protein, the M2 protein, is present in small quantities in influenza A viruses. By functioning as an ion channel, this protein regulates the internal pH of the virus, which is essential for uncoating of the virus during the early stages of viral replication. This function is blocked by the antiviral drugs amantadine and rimantadine.

The genome of influenza viruses is segmented, consisting of 8 single-stranded, negative sense RNA molecules, which encode 10 proteins. The RNA segments are contained within the viral envelope in association with the nucleoprotein (NP) and three subunits of viral polymerase (PA, PB1, and PB2), which together form the ribonucleoprotein (RNP) complex responsible for RNA replication and transcription. Additional proteins contained within the virion include M2 and the viral nuclear export protein (NEP), which function in assembly and budding, and export of RNP from the nucleus, respectively.

Based on antigenic differences in NP and M proteins, influenza viruses are classified as types A, B, and C. Influenza B and C viruses are not divided into subtypes. All avian influenza viruses are classified as type A. Further subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins HA and NA. To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) of influenza A viruses have been identified (Fouchier et al., 2005). The standard nomenclature for influenza viruses includes the influenza type, the host of origin (excluding humans), the place of isolation, the strain number, the year of isolation, and finally the influenza A subtype in parentheses (e.g. A/Duck/Vietnam/11/04 (H5N1)).

The natural reservoir of influenza A viruses are aquatic birds, in which the viruses appear to have achieved an optimal level of host adaptation (Webster et al., 1992). Transmission between birds occurs directly or indirectly through fecally contaminated aerosols, water, feed, and other materials. The spectrum of disease in birds ranges from asymptomatic infection, to mild respiratory illness, to severe and rapidly fatal systemic disease. Most avian influenza viruses isolated from birds are avirulent, i.e. result in asymptomatic infection or only mild disease. Avian influenza viruses capable of causing outbreaks of severe disease (fowl plague) in chickens or turkeys are classified as highly pathogenic, and are currently restricted to H5 and H7 subtypes. Infection of poultry by highly pathogenic avian influenza viruses is characterized by disseminated infection, and clinically manifested by decreased egg production, respiratory signs, excessive lacrimation, edema of the head, diarrhea, neurological symptoms, and death.

From the principal reservoir of aquatic birds, viruses are occasionally transmitted to other animals, including mammals and domestic poultry, causing transitory infections and outbreaks. Through adaptation by mutation or genetic reassortment, some of these viruses may establish species-specific permanent lineages of influenza A viruses, and cause epidemics or epizootics in the new host. In the human population, the establishment of these lineages in the 20th century was preceded by influenza pandemics. Transmission of viruses and transitory infections may also occur among the new hosts, e.g. between humans and pigs or chickens and humans.

Although all HA and NA subtypes are found in aquatic birds, the number of subtypes that have crossed the species barrier and established stable lineages in mammals is limited. Only three HA and two NA subtypes (i.e. H1–3 and N1–2)) have circulated in humans since 1918. In horses, only two influenza A subtypes (H7N7 and H3N8) are found, while, despite susceptibility to all avian subtypes in experimental settings, the only subtypes recovered from pigs in nature are H1, H3, N1, and N2. The molecular, biological or ecological factors determining the apparent subtype-specific ability of viruses to cross species barriers and spread among a range of hosts remain largely unresolved.

While interspecies transmission does occur at times, there certainly are host range restrictions. Human influenza strains preferentially bind to sialic acid residues linked to galactose by the α2,6 linkage, while avian and equine influenza strains recognize sialic acid linked to galactose by α2,3 linkage (Connor et al., 1994; Gambaryan et al., 1997; Matrosovich

et al., 1997, 2004; Rogers and D'Souza, 1989; Rogers and Paulson, 1983; Rogers et al., 1983). Correspondingly, human respiratory epithelial cells predominantly contain α2,6 sialic acid–galactose linkages, while the host cells in birds and horses mainly contain α2,3 linkages (Couceiro et al., 1993; Ito et al., 1998; Matrosovich et al., 2004). Respiratory epithelial cells in the pig contain both α2,3- and α2,6 linkages, which explains why this animal is susceptible to both human and avian influenza viruses (Ito et al., 1998). Because of this trait, the pig is widely regarded as a potential source of new pandemic strains, since it could serve as a non-selective host in which mixed infection of avian and human strains efficiently occurs, potentially resulting in new reassortant viruses, or in which purely avian strains can adapt to human receptor recognition.

3. Avian influenza viruses and pandemic influenza

Introduction of an influenza A virus with a novel HA gene in a population which lacks immunity to this HA has the potential to cause a pandemic when the virus posesses the ability to spread efficiently among humans. During the 20th century, this has happened three times, in 1918, 1957, and 1968, killing millions of people worldwide. In all three pandemics, the viruses originated from avian influenza viruses.

The virus strains responsible for the influenza pandemics of 1957 and 1968 both first emerged in southeastern Asia, and both arose through reassortment of genes between avian viruses and the prevailing human influenza strain (Scholtissek et al., 1978). The "Asian influenza" pandemic of 1957 was caused by an H2N2 virus that had acquired three genes (H2, N2, and PB1) from avian viruses infecting wild ducks, in a backbone of the circulating H1N1 human influenza strain. As the Asian flu strain emerged and established a permanent lineage, the H1N1 strains soon disappeared from the human population for unclear reasons. Similarly, the H3N2 virus causing the "Hong Kong influenza" pandemic of 1968 consisted of two genes from a duck virus (H3 and PB1) in a background of the human H2N2 strain circulating at that time. The latter virus disappeared with the emergence of the H3N2 virus and since then has not been detected in humans. Sequence analysis of the hypothetical precursor strain, which immediately preceded the pandemic H3N2 virus suggested that fewer than six amino acids in HA had changed during the avian-to-human transition (Bean et al., 1992). Interestingly, a number of these changes may reflect adaptation to the new host since they modified the area surrounding the receptor-binding pocket of HA, including a Glu to Leu change at position 226 which is particluarly implicated in determining specificity for human receptors. The fact that, beside one or two novel surface glycoproteins, both pandemic strains also posessed a PB1 gene of avian origin is intriguing and may suggest a role of this gene in interspecies transmission (Kawaoka et al., 1989).

