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Review

An Overview of the Highly Pathogenic H5N1 Influenza Virus*

Jingchuan Yin, Shi Liu and Ying Zhu ✉

The State Key laboratory of Virology and College of Life Sciences, Wuhan University, Wuhan 430072, China

Since the first human case of H5N1 avian influenza virus infection was reported in 1997, this highly pathogenic virus has infected hundreds of people around the world and resulted in many deaths. The ability of H5N1 to cross species boundaries, and the presence of polymorphisms that enhance virulence, present challenges to developing clear strategies to prevent the pandemic spread of this highly pathogenic avian influenza (HPAI) virus. This review summarizes the current understanding of, and recent research on, the avian influenza H5N1 virus, including transmission, virulence, pathogenesis, clinical characteristics, treatment and prevention.

Avian influenza; H5N1; Transmission; Virulence; Pathogenesis

AVIAN INFLUENZA VIRUS

Influenza virus belongs to the family *Orthomyxoviridae* (Nelson M I, et al., 2007), which contains three different genera of influenza virus: type A, type B, and type C (Shaw M, et al., 1992). Influenza viruses are enveloped RNA viruses with a genome of seven or eight single-stranded negative-sense RNAs consisting of from 890 to 2,341 nucleotides (Noda T, et al., 2006). Avian influenza virus (AIV) is a type A virus that has eight gene segments encoding 11-12 proteins: hemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2), non-structural proteins (NS1 and NS2), nucleocapsid proteins (NP), and three polymerases, polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) proteins. Another protein, N40, which is encoded by the PB2 segment, has been identified recently (Medina R A, et al., 2011; Webster R G, et al., 1992; Wise H M, et al., 2009). Some H5N1 viruses also express the pro-apoptotic protein PB1-F2, which is encoded by the PB1 segment (Chen W, et al., 2001; Medina R A, et al., 2011) (Table 1).

The type A influenza viruses are divided into subtypes by two envelope proteins: hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA subtypes (H1 to H16) and nine NA subtypes (N1 to N9) have been detected in birds (Webster R G, et al., 1992). Many species of shorebirds and other waterfowl (e.g., wild geese, ducks) are natural reservoirs of AIV (Webster R G, et al., 1992). Most AIV subtypes appear non-virulent to birds, since infected birds generally show no clinical symptoms of infection (de Jong M D, et al., 2006; Webster R G, et al., 1992). Although the majority of the known avian influenza A virus subtypes have only been isolated from wild birds, they have spread among marine mammals, including seals and whales, swine, dogs, cats, and horses (Loeffelholz M J, 2010; Suarez D L, et al., 2000). Currently, five AIV subtypes [H1N1, H3N2, H2N2, H1N2 and the highly pathogenic H5N1] replicate efficiently in human cells, resulting in greater potential for an AIV pandemic among humans (Loeffelholz M J, 2010).

THE H5N1 VIRUS

Emergency

Outbreaks of highly pathogenic avian influenza (HPAI) H5N1 virus infections were first recorded in poultry in Guangdong, China in 1996. It is now suspected that this virus contained only a few HA gene mutations that were not present in the HA of a low pandemic avian influenza

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✉ Corresponding author.
Phone: +86-027-68754819, Fax: +86-027-68754592 ,
Email: yingzhu@whu.edu.cn

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Table 1. The function of each protein that is encoded by influenza A virus.

Protein	Function
HA	Receptor-binding protein
NA	Sialic acid-degrading enzyme; exit of the virus from the cell in preparation for another round of infection
NP	Major nucleocapsid structural component, encapsidates the RNA, serve as templates for *de novo* virus RNA synthesis
NS1	Non-structure protein 1 involved in the post-transcription , host antiviral response antagonist
NS2(NEP)	Nuclear export protein which regulates the virus transcription and replication
PB1	Core subunit of RNA-dependent RNA polymerase involved in RNA transcription and replication; contains the polymerase activity
PB2	Component of the RNA-dependent RNA polymerase involved in RNA transcription; has the temperature-dependent replication competence; recognizes capped cellular mRNA
N40	Unknown
PB1-F2	Induction of apoptosis; promotion of secondary bacterial infection
PA	Component of the RNA-dependent RNA polymerase involved in RNA replication; possesses an endonuclease activity
M1	Membrane matrix protein
M2	Membrane ion channel protein