Although millions of people died during the 1957 and 1968 pandemics, the viruses involved did not appear particularly virulent, suggesting that lack of immunity was the main reason for the excess mortality. This was different during the "Spanish flu" pandemic of 1918, in which lack of immunity in the human population was combined with an apparent extremely high virulence of the virus, resulting in the demise of up to 100 million people worldwide. Because the 1918 pandemic occurred before viruses were identified as the causative agents, no intact virus has been available for analysis. This and the similar lack of available human and animal influenza strains circulating before 1918 has made it difficult to determine the exact origin of the pandemic H1N1 virus and the reason for its extreme virulence. However, valuable insight has been provided by the recovery of fragments of viral RNA isolated from archived autopsy specimens and tissue from Alaskan flu victims buried in the permafrost (Taubenberger et al., 1997). This enabled sequence analysis of five of the eight genes (HA, NA, NP, M, and NS) (Reid et al., 2004). Phylogenetic analyses of these genes suggest that the 1918 H1N1 virus may not have arisen by the same mechanism as the 1957 and 1968 pandemic viruses, i.e. by reassortment of avian and human influenza viruses, but perhaps by direct transmission from an avian source after adaptation in humans or another permissive mammalian host, such as the pig (Reid et al., 2004). This is supported by the observation that the 1918 pandemic strain retained the amino acid residues at positions 226 and 228 of HA predictive for binding to avian receptors (Taubenberger et al., 1997). Recent chrystallographic studies showed that structural changes in the H1 HA allowed the virus to recognize human receptors despite the presence of these avian-like residues, which may explain why the virus could nevertheless efficiently infect and spread among humans (Gamblin et al., 2004; Stevens et al., 2004). The possibility that the 1918 strain had retained the structure and biological properties of its avian ancestors while acquiring the ability to recognize and efficiently infect human cells may explain the high virulence of this virus. Mathematical modelling studies have suggested that the transmissability of the 1918 virus was not remarkably different than regular human influenza strains, suggesting that extremely efficient spread did not account for the high morbidity and mortality (Mills et al., 2004). While part of the high mortality of the 1918 pandemic could be explained by the lack of antibiotics to treat secondary bacterial pneumonia and poor living conditions, the extremely rapid and severe clinical course implies high pathogenicity of the virus as the major cause. The molecular basis for this high virulence remains unclear. The 1918 H1 HA lacks the multibasic cleavage site characteristic of highly pathogenic avian influenza viruses (Reid et al., 1999; Taubenberger et al., 1997). There are conflicting observations concerning the role of the NS gene in the 1918 pandemic strain. In mice, the presence of the complete NS or only the NS1 segment seemed to confer decreased, rather than enhanced pathogenicity of reassortant H1N1 viruses (Basler et al., 2001). In contrast,

in vitro experiments in human lung cells suggested more efficient inhibition of interferon-regulated genes by H1N1 virus in the presence of the 1918 NS gene (Geiss et al., 2002). The most convincing evidence implicates HA as an important determinant of the high virulence. The presence of HA of the 1918 virus conferred high pathogenicity in mice to human strains that were otherwise non-pathogenic in this host (Kobasa et al., 2004). Furthermore, these infections were associated with severe hemorrhagic pneumonia and the induction of high levels of macrophage-derived cytokines and chemokines, strikingly reminiscent of clinical observations in humans during the Spanish flu pandemic, as well as of recent in vitro and in vivo observations of infections with highly pathogenic avian influenza H5N1 viruses (Cheung et al., 2002; Oxford, 2000; Peiris et al., 2004; To et al., 2001).

4. Human infections with influenza A (H5N1) viruses

In recent years, it has become clear that human infections with highly pathogenic influenza H5N1 viruses are associated with severe, often fatal disease. In May 1997, following outbreaks of influenza H5N1 among poultry on three farms in the New Territories of Hong Kong, an influenza H5N1 virus was isolated from a 3-year-old boy in Hong Kong, who died of severe pneumonia complicated by acute respiratory distress syndrome and Reye's syndrome (Subbarao et al., 1998). In November and December of the same year, concomittant with outbreaks of influenza H5N1 among chickens in poultry markets and on farms in Hong Kong, 17 additional cases of human H5N1 infections were identified, 5 of which were fatal (Chan, 2002; Yuen et al., 1998). The outbreak was contained after the slaughtering of all 1.5 million chickens in Hong Kong. In response to the outbreak, influenza surveillance in poultry was intensified permitting early recognition of other outbreaks of avian influenza in 2001 and 2002. No further human H5N1 infections were reported until February 2003, when two laboratory-confirmed cases and one probable case were identified in one family from Hong Kong (Peiris et al., 2004). The daughter died of an undiagnosed respiratory infection while visiting Fujian Province in mainland China. Upon their return to Hong Kong, the father and son developed severe respiratory illnesses of which the father died. H5N1 virus was isolated from both patients.

In December 2003, an outbreak of highly pathogenic H5N1 virus was identified among poultry in the Republic of Korea (Lee et al., 2005). Subsequently, outbreaks by antigenically related viruses were reported among poultry in Thailand, Viet Nam, Japan, China, Cambodia, Laos, Malaysia, and Indonesia. The reason for this apparent simultaneous occurrence of H5N1 outbreaks in many Asian countries remains unclear. However, H5N1 viruses have also been found in dead migratory birds, which may suggest a role of wild birds in the maintenance and spread of H5N1 viruses in the region (Chen et al., 2005; Li et al., 2004).

Human infections during the southeast Asian outbreaks were first reported in early 2004 from Viet Nam and Thailand, followed by still ongoing resurgences of human cases in Viet Nam, Cambodia, and Indonesia from then onwards (Chotpitayasunondh et al., 2005; Tran et al., 2004). At the time of this writing (August 2005), the total number of confirmed human cases of influenza H5N1 in the 4 countries amounts to 112 (Thailand: 17; Cambodia: 4; Indonesia: 1; Viet Nam: 90), of which 57 were fatal (WHO, 2005). It cannot be excluded and may even be likely that additional cases have gone unnoticed in these and other affected countries due to a lack of clinical awareness, active surveillance, or diagnostic facilities (Hien et al., 2004).

While many countries initially affected by poultry outbreaks in 2004 have been declared free of the virus, H5N1 virus seems to have reached endemic levels in poultry and aquatic birds in several Asian countries, despite attempts to contain the outbreak by extensive culling of poultry. In these countries, continuing occurrences of bird-to-human transmissions increase the opportunity of the virus to adapt to humans and acquire the ability to spread between humans. In addition, continuing cocirculation of avian and human viruses in these countries, where humans live in close proximity with poultry and pigs, increases the risk of reassortment between both in co-infected humans or other mammalian hosts, such as the pig. The recent isolation of H5N1 viruses from pigs in China (Chen et al., 2004), and, albeit at low prevelance, the detection of H5N1 antibodies in Vietnamese pigs (Choi et al., 2005), are concerning in this respect. For all these reasons, the current developments in southeast Asia seem to justify the global concern that, similar to 1957 and 1968, a new pandemic influenza strain may emerge from this region in the near future.

5. The clinical spectrum of human H5N1 infections

At presentation, most cases of human H5N1 infections were characterized by a severe influenza syndrome, clinically indistinguishable from severe human influenza, with symptoms of fever, cough and shortness of breath, and radiological evidence of pneumonia (Chotpitayasunondh et al., 2005; Tran et al., 2004; Yuen et al., 1998). Abnormalities on chest radiographs at presentation included extensive, usually bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms. Radiological evidence of pulmonary damage could still be observed in surviving patients several months after the illness. Beside respiratory symptoms, a large proportion of patients also complained of gastrointestinal symptoms such as diarrhea, vomiting, and abdominal pain, which are common in children with human influenza, but not in adults. In some cases, diarrhea was the only presenting symptom, preceding other clinical manifestations (Apisarnthanarak et al., 2004; de Jong et al., 2005). Unlike human infections with H7 or H9 viruses, conjunctivitis was not prominent in H5N1-infected patients. The clinical course of the illness

in severe cases was characterized by rapid development of severe bilateral pneumonia necessitating ventilatory support within days after onset. Complications included acute respiratory distress syndrome, renal failure, and multi-organ failure. Evidence that the clinical spectrum of human H5N1 infections is not restricted to pulmonary symptoms was provided by a reported case of possible central nervous system involvement in a Vietnamese boy who presented with diarrhea, followed by coma and death. Influenza H5N1 virus was isolated from throat, rectal, blood, and cerebrospinal fluid specimens, suggesting widely disseminated viral replication (de Jong et al., 2005). His sister had died of a similar illness 2 weeks earlier, but no diagnostic specimens were obtained. Although highly virulent H5N1 viruses have shown neurotropism in mammals such as mice and cats (Keawcharoen et al., 2004; Lipatov et al., 2003; Tanaka et al., 2003), these cases may be similarly rare as central nervous system manifestations associated with human influenza (Morishima et al., 2002; Sugaya, 2002). Genetic predisposition of the host to such manifestations may play a role.

Striking routine laboratory results in H5N1-infected patients, especially in severe cases, were an early onset of lymphopenia, with a pronounced inversion of the CD4+/CD8+ ratio, thrombocytopenia and increased levels of serum transaminases (Chotpitayasunondh et al., 2005; Tran et al., 2004; Yuen et al., 1998). High levels of cytokines and chemokines have been observed in several H5N1-infected patients, suggesting a role of immune-mediated pathology in the pathogenesis of H5N1 infections (Peiris et al., 2004; To et al., 2001). This was supported by pathological examination in two patients who died during the outbreak in Hong Kong, which showed reactive hemophagocytosis as the most prominent feature (To et al., 2001). Other findings included diffuse alveolar damage with interstitial fibrosis, hepatic central lobular necrosis, acute renal tubular necrosis, and lymphoid depletion. Although the gastrointestinal, hepatic, renal, and hematologic manifestations could suggest wider tissue tropism, there was no evidence of viral replication in organs outside the respiratory tract (To et al., 2001). However, viral replication in the gastrointestinal is strongly suggested by reported virus isolation and detection of positive strand viral RNA from fecal specimens (de Jong et al., 2005; Uiprasertkul et al., 2005).

While many laboratory-confirmed H5N1 infections were associated with severe, often fatal disease, milder cases have also been reported, especially during the outbreak in Hong Kong (Chan, 2002; Yuen et al., 1998). An increasing number of milder cases also seemed to occur in Viet Nam, as the outbreak progressed in 2005 (WHO, 2005). While increased clinical awareness and surveillance may account for such observations, progressive adaptation of the virus to humans is the dreaded alternative explanation. The occurrence of mildly symptomatic and asymptomatic infections have also been suggested during the outbreak in Hong Kong by seroepidemiological studies in household members of H5N1-infected patients and health care workers. In these studies, 8 of 217 exposed and 2 of 309 non-exposed healthcare workers were seropositive for H5N1-specific antibodies (Bridges et al., 2002). Seroconversion was documented in two exposed nurses, one of whom reported a respiratory illness 2 days after exposure to an H5N1-infected patient. More importantly than showing the occurrence of asymptomatic infections, these data indicated that nosocomial person-to-person transmission had occurred, albeit limited to a few cases. An additional case of possible human-to-human transmission during the Hong Kong outbreak was suggested by H5N1-seropositivity in a household contact of a patient, who had no history of poultry exposure (Katz et al., 1999). Seroepidemiological studies in health care workers involved in the care of H5N1-infected patients in Thailand and Viet Nam in 2004 have not shown evidence of person-to-person transmission, despite the absence of adequate infection control measures in the Vietnamese cohort at the time of study (Apisarnthanarak et al., 2005; Liem and Lim, 2005; Schultsz et al., 2005). During the outbreak in Thailand in 2004, extensive epidemiological investigations have suggested person-to-person transmission from a child, who died of presumed H5N1 infection, to her mother who had no history of exposure to poultry and had provided prolonged unprotected nursing care to her daughter (Ungchusak et al., 2005). An aunt of the child may have been infected by the same route since her last exposure to poultry before infection had been 17 days, considerably longer than the estimated incubation period of 2–10 days. There have been several similar family clusters of H5N1 cases in Viet Nam, which have all ignited concerns about the possibility of human-to-human transmission, but most of which could be explained by common exposure to poultry. While there has been no evidence of efficient transmission of influenza H5N1 virus between humans to date, caution and detailed investigations remain warranted in case of any cluster of infections, especially in view of the relatively rapid evolution H5N1 viruses have exhibited in recent years.

6. The evolution of H5N1 viruses, 1997–2004

In 1996, an H5N1 virus was isolated from geese during an outbreak in Guangdong Province in China (influenza A/Goose/Guangdong/1/96 (A/G/Gd/96)) (Xu et al., 1999). This virus proved to be the donor of the HA gene of the reassortant H5N1 viruses causing the outbreak among poultry and humans in Hong Kong in 1997. The internal genes of the Hong Kong H5N1 viruses were closely related to those of an H9N2 virus isolated from quail (Guan et al., 1999). The origin of the NA gene remains unclear, but was notable for a 19-amino acid deletion in the stalk region (Subbarao et al., 1998). Such deletions may be associated with adaptation of influenza viruses to land-based poultry (Matrosovich et al., 1999). The HA gene contained multibasic sequences at the cleavage site, in accordance with its classification as a highly pathogenic strain (Claas et al., 1998; Matrosovich et al., 1999).