The table lists all 12 proteins encoded by influenza A virus genome: the receptor-binding protein hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), non-structural protein 1 (NS1), nuclear export protein (NEP, also called NS2), matrix protein (M1), ion channel M2, the components of the RNA-dependent RNA polymerase complex (PB1, PB2, and PA), and the newly identified N40 protein. In addition, some viruses express the pro-apoptotic protein PB1-F2.

(LPAI) H5N1 virus isolated from geese (Medina R A, et al., 2011; Mukhtar M M, et al., 2007). In March 1997, the previously isolated HPAI H5N1 outbreaks exploded into a massive outbreak in chickens in Hong Kong (Centers for Disease Control, et al., 1997). In contrast to 1996, the H5N1 outbreak among the Hong Kong poultry markets spread to humans, with the isolation of an H5N1 influenza virus strain from a 3-year-old boy in Hong Kong (Subbarao K, et al., 1998). The patient died of severe pneumonia complicated by acute respiratory distress syndrome and Reye’s syndrome (Subbarao K, et al., 1998). In 1997, a total of 18 human cases were reported, and of those, 6 were fatal. In 1998, a different avian H5N1 strain emerged (Li K S, et al., 2004; Yuen K Y, et al., 1998). After a few years’ silence, in February 2003, H5N1 infections were once again diagnosed among humans (Li K S, et al., 2004). The occurrence of frequent outbreaks of the HPAI H5N1 virus among poultry since it emerged in 1996 is a cause of concern.

In May 2005, more than 6,000 infected wild birds on Lake Qinghai in Western China were infected and, during their seasonal migrations, spread the H5N1 virus westward to Europe and Africa in the years following (Lei F, et al., 2011; Liu J, et al., 2005; Peiris J S, et al., 2007). A new H5 sub-lineage, which had an increased binding affinity for human receptors, was isolated in Egypt in 2008 (Watanabe Y, et al., 2012). Studies showed that the dominant haemagglutinin clade of the H5N1 virus has changed from clade 0 to clade 2.3， since it emerged in 1996, apparently as a result of antigenic shift (Pfeiffer D U, et al., 2011). Although in the H5N1 infections that occurred in Egypt, the morbidity was not as high as for seasonal influenza strains, the mortality rate of approximately 60% was substantially higher than for seasonal influenza (Peiris J S, et al., 2010). As of December 17, 2012, according to the World Health Organization (WHO), 610 confirmed cases of human H5N1 infection have been reported since 2003. These infections have been reported from 15 different countries and resulted in 360 deaths. These statistics demonstrate that a global H5N1 pandemic would have devastating effects, and that this virus represents a serious public health threat.

Transmission

Most H5N1 influenza viruses were isolated during the winter months (Peiris J S, et al., 2007; Suarez D L, 2010). The natural spread of the H5N1 virus results, in part, from the migration of wild birds, especially long-distance migratory birds (Ndung'u T, et al., 2006). The risk of HPAI H5N1 infection in wild birds, and the risk of transmission between wild birds and domestic poultry may be similar at various times of the year (Reperant L A, et al., 2011). Another major factor in transmission is the local commercial poultry industry. There, large numbers of infected and uninfected birds are housed together,

sometimes for extended periods, thus increasing the likelihood of virus transmission (Abdelwhab E M, et al., 2011; Ndung'u T, et al., 2006).

Wider spread of the virus among birds, from birds to mammals (including humans), and subsequent spread among humans requires adaptations that favor virus transmission rates (Gutierrez R A, et al., 2011; Vandegrift K J, et al., 2010). Most human cases seem to be the result of exposure to sick poultry or the butchering of birds. However, a number of confirmed human cases show that H5N1 can spread by close contact with an infected patient (e.g., spread to caretakers of an infected family member) (Ungchusak K, et al., 2005; Van Kerkhove M D, et al., 2011). Although rare, human-to- human transmission of the H5N1 virus has been found in several household groups in tribal communities (Van Kerkhove M D, et al., 2011). Consuming food products from infected poultry is not likely to cause a human infection, but there is evidence that cats have become infected by eating raw meat from infected chickens, and that cats could, in turn, transmit the virus to their owners or to other household mammals, such as hamsters (Abdelwhab E M, et al., 2011; Thiry E, et al., 2007). Other studies performed since the emergence of HPAI H5N1 show that the virus can be transmitted through contact with contaminated water and sewage, a risk factor that must be guarded against (Beigel J H, et al., 2005; Van Kerkhove M D, et al., 2011). Hence, interactions of current hosts and their environment influence the ability of H5N1 influenza viruses to spread to new and different hosts (Lowen A C, et al., 2007; Park A W, et al., 2007).

Viral genetic characterization

Genetic characterization of the 1997 H5N1 strain showed that it is a multiple reassortant virus from different progenitors. The HA gene of the 1997 H5N1 strain is derived from the A/Goose/Guangdong/1/96 strain, which was found to have close genomic relationships with H3N8 and H7N1 strains, as well as with H5N3 strains (Li K S, et al., 2004; Mukhtar M M, et al., 2007). An H6N1 virus contributed the NA and some of the internal genes of the 1997 H5N1, and the H9N2 virus contributed the remaining internal genes (Cauthen A N, et al., 2000; Guan Y, et al., 1999; Hoffmann E, et al., 2000). The HPAI H5N1 virus became lethal in poultry, mammals, and human beings soon after the outbreak among wild birds. Recent data suggest that there are more than 15 H5N1 genotypes distributed globally, with a newly emerged genotype, named genotype P (Watanabe Y, et al., 2012). Among all the circulating genotypes in China, genotype V became the dominant H5N1 representative in 2005, rather than genotype B and genotype Z (Duan L, et al., 2008).

Determinants of viral pathogenesis

Inchoate studies indicate that HA is the major protein involved in AIV binding to its receptor on the cell surface (Suksatu A, et al., 2009). Six of the 16 known influenza A HA serotypes (H1, H2, H3, H5, H7, and H9) have been isolated from humans. It is widely accepted that α-2,6-linked sialic acids (α-2,6-SA) are the primary HA receptors in the upper respiratory tract of humans. In contrast, α-2,3-linked sialic acids (α-2,3-SA) that can be detected in duck intestinal epithelium are abundantly distributed on the cells of birds (van Riel D, et al., 2007). Although infection is infrequent, current research shows that H5N1 viruses do infect humans. Various studies have described mechanisms to account for this phenomenon. Cells in the lower respiratory tract of humans, such as type II pneumocytes, alveolar macrophages and the non-ciliated cuboidal epithelial cells of terminal bronchioles express α-2,3-SA moieties (Nicholls J M, et al., 2007; van Riel D, et al., 2006). Additionally, amino acid mutations in the HA receptor-binding site have been identified that may change or broaden attachment behavior and alter the inflammatory response of the host or the virulence of the virus (Ayora-Talavera G, et al., 2009; Gao Y, et al., 2009; Manz B, et al., 2010; Ramos I, et al., 2011; Stevens J, et al., 2006; Suksatu A, et al., 2009). For example, mutation of the G225D and E190D receptors in the H5N1 virus HA protein greatly decreases its binding affinity for α-2,3-SA without changing its affinity for α-2,6-SA (Stevens J, et al., 2006). These data indicate that changes in HA dictate the receptor-binding specificity for H5N1 viruses (Medina R A, et al., 2011; Stevens J, et al., 2006). Further, the relative expression of HA and NA in the virus envelope can be disrupted by mutations in HA, which changes the replication efficiency of the virus (Chutinimitkul S, et al., 2010). Since pigs and some birds have both α-2,3-SA and α-2,6-SA receptors (and some have a third receptor), co-infection of these animals could serve as an incubator to generate novel reassortant viruses with increased ability to spread from animals to humans and humans to humans (Gambaryan A S, et al., 2008; Ito T, et al., 1998; Kimble B, et al., 2010; Li C, et al., 2010; Wan H, et al., 2006). The HA protein has also been suspected of inducing autophagy of epithelial lung cells, which would enable the virus to spread to other tissues and cause