After the eradication of the 1997 Hong Kong strain, the goose precursor viruses continued to circulate in geese in southeastern China (Cauthen et al., 2000; Webster et al., 2002). Through reassortment between this virus and other avian viruses, multiple antigenically similar genotypes, that were highly pathogenic in chickens but not in ducks, emerged and again were eradicated in Hong Kong in 2001 and 2002 (Guan et al., 2002). Then, in late 2002, H5N1 strains isolated from wild migratory birds and resident waterfowl in two Hong Kong parks showed marked antigenic drift and exhibited high pathogenicity in ducks (Guan et al., 2004; Sturm-Ramirez et al., 2004). The latter property is rarely found in nature, and had not been observed in strains isolated during previous years. An antigenically and molecularly similar virus caused the two confirmed human infections in early 2003 in a family from Hong Kong (Guan et al., 2004; Peiris et al., 2004).

H5N1 influenza viruses isolated from healthy ducks in southern China between 1999 and 2002 were all antigenically similar to the precursor influenza A/G/Gd/96 virus (Chen et al., 2004). It is thought that these ducks played a central role in the generation of the virus responsible for the outbreaks in southeast Asia since 2003. Detailed genetic analyses of H5N1 strains isolated during the period 2000–2004 from poultry and humans in China, Hong Kong, Indonesia, Thailand, and Viet Nam, demonstrated that a series of genetic reassortment events, all traceable to the A/G/Gd/96-precursor virus, ultimately gave rise to a dominant H5N1 genotype (genotype Z) in chickens and ducks (Li et al., 2004). This genotype is implicated in the human cases in Hong Kong in 2003 and the outbreaks among poultry and humans since 2004.

The evolution of H5N1 viruses in recent years has been associated with increasing virulence and an expanding host range, which beside terrestrial poultry and wild birds, also includes mammals. While all H5N1 viruses isolated from ducks in China between 1999 and 2002 were highly pathogenic in chickens, an increasing level of pathogenicity was observed in mice with the progression of time: virus isolated in 1999 and 2000 were less pathogenic than those isolated in 2001 and 2002 (Chen et al., 2004). It has been suggested that the increasing ability to replicate in mammals has resulted from transmission between ducks and pigs. The expanding host range is also illustrated by successful experimental infection of domestic cats, and natural infections of tigers and leopards with recent H5N1 strains (Keawcharoen et al., 2004; Kuiken et al., 2004).

In summary, continued evolution of H5N1 viruses since 1997, involving multiple genetic reassortment events between A/G/Gd/96-like viruses and other avian viruses and perhaps transmission between birds and pigs or other mammalian hosts, have resulted in a highly virulent genotype with an expanded host range which is now causing widespread outbreaks among poultry and humans in southeast Asia. While transmission between birds and humans at present still seems inefficient, as does transmission between humans, this may change when the virus is allowed to continue its evolution through adaptation and reassortment.

7. Laboratory diagnosis of influenza H5N1

Although virus isolation remains the gold standard of diagnosis and indispensable for virus characterization, rapid laboratory confirmation of suspected human influenza in routine diagnostic laboratories is usually performed by immunochromatographic or immunofluorescent detection of influenza virus antigens, or reverse transcriptase (RT) PCR detection of viral nucleic acids in respiratory specimens. In addition, serological evidence of human influenza A virus infection can be obtained by commercially available elisa kits which detect antibodies to conserved viral antigens, such as the nucleoprotein. In the absence of cocirculating avian influenza strains in the human population, further subtyping of influenza viruses or detection of subtype-specific antibodies are usually not done by routine diagnostic laboratories, but are restricted to reference laboratories involved in epidemiological analyses and planning of vaccine strains. However, in case of an outbreak of avian influenza, efforts to further subtype the virus, e.g. by subtype-specific RT PCR methods, should be made by routine laboratories since immediate knowledge about the infecting influenza subtype is essential for infection control and timely epidemiological investigations. Dependence on reference laboratories, which in the case of many southeast Asian countries affected by avian influenza outbreaks, are situated abroad, potentially results in unacceptable delays and hampers timely recognition of outbreaks and institution of adequate control measures (Hien et al., 2004). However, the reality is that diagnostic facilities in many affected countries are scarce and often not sufficiently equipped for virological diagnostics, let alone subtyping of influenza viruses. Global efforts to improve diagnostic capacity in resource-poor countries may prove an important step towards the prevention and control of pandemic influenza (Hien et al., 2004).

7.1. Virus isolation

Similar to human influenza viruses, avian viruses can be isolated in embryonated eggs or in cell culture, using permissive cells such as Madin Darby canine kidney (MDCK) cells or rhesus monkey kidney (LLC-MK2) cells. Unlike human strains and avirulent avian strains and in accordance with their promiscuity for cellular proteases, highly pathogenic avian viruses do not require the addition of exogenous trypsine for efficient replication in cell culture. For safety purposes, the isolation of highly pathogenic avian influenza virus requires biosafety level 3 laboratory facilities or higher. Cytopathic effects in cell culture are non-specific. Initial identification of influenza A virus can be performed by immunofluorescent staining with monoclonal antibodies against the nucleoprotein. Further HA and NA subtyping is performed by subtype-specific RT PCRs of culture supernatant or hemag-

glutination inhibition and neuraminidase inhibition assays using a panel of reference antisera against various subtypes. In human infections, avian influenza viruses have mostly been isolated from conjunctival swabs and respiratory specimens such as throat or nasal secretions or washings (Fouchier et al., 2004; Tran et al., 2004; Yuen et al., 1998). In one reported case of H5N1 infection, virus was also isolated from serum, cerebrospinal fluid, and a rectal swab (de Jong et al., 2005).

7.2. Antigen detection

Detection of influenza A viral antigens in clinical specimens by direct immunofluorescence or by rapid immunochromatographic assays are widely used for diagnosis of human influenza because of their ability for rapid diagnosis. However, in patients with avian influenza, the usefulness of these assays seems limited due to low sensitivity (Peiris et al., 2004; Yuen et al., 1998). In addition, some rapid antigen detection kits do not distinguish between influenza types A and B, and none of the currently available immunofluoresent and immunochromatographic assays distinguish between influenza A subtypes. However, developments of H5N1-specific rapid antigen detection tests are ongoing (Xu et al., 2005).

7.3. Reverse transcriptase PCR

RT PCR methods allow for sensitive and specific detection of viral nucleic acids and have shown to increase the diagnostic sensitivity for many viral pathogens when compared to culture or antigen detection methods. During the H5N1 outbreaks in Hong Kong and southeast Asia, RT PCR methods for specific detection of H5N1 viral nucleic acids have proven valuable and seem the diagnostic methods of choice in case of an outbreak of avian influenza (Chotpitayasunondh et al., 2005; Tran et al., 2004; Yuen et al., 1998). Especially when using real-time PCR technology, a reliable subtype-specific diagnostic result can be generated within a few hours after specimen collection. A disadvantage of RT PCR methods is its proneness for contamination and the consequent risk of false-positive results, which should be be minimized by proper precautions, including physical separation of laboratories for PCR preparation and amplification, and the use of the uracil-*n*-glycosylase system to prevent contamination by carryover of amplimers. In addition, the inclusion of an internal control in RT PCR assays is highly desirable to monitor for false-negative results due to inefficient nucleic acid extraction, cDNA synthesis, or amplification.