further damage (Manz B, et al., 2010; Sun Y, et al., 2012; Tolnay A E, et al., 2010). Other mechanisms that could enable avian-to-human transmission of H5N1 viruses include the existence of currently unknown novel binding sites on HA that recognize the topology of sialylated pentasaccharides found on human cells (Chutinimitkul S, et al., 2010; Medina R A, et al., 2011; Nicholls J M, et al., 2007).

No single viral protein by itself ensures successful infection and robust replication within a host; instead, the overall constellation of genes reprograms host cells for efficient propagation and spread. HPAI H5N1 is no exception, as several of the viral non-structural genes have been reported to be important for replication and pathogenesis. It has been confirmed that the NS gene contributes to viral pathogenesis in cell cultures and animal models (Imai H, et al., 2010). The NS1 protein inhibits nuclear export of mRNAs to the cytoplasm and binds to the cellular protein phosphatidylinositol-3-kinase (PI3K) (Hale B G, et al., 2010; Robb N C, et al., 2012). Avian viruses that have the HPAI H5N1 NS1 gene efficiently block innate immune responses, including reduced interferon (IFN) and inducible phosphorylation of signal transducer and activator of transcription (STAT) proteins (Jia D, et al., 2010; Seo S H, et al., 2002). Disrupted cytokine and chemokine expression, especially IFNs and TNF, and decreased antiviral activity of innate immune response mediators, such as protein kinase resource and retinoic acid-inducible gene product I (RIG-I), directly lead to increased virulence of the virus (Haye K, et al., 2009; Jackson D, et al., 2008; Lipatov A S, et al., 2005; Munir M, et al., 2011). Further, it has been suggested that the PDZ domain binding motif, comprised of the four C-terminal residues of NS1, may influence pathogenicity through an IFN-independent pathway (Jackson D, et al., 2008). Mutations of a single residue or short sequence of NS1 have enabled the virus to spread across species with increased virulence to the host (Jackson D, et al., 2008; Li W, et al., 2010; Long J X, et al., 2008; Zhu Q, et al., 2008). A deletion at position 80-84 or 191-195, and an aspartic acid to glutamic acid substitution at NS1 residue 92 (D92E), also enhance the virulence of H5N1 by influencing the virus titers and the expression of pro-inflammatory cytokines (e.g., IFNs and TNF-α) (Li W, et al., 2010; Lipatov A S, et al., 2005; Zhu Q, et al., 2008). The E92 residue was found in viruses that were isolated during the 1997 H5N1 outbreak (Lipatov A S, et al., 2005). This confirmed the presumption that NS1 residue E92 of the HPAI H5N1 may be crucial for pathogenicity (Medina R A, et al., 2011; Seo S H, et al., 2002). Furthermore, the existence of other NS1 gene alleles exhibiting versatile abilities to inhibit the IFN responses underscores the importance of NS1 for H5N1 pathogenesis (Munir M, et al., 2011).

Other AIV proteins, such as PA, PB1, PB2, M1, and M2 can also alter virus virulence and transmission, increasing the pandemic potential for this virus. (Gao Y, et al., 2009; Guan Y, et al., 2002; Tafforeau L, et al., 2011; Thanh T T, et al., 2008; Wasilenko J L, et al., 2008; Zamarin D, et al., 2006). A study has shown that PB1-F2 interacts with two mitochondrial proteins, adenine nucleotide translocator 3 and voltage-dependent anion channel 1, which induces apoptosis (Zamarin D, et al., 2005). Silencing of PB1-F2 protein expression by short interfering RNA diminished AIV pathogenicity and mortality in mice without affecting viral replication kinetics in cell culture (Zamarin D, et al., 2006). Another study showed that mutation of PB1-F2 residue N66S delayed activation of type I IFN pathway genes (Conenello G M, et al., 2011). Similar studies have confirmed that mutations at single residues in multiple AIV proteins were associated with increased (or decreased) virulence. For example, residue K627 in PB2 has been detected in human HPAI H5N1 viruses and shown to be a host range determinant, but a D701N mutation of PB2 can also enable AIV to infect mammalian cells (de Jong M D, et al., 2006; Gabriel G, et al., 2005; Subbarao E K, et al., 1993). In addition, some poultry-derived H5N1 viruses can cross the species barrier and replicate in mammalian cells without additional mutations, indicating that mammal-adapted AIVs may exist in nature (Suksatu A, et al., 2009). The malleable nature of influenza virus, and the complex biological characteristics of the H5N1 avian virus pose a challenge for the development of vaccines and antiviral drugs needed to counter pandemic spread of this influenza virus (Guan Y, et al., 2002).

CLINICAL FEATURES AND HOST RESPONSE

Clinical spectrum

The time from H5N1 virus exposure to onset of symptoms ranges from one to seven days. The majority of reported cases have been in infants and young children (Beigel J H, et al., 2005). The time from onset of illness to death can be as short as few days to as long as one month, most likely reflecting different environmental conditions and characteristics of individual virus strains

(Cattoli G, et al., 2009). Human H5N1 infection is characterized by high pharyngeal virus loads and presence of viral RNA in the feces and blood (de Jong M D, et al., 2006; Wang H, et al., 2009). The distribution of virus isolates and clinical features of infection vary considerably among host species (Gao P, et al., 1999; Kuiken T, et al., 2010). For example, although H5N1 virus can be found in the brain of experimentally infected mice, it is more likely to infect the heart and lungs of cynomolgus macaques (Rimmelzwaan G F, et al., 2001). In humans, the clinical spectrum of H5N1 infection ranges from asymptomatic to severe or fatal disease. Despite some features shared with seasonal influenza viruses, such as high fever (> 38°C), headache, myalgia, and cough, a large number of patients also have severe respiratory involvement including dyspnea and pneumonia, as well as manifestations of acute respiratory distress syndrome (Beigel J H, et al., 2005; Cameron M J, et al.; Hui D S, 2008).

Hospital records indicate that rather than upper respiratory tract symptoms, patients with H5N1 are more likely to develop lower respiratory tract symptoms within days of admission (Hui D S, 2008). Respiratory epithelial cells, especially the type II pneumocytes, are key target cells of the AIV in the human lung in humans, and respond by abundant release of inflammatory chemokines and cytokines (Chan R W, et al., 2010; Peiris J S, et al., 2010; Snelgrove R J, et al., 2011; Wang J, et al., 2009; Yu W C, et al., 2011). A similar preference for type II pneumocytes was observed in animal models, including the cat, mouse, and ferret (van Riel D, et al., 2006). Alveolar epithelium cells amplify the H5N1 virus-induced cytokine cascade, which is an important component of H5N1 pathogenesis (Snelgrove R J, et al., 2011). One of the primary clinical outcomes is acute lung injury (ALI), presumed to be a consequence of autophagic cell death within the alveolar epithelium. Severe ALI appears to be major cause of fatality (Sun Y, et al., 2012; WHO, 2008).

Numerous studies indicate that H5N1 is more pathogenic than seasonal influenza. Tests in mice and primates showed more severe lung inflammation and higher virus yields following infection with the H5N1 virus than with seasonal virus, or a reassortant 1918 H1N1 virus (Baskin C R, et al., 2009; Sun Y, et al., 2012). However, similar levels of apoptosis were seen in A549 cells, with undetectable differences in virus yield, indicating that higher virus load does not necessarily result in more severe disease (Sun Y, et al., 2012). Chest radiographs revealed that although multifocal broncho-pneumonia in the parahilar of both lungs can been seen predominantly when infected with pneumococcus, H5N1 pneumonia presented with bilateral interstitial infiltration (Loeffelholz M J, 2010).