7.4. Serology

During outbreaks of avian influenza, the detection of subtype-specific antibodies is particularly important for epidemiological investigations. Hemagglutination inhibition (HI) assays are the gold standard for detection of antibodies against human influenza viruses. However, their usefulness for detection of antibodies against avian viruses in mammalian species, including humans, seems limited (Beare and Webster, 1991; Hinshaw et al., 1981; Kida et al., 1994). Several studies have shown a failure to detect HI antibodies against avian viruses in mammals, even in cases where infection was confirmed by virus isolation. Possible reasons for this failure include poor immunogenicity of some avian viruses and lack of sensitivity to detect low titered or less avid antibodies induced by avian viruses (Hinshaw et al., 1981; Kida et al., 1994; Lu et al., 1982; Rowe et al., 1999). It has been demonstrated that HI testing with subunit HA, but not with intact virus, could detect antibodies against an avian H2N2 virus (Lu et al., 1982). However, neutralizing antibodies against this virus could readily be detected with intact virus. A direct comparison of HI testing with a microneutralization assay in H5N1-infected persons from the 1997 Hong Kong outbreak indeed showed the latter to be more sensitive (Rowe et al., 1999). While an indirect ELISA assay using recombinant HA from H5N1/97 showed at least equal sensitivity as the microneutralization assay, the specificity in adult sera was inferior, possibly due to the presence of cross-reactive epitopes common to all HAs (Rowe et al., 1999). Based on these observations, neutralization assays are the methods of choice for detection of antibodies against avian viruses in humans.

Using these assays, it has been shown that the kinetics of the antibody response against H5N1 virus in patients infected during the Hong Kong outbreak are similar to the primary response to human influenza viruses (Katz et al., 1999). Neutralizing antibodies were generally detected 14 or more days after the onset of symptoms and titers equal to or higher that 1:640 were observed 20 or more days after onset.

8. Treatment and prevention

8.1. Antiviral treatment

Currently, two classes of drugs are available with antiviral activity against influenza viruses: inhibitors of the ion channel activity of the M2 membrane protein, amantadine and rimantadine, and inhibitors of the neuraminidase, oseltamivir, and zanamivir. The therapeutic efficacy of amantadine in human influenza is unclear due to a paucity of reliable clinical studies, but reductions of fever or illness by 1 day have been observed in adults and children (Nicholson et al., 2003). Major disadvantages of amantadine include neurotoxicity and a rapid development of drug resistance during treatment. Resistance is conferred by single nucleotide changes resulting in amino acid substitutions at positions 26, 27, 30, 31, or 34 of the M2 protein. Rimantadine causes less neurological side effects but is not available in most parts of the world. Although several H5N1-infected patients have been treated with amantadine during the 1997 H5N1 outbreak in Hong Kong, the numbers were too small to draw any meaningful conclusions concerning its activity against this virus

(Yuen et al., 1998). In vitro sensitivity testing of virus isolated from the first patient during this outbreak showed normal susceptibility to amantadine (Subbarao et al., 1998). Strikingly, genotype Z H5N1 viruses isolated from poultry and humans in Thailand and Viet Nam in 2004 invariably showed an amantadine-resistance conferring amino acid substitution at position 31 of the M2 protein, indicating that amantadine treatment is not an option during the ongoing outbtreak in southeast Asia (Li et al., 2004; Puthavathana et al., 2005).

Both oseltamivir and zanamivir have proven efficacy in the treatment of human influenza when started early during the course of illness, and are particularly effective as seasonal or postexposure prohylaxis (Nicholson et al., 2003). Zanamivir has poor oral availability and is therefore administered by inhalation, which has limited its use in the elderly and may induce bronchospasm. Oseltamivir can be given orally. The development of drug resistance during treatment has been reported for both drugs and is associated with mutations in the active site of neuraminidase or in the hemaglutinin. The latter mutations decrease the affinity of HA for the cellular receptor, thereby obviating the need for neuraminidase to escape the cells.

Data on the efficacy of neuraminidase inhibitors in avian influenza virus are scarce. The H5N1 strains implicated in the 1997 Hong Kong outbreak were susceptible in vitro to oseltamivir and zanamivir (Govorkova et al., 2001; Leneva et al., 2000). Oral oseltamivir and topical zanamivir also showed therapeutic and protective activities against Hong Kong H5N1 isolates in murine animal models (Gubareva et al., 1998; Leneva et al., 2001). Recent murine studies suggest that, perhaps due to higher virulence, higher doses of oseltamivir and longer durations of treatment are necessary to achieve antiviral effects in mice against H5N1 strains causing the southeast Asian outbreak since 2004, when compared to the 1997 Hong Kong H5N1 strain (Yen et al., 2005). Oseltamivir treatment has been given to several H5N1-infected patients, but no conclusions can be made concerning its efficacy. However, the timing of antiviral treatment may not have been optimal in many cases of avian influenza so far. Beneficial effects of antiviral treatment in human influenza are optimal when started within 48 h after onset of the illness. During the H5N1 outbreak in Viet Nam in 2004, H5N1-infected patients were admitted 5 days or later after onset of symptoms (Tran et al., 2004). Earlier recognition of avian influenza in humans may improve the efficacy of antiviral treatment.

While several H5N1-infected patients have received steroids in addition to oseltamivir, the potential benefits of this need formal evaluation in clinical studies (Tran et al., 2004). Considering the observed cytokine dysregulation in H5N1-infected animals and humans, a beneficial effect of immunomodulating agents could be hypothesized and perhaps requires further study. Finally, neutralizing monoclonal antibodies have been shown effective in treating established influenza A virus infection in mice with severe combined immunodeficiency (Palladino et al., 1995). Although mice are not men, this strategy deserves attention in the treatment of a severe illness such as influenza H5N1.

8.2. Infection control and prophylaxis

Birds infected with avian influenza excrete large amounts of virus in feces and other secretions, which contaminate the direct environment, such as dust, soil, water, cages, tools, and other fomites. Avian influenza virus may remain infectious in soil, water, or contaminated equipment for weeks to months, depending on the temperature and humidity (i.e. longer in colder climates). Illness in birds caused by highly pathogenic avian influenza viruses results in systemic replication and the presence of infectious virus in their eggs and many tissues and organs. Transmission of avian influenza viruses between birds occurs directly or indirectly through contact with fecally contaminated aerosols, water, feed, and other materials. Bird-to-human transmission likely occurs via the same route, i.e. direct contact with birds or contaminated fomites.

Most, but not all human infections with avian influenza viruses involved handling of affected poultry or direct exposure to live poultry in the week before onset of the illness (Koopmans et al., 2004; Mounts et al., 1999; Tran et al., 2004). Case–control studies during the 1997 H5N1 outbreak in Hong Kong identified visiting a stall or market selling live poultry during the week before the illness as a risk factor, whereas eating or preparing poultry products were not (Mounts et al., 1999). In cases in which no apparent direct exposure to poultry could be identified, contact with contaminated environment, such as water, has been suggested (de Jong et al., 2005). Of note, it has been shown that ducks infected by the currently circulating H5N1 strain in southeast Asia remain healthy but excrete large amounts of virus for prolonged periods of time (Hulse-Post et al., 2005). Since water in ponds and canals in which large flocks of ducks reside, is widely used for bathing and drinking in rural areas of many southeast Asian countries, it may not be unlikely that such water represents a source of transmission when contaminated by infected ducks. In fact, contact with contaminated water is regarded as the most important mode of transmission between aquatic birds.

A limited number of possible human-to-human transmissions of influenza H5N1 have been reported, which involved prolonged, close and unprotected contact with infected patients (Katz et al., 1999; Koopmans et al., 2004; Ungchusak et al., 2005). Similar to human influenza, droplet and contact transmission are probably the most effective means of transmission of avian influenza virus between humans, should the virus acquire the ability for efficient spread, but airborne transmission remains a possibility. The occurrence of diarrhea in H5N1-infected patients, which may contain infectious virus, represents a potential non-respiratory route of transmission, which needs to be considered in infection control practices (Apisarnthanarak et al., 2004; de Jong et al., 2005; Tran et al., 2004). Data concerning excretion patterns and periods of potential infectivity are lacking for human

infections with avian influenza viruses. Based on exposure histories, the incubation time for human H5N1-infections has been estimated 2–10 days, but it is not known whether excretion of virus occurs during this time (Tran et al., 2004; Yuen et al., 1998). Based on the current (lack of) knowledge, infection control measures during contact with potentially infected birds or environment, or with patients with suspected or confirmed infection should prevent contact, droplet, and airborne transmission. These measures include mask (preferably high efficiency masks, with surgical masks as a second alternative), gown, face shield, or goggles and gloves.

The efficacy of neuraminidase inhibitors as seasonal or postexposure prohylaxis against human influenza is high (Nicholson et al., 2003). Offering prophylactic treatment to potentially exposed people in the setting of a poultry outbreak of avian influenza, as has been done during H7-outbreaks in the Netherlands and Canada (Koopmans et al., 2004; Tweed et al., 2004), is rational but hardly feasible during the ongoing outbreak in southeast Asia for logistical and financial reasons. Postexposure prophylaxis to unprotected healthcare workers and close contacts of infected patients is advisable. The potential use of specific monoclonal antibodies for prophylaxis warrants further investigation.

Eliminating the source of infection, i.e. infected birds, remains the most effective infection control measure. Culling of all infected poultry have proven succesful during avian influenza outbreaks in Hong Kong, the Netherlands and Canada (Chan, 2002; Koopmans et al., 2004; Tweed et al., 2004). However, considering the geographic extensiveness of the outbreak in southeast Asia, the different farming practices, and the reported H5N1 infections in migratory birds (Chen et al., 2005), it seems very unlikely that culling of poultry alone will contain the outbreak in that region.

8.3. Vaccination

The bulk of human influenza vaccines are produced from inactivated viruses grown in embryonated eggs. Vaccine production against highly pathogenic avian influenza viruses is complicated because of the requirement for high biosafety containment facilities, and the difficulty, in some cases, to obtain high virus yields in embryonated eggs because of the virus' pathogenicity (Stephenson et al., 2004; Wood and Robertson, 2004). Several other approaches have been used in an attempt to overcome these obstacles, including the use of reverse genetics techniques, generation of recombinant hemagglutinin, DNA vaccination and the use of related apathogenic H5 viruses with and without different adjuvants (Nicholson et al., 2003; Stephenson et al., 2004; Webby et al., 2004; Wood and Robertson, 2004). Experimental H5N1 vaccines in which important virulence determinants were altered using plasmid-based reverse genetics, have shown protective efficacy to homologous and heterologous H5 strains in animal models and may prove an attractive approach (Li et al., 1999; Lipatov et al., 2005; Takada et al., 1999). Studies in humans using an H5N3 vaccine developed from a 1997 apathogenic avian virus showed high rates of seroconversions to the vaccine strain and heterologous H5N1 strains after three doses, but only when the vaccine was given with the adjuvans MF59 (Stephenson et al., 2005). In animal models, baculovirus-derived recombinant H5 vaccines were immunogenic and protective, but results in humans were disappointing even when using high doses (Crawford et al., 1999; Treanor et al., 2001). H5 DNA vaccines protected mice from infection by homologous, but not by heterologous H5N1 viruses (Epstein et al., 2002; Kodihalli et al., 1999).

9. Pandemic preparedness and future directives

The increasing frequency of outbreaks with highly pathogenic avian influenza viruses among poultry and direct transmission of these viruses to humans, culminating in the ongoing extensive H5N1 outbreak in southeast Asia, has ignited grave concerns about an imminent influenza pandemic. Indeed, two of three prerequisites for a human pandemic have been met in the southeast Asian H5N1 outbreak: the emergence of an antigenically novel strain to which the population has no immunity, and the transmission of this strain to humans in whom it can cause severe disease. To date, there is fortunately no evidence of efficient spread of H5N1 virus between humans, but continued circulation of this strain, which now has reached levels of endemicity among poultry in several southeast Asian countries, increases the opportunity to adapt to humans through mutation or genetic reassortment in humans or intermediate mammalian hosts. As suggested by the "Spanish flu" pandemic of 1918, extremely high transmissability is no prerequisite for a severe pandemic killing tens of millions of people, and as shown by the Severe Acute Respiratory Syndrome (SARS) virus epidemic in 2003, viruses can rapidly spread across the globe in the current age of intense global travel. As a consequence of all this, pandemic preparedness has become an important issue worldwide. Pandemic plans, which include stock-piling of antivirals and candidate vaccines, are being developed by an increasing number of countries worldwide, and alternative methods for rapid vaccine production and potential methods enabling dose reduction of vaccines are increasingly propagated (Schwartz and Gellin, 2005; Stephenson et al., 2004; Webby et al., 2004; Webby and Webster, 2003; Wood and Robertson, 2004).