Central nervous system (CNS) manifestations are infrequent with seasonal influenza virus, but been observed in H5N1-virus-infected patients (de Jong M D, et al., 2005). Other studies showed that H5N1 infection of the CNS can induce Parkinsonian symptoms as well as substantia nigra pars compacta dopaminergic neuron degeneration (Jang H, et al., 2009). Animal studies show a similar spectrum of CNS disease and encephalopathy after infection with HPAI H5N1 virus, with high levels of pro-inflammatory cytokines including TNF-α, IFNs, IL-1 and IL-6 (Thiry E, et al., 2007; Tolnay A E, et al., 2010). In the mouse model, fatal H5N1 infections were associated with the appearance of CD3+ T cells and TUNEL+ cells in the CNS, as well as the absence of perineuronal nets in the brain (Bissel S J, et al., 2012). Nevertheless, IFN levels, especially IFN-γ, were not as high in the CNS as in the lungs (Bissel S J, et al., 2012). The mechanism of H5N1 access of the CNS is not fully understood, but one hypothesis is that the E223K mutation of HA facilitates virus spread to the brain and binding to neurons (Manz B, et al., 2010).

In contrast with seasonal influenza strains, a large proportion of H5N1-infected patients (both children and adults) have gastrointestinal symptoms such as watery diarrhea, vomiting, and abdominal pain, which occur rarely in adults with human seasonal influenza (Kandun I N, et al., 2008; Wang H, et al., 2009). The relative increase in frequency of pneumonia and gastrointestinal symptoms is an important feature of avian influenza, and may help to distinguish avian from seasonal influenza in the clinic. Multi-organ failure involving the kidneys, liver, and many other non-respiratory organs has been common (Thanh T T, et al., 2008). Fatal cases of H5N1 during pregnancy, have also been reported (Shu Y, et al., 2006). Compared with H9 and H7 virus strains, conjunctivitis has not been prominent in H5N1-infected patients (de Jong M D, et al., 2006; Hui D S, 2008). Only rare H5N1 cases are associated with secondary bacterial infections, which are commonly observed with H1N1 infections however, secondary bacterial infections were observed in H5N1 cases with hypercytokinemia (Gill J R, et al., 2010; Peiris J S, et al., 2009; Peiris M, 2006).

Prominent lymphopenia, and thrombocytopenia have also been observed in H5N1-infected patients (Loeffelholz M J, 2010). Laboratory testing has frequently revealed

elevated alanine aminotransferase, aspartate aminotransferase, creatinine kinase, and lactate dehydrogenase in H5N1 infection (de Jong M D, et al., 2006). Increased levels of lactate dehydrogenase and creatinine phosphokinase, prolonged prothrombin time, and activated partial thromboplastin time, as well as hypo-albuminemia, are also common (Hui D S, 2008). In severe cases, insufficient expression of perforin in CD8+ T cells and hyper-production of IFN-γ has occurred, leading to potential hyper-cytokinemia and hemophagocytosis (Hsieh S M, et al., 2006). Finally, production of pulmonary surfactant protein D was found to be down-regulated in H5N1 infections (Kongchanagul A, et al., 2011).