Notwithstanding the importance of these efforts to prepare for a possible H5N1 pandemic, more structural and longer term global efforts are needed to allow for early recognition of novel influenza viruses or other emerging pathogens infecting humans in the future. In 2002, a WHO Global Agenda for Influenza Surveillance and Control has been adopted, of which the main objectives are to strengthen surveillance, improve knowledge of the disease burden, increase vaccine use, and accelerate pandemic preparedness (Stohr, 2003). It is essential that these objectives are increasingly focused on the southeast Asian region, which has been the source of

previous pandemics and is the epicentre of the current pandemic threat. Many southeast Asian countries currently lack the expertise, financial means, and infrastructure for human and animal diagnostics and surveillance. Global investments to improve public health care infrastructures and laboratory facilities, and to transfer clinical, epidemiological, and technical knowledge to these countries are much needed (Hien et al., 2004). The window of opportunity in the era of global travel is narrow. Local capacity, and less dependence on foreign laboratories and expertise, will allow for earlier recognition and quicker responses to epidemics. In addition, local availability of clinical, scientific, and laboratory capacity facilitates and expedites clinical, virological, and epidemiological analyses needed to optimize outbreak control, infection control, and clinical managment. It also guarantees the timely availability of virus strains for monitoring virus evolution and planning of vaccines by reference laboratories. Such global investments to enhance local infrastructure and expertise will increase the chances of success of containing an influenza pandemic at the source by antiviral prophylaxis and other preventive measures suggested by recent mathematical modelling studies (Ferguson et al., 2005; Longini et al., 2005).

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References

Apisarnthanarak A, Erb S, Stephenson I, Katz JM, Chittaganpitch M, Sangkitporn S, et al. Seroprevalence of anti-H5 antibody among Thai health care workers after exposure to Avian influenza (H5N1) in a tertiary care center. Clin Infect Dis 2005;40:e16–8.

Apisarnthanarak A, Kitphati R, Thongphubeth K, Patoomanunt P, Anthanont P, Auwanit W, et al. Atypical avian influenza (H5N1). Emerg Infect Dis 2004;10:1321–4.

Basler CF, Reid AH, Dybing JK, Janczewski TA, Fanning TG, Zheng H, et al. Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. Proc Natl Acad Sci USA 2001;98:2746–51.

Bean WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, et al. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. J Virol 1992;66:1129–38.

Beare AS, Webster RG. Replication of avian influenza viruses in humans. Arch Virol 1991;119:37–42.

Bridges CB, Lim W, Hu-Primmer J, Sims L, Fukuda K, Mak KH, et al. Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997–1998. J Infect Dis 2002;185:1005–10.

Cauthen AN, Swayne DE, Schultz-Cherry S, Perdue ML, Suarez DL. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J Virol 2000;74:6592–9.

Chan PK. Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. Clin Infect Dis 2002;34(Suppl. 2):S58–64.

Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, et al. The evolution of H5N1 influenza viruses in ducks in southern China. Proc Natl Acad Sci USA 2004;101:10452–7.

Chen H, Smith GJ, Zhang SY, Qin K, Wang J, Li KS, et al. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 2005;436:191–2.

Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, et al. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 2002;360:1831–7.

Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, et al. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 2005;79:10821–5.

Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W, Chunsuthiwat S, Sawanpanyalert P, Kijphati R, et al. Human disease from influenza A (H5N1) Thailand, 2004. Emerg Infect Dis 2005;11:201–9.

Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–7.

Connor RJ, Kawaoka Y, Webster RG, Paulson JC. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 1994;205:17–23.

Couceiro JN, Paulson JC, Baum LG. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 1993;29:155–65.

Crawford J, Wilkinson B, Vosnesensky A, Smith G, Garcia M, Stone H, et al. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine 1999;17:2265–74.

de Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med 2005;352:686–91.

Epstein SL, Tumpey TM, Misplon JA, Lo CY, Cooper LA, Subbarao K, et al. DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. Emerg Infect Dis 2002;8:796–801.

Ferguson NM, Cummings DA, Cauchemez S, Fraser C, Riley S, Meeyai A, et al. Strategies for containing an emerging influenza pandemic in Southeast Asia. Nature 2005;437:209–14.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79:2814–22.

Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA 2004;101:1356–61.

Gambaryan AS, Tuzikov AB, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, et al. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl(*N*-acetyllactosamine). Virology 1997;232:345–50.

Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, et al. The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science 2004;303:1838–42.

Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, et al. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc Natl Acad Sci USA 2002;99:10736–41.

Govorkova EA, Leneva IA, Goloubeva OG, Bush K, Webster RG. Comparison of efficacies of RWJ-270201, zanamivir, and oseltamivir against H5N1, H9N2, and other avian influenza viruses. Antimicrob Agents Chemother 2001;45:2723–32.

Guan Y, Peiris JS, Lipatov AS, Ellis TM, Dyrting KC, Krauss S, et al. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc Natl Acad Sci USA 2002;99:8950–5.

Guan Y, Poon LL, Cheung CY, Ellis TM, Lim W, Lipatov AS, et al. H5N1 influenza: a protean pandemic threat. Proc Natl Acad Sci USA 2004;101:8156–61.

Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci USA 1999;96:9363–7.

Gubareva LV, McCullers JA, Bethell RC, Webster RG. Characterization of influenza A/HongKong/156/97 (H5N1) virus in a mouse model and protective effect of zanamivir on H5N1 infection in mice. J Infect Dis 1998;178:1592–6.

Hien TT, de Jong M, Farrar J. Avian influenza—a challenge to global health care structures. N Engl J Med 2004;351:2363–5.

Hinshaw VS, Webster RG, Easterday BC, Bean Jr WJ. Replication of avian influenza A viruses in mammals. Infect Immun 1981;34:354–61.

Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, et al. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci USA 2005;102:10682–7.

Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 1998;72:7367–73.

Katz JM, Lim W, Bridges CB, Rowe T, Hu-Primmer J, Lu X, et al. Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. J Infect Dis 1999;180:1763–70.

Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 1989;63:4603–8.

Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, et al. Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis 2004;10:2189–91.

Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, et al. Potential for transmission of avian influenza viruses to pigs. J Gen Virol 1994;75(Pt 9):2183–8.

Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, et al. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 2004;431:703–7.

Kodihalli S, Goto H, Kobasa DL, Krauss S, Kawaoka Y, Webster RG. DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. J Virol 1999;73:2094–8.

Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, et al. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 2004;363:587–93.

Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, et al. Avian H5N1 influenza in cats. Science 2004;306:241.

Lee CW, Suarez DL, Tumpey TM, Sung HW, Kwon YK, Lee YJ, et al. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J Virol 2005;79:3692–702.

Leneva IA, Goloubeva O, Fenton RJ, Tisdale M, Webster RG. Efficacy of zanamivir against avian influenza A viruses that possess genes encoding H5N1 internal proteins and are pathogenic in mammals. Antimicrob Agents Chemother 2001;45:1216–24.

Leneva IA, Roberts N, Govorkova EA, Goloubeva OG, Webster RG. The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses. Antiviral Res 2000;48:101–15.

Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 2004;430:209–13.

Li S, Liu C, Klimov A, Subbarao K, Perdue ML, Mo D, et al. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. J Infect Dis 1999;179:1132–8.

Liem NT, Lim W. Lack of H5N1 avian influenza transmission to hospital employees, Hanoi, 2004. Emerg Infect Dis 2005;11:210–5.

Lipatov AS, Krauss S, Guan Y, Peiris M, Rehg JE, Perez DR, et al. Neurovirulence in mice of H5N1 influenza virus genotypes isolated from Hong Kong poultry in 2001. J Virol 2003;77:3816–23.

Lipatov AS, Webby RJ, Govorkova EA, Krauss S, Webster RG. Efficacy of h5 influenza vaccines produced by reverse genetics in a lethal mouse model. J Infect Dis 2005;191:1216–20.

Longini Jr IM, Nizam A, Xu S, Ungchusak K, Hanshaoworakul W, Cummings DA, et al. Containing pandemic influenza at the source. Science 2005;309:1083–7.

Lu BL, Webster RG, Hinshaw VS. Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect Immun 1982;38:530–5.

Matrosovich M, Zhou N, Kawaoka Y, Webster R. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 1999;73:1146–55.

Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, Lvov DK, et al. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology 1997;233:224–34.

Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci USA 2004;101:4620–4.

Mills CE, Robins JM, Lipsitch M. Transmissibility of 1918 pandemic influenza. Nature 2004;432:904–6.

Morishima T, Togashi T, Yokota S, Okuno Y, Miyazaki C, Tashiro M, et al. Encephalitis and encephalopathy associated with an influenza epidemic in Japan. Clin Infect Dis 2002;35:512–7.

Mounts AW, Kwong H, Izurieta HS, Ho Y, Au T, Lee M, et al. Case–control study of risk factors for avian influenza A (H5N1) disease, Hong Kong, 1997. J Infect Dis 1999;180:505–8.

Nicholson KG, Wood JM, Zambon M. Influenza. Lancet 2003;362:1733–45.

Oxford JS. Influenza A pandemics of the 20th century with special reference to 1918: virology, pathology and epidemiology. Rev Med Virol 2000;10:119–33.

Palladino G, Mozdzanowska K, Washko G, Gerhard W. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J Virol 1995;69:2075–81.

Peiris JS, Yu WC, Leung CW, Cheung CY, Ng WF, Nicholls JM, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004;363:617–9.

Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, et al. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol 2005;86:423–33.

Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc Natl Acad Sci USA 1999;96:1651–6.

Reid AH, Taubenberger JK, Fanning TG. Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. Nat Rev Microbiol 2004;2:909–14.

Rogers GN, D'Souza BL. Receptor binding properties of human and animal H1 influenza virus isolates. Virology 1989;173:317–22.

Rogers GN, Paulson JC. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 1983;127:361–73.

Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature 1983;304:76–8.

Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol 1999;37:937–43.

Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 1978;87:13–20.

Schultsz C, Dong VC, Chau NVV, Le NTH, Lim W, Thanh TT, et al. Avian influenza H5N1 and healthcare workers. Emerg Infect Dis 2005;11:1158–9.

Schwartz B, Gellin B. Vaccination strategies for an influenza pandemic. J Infect Dis 2005;191:1207–9.

Stephenson I, Bugarini R, Nicholson KG, Podda A, Wood JM, Zambon MC, et al. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. J Infect Dis 2005;191:1210–5.

Stephenson I, Nicholson KG, Wood JM, Zambon MC, Katz JM. Confronting the avian influenza threat: vaccine development for a potential pandemic. Lancet Infect Dis 2004;4:499–509.

Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 2004;303:1866–70.

Stohr K. The global agenda on influenza surveillance and control. Vaccine 2003;21:1744–8.

Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, Rehg JE, et al. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol 2004;78:4892–901.

Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 1998;279:393–6.

Sugaya N. Influenza-associated encephalopathy in Japan. Semin Pediatr Infect Dis 2002;13:79–84.

Takada A, Kuboki N, Okazaki K, Ninomiya A, Tanaka H, Ozaki H, et al. Avirulent Avian influenza virus as a vaccine strain against a potential human pandemic. J Virol 1999;73:8303–7.

Tanaka H, Park CH, Ninomiya A, Ozaki H, Takada A, Umemura T, et al. Neurotropism of the 1997 Hong Kong H5N1 influenza virus in mice. Vet Microbiol 2003;95:1–13.

Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG. Initial genetic characterization of the 1918 “Spanish” influenza virus. Science 1997;275:1793–6.

To KF, Chan PK, Chan KF, Lee WK, Lam WY, Wong KF, et al. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol 2001;63:242–6.

Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004;350:1179–88.

Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O’Brien D, et al. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine 2001;19:1732–7.

Tweed SA, Skowroski DM, David ST, Larder A, Petric M, Lees W, et al. Human illness from avian influenza H7N3, British Columbia. Emerg Infect Dis 2004;10:2196–9.

Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, et al. Influenza H5N1 replication sites in humans. Emerg Infect Dis 2005;11:1036–41.

Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med 2005;352:333–40.

Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, et al. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 2004;363:1099–103.

Webby RJ, Webster RG. Are we ready for pandemic influenza? Science 2003;302:1519–22.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–79.

Webster RG, Guan Y, Peiris M, Walker D, Krauss S, Zhou NN, et al. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. J Virol 2002;76:118–26.

WHO. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO; 2005. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2005_06_08/en/index.html [accessed 10 August 2005].

Wood JM, Robertson JS. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat Rev Microbiol 2004;2:842–7.

Xu X, Jin M, Yu Z, Li H, Qiu D, Tan Y, et al. Latex agglutination test for monitoring antibodies to avian influenza virus subtype H5N1. J Clin Microbiol 2005;43:1953–5.

Xu X, Subbarao K, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 1999;261:15–9.

Yen HL, Monto AS, Webster RG, Govorkova EA. Virulence may determine the necessary duration and dosage of oseltamivir treatment for highly pathogenic A/Vietnam/1203/04 (H5N1) influenza virus in mice. J Infect Dis 2005;192:665–72.

Yuen KY, Chan PK, Peiris M, Tsang DN, Que TL, Shortridge KF, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 1998;351:467–71.