Host immune responses

In humans, the H5N1 virus triggers a massive pro-inflammatory cytokine and chemokine response that contributes to systemic tissue damage and morbidity (de Jong M D, et al., 2006; Loeffelholz M J, 2010; Suksatu A, et al., 2009; Szretter K J, et al., 2007). Data show that those who died from H5N1 infection had significantly higher levels of cytokine and chemokine expression than survivors. IFN-αIFN-β, IFN-λ1, TNF-α, and RANTES were the primary cytokines induced by the H5N1 virus in macrophages, accompanied by up-regulation of IRF3, the key transcription factor for IFN gene regulation (Lee D C, et al., 2005; Lee D C, et al., 2012). Further, up-regulation of cyclooxygenase 2 (COX-2), followed by IL-32, inducible nitric oxide synthase and prostaglandin E2 production and accumulation were have been observed in cell cultures and in patients with H5N1 infections (Li W, et al., 2010; Li W, et al., 2009; Li W, et al., 2008). The stimulation of COX-2, an inducer of IL-27 and IFN-λ1, acts as an important mediator of the inflammatory response during influenza virus infection (Fang J, et al., 2011; Liu L, et al., 2012). Although results of previous studies using different model systems (both animals and cultured cells) did not completely agree, they did lead to a consensus that H5N1 virus induces a 'cytokine storm' (Yu W C, et al., 2011). Thus, patients with H5N1 disease typically present with a hyper- induced systemic inflammatory response syndrome and a higher and a more prolonged viral load in respiratory specimens than other seasonal influenza virus like human H1N1 viruses (Chan M C, et al., 2005; Kuiken T, et al., 2010; Lee D C, et al., 2012; Peiris J S, et al., 2010; Sandbulte M R, et al., 2008). The outcomes of this imbalance are always associated with fulminant viral pneumonia followed by acute respiratory distress syndrome, multi-organ failure, and/or often death (de Jong M D, et al., 2006; Szretter K J, et al., 2007). Most pro-inflammatory cytokines and chemokines reach higher levels in the serum of H5N1-infected individuals than comparable samples from seasonal human influenza virus-infected individuals (Lee S M, et al., 2009). In H5N1-infected primary human macrophages, many genes showed at least a 1.5-fold greater response than the response observed in seasonal H1N1-infected cells at 6 hours post-infection. Of those genes, TNF, CCL4L1, IFIT2, and PMAIP1 were also up-regulated to a greater degree at 3 hours post-infection (Lee S M, et al., 2009). The negative effector of Fas (Nef) and TNF-α seem to be unique to H5N1 infection (Lee S M, et al., 2009). Despite the increased expression of IFN-α and IFN-β, an up-regulation of IL-29, which was not detected during H1N1 infections, has also been observed following H5N1 infection, leading to enhanced signaling through the Jak-STAT pathway (Lee S M, et al., 2009). Further, avian H5N1 infection triggered a delayed onset of caspase- and poly (ADP-ribose) polymerase (PARP)-mediated apoptotic pathways, resulting in a decreased percentage of cell death and expression level of the PARP fragment than that observed with the H1N1 virus (Mok C K, et al., 2007). Similar results have also been seen in previous studies using other types of human cells and animal models. Much stronger natural killer (NK) cell activation, followed by up-regulation of CD69 and CD107a, significantly enhanced expression of IFN-γ production, and down-regulation of NKp46 have also been observed in H5N1 infection (Du N, et al., 2010). H5N1 virus can also hyper-induce pro-inflammatory cytokines including TNF-α, with enhanced TNF-related apoptosis-inducing ligand-induced apoptosis in Jurkat T cells (Lee D C, et al., 2005; Mok C K, et al., 2007). However, in contrast with findings from human macrophage studies, differences between H5N1 and H1N1 induced secretion of pro-inflammatory cytokines TNF-α and IP-10 were less pronounced in human plasmacytoid dendritic cells, although H5N1 also showed higher expression of IFN-α than did H1N1 and H3N2 viruses (Sandbulte M R, et al., 2008). Despite significant diversity in the induction profile of pro-inflammatory cytokines and chemokines in H5N1 as compared with seasonal H1N1, the magnitude of the pro-inflammatory response triggered by H5N1 is also much stronger than that triggered by other human influenza viruses (Bel M, et al., 2011; Sandbulte M R, et al., 2008; Tolnay A E, et al., 2010; Zeng H, et al., 2007). The cytokine storm caused by the induction (or suppression) of gene expression and signaling pathways

illustrates the extreme and serious consequences of H5N1 infection. Therefore, identifying these pathways will help to find effective methods of fighting this highly pathogenic virus.

DIAGNOSIS AND THERAPY

Diagnosis

Although high virus titers were often detected in many tissues and blood during H5N1 infections, throat swabs and lower respiratory samples are the preferred and recommended specimen for virus detection. H5N1 virus titers are higher in pharyngeal and bronchial alveolar lavages and tracheal aspirates than in nasal epithelial cells or aspirates (Beigel J H, et al., 2005; de Jong M D, et al., 2006; Loeffelholz M J, 2011; Wang H, et al., 2009; WHO, 2008; Yu W C, et al., 2011). Patient samples should be stored carefully (e.g., kept on ice) before testing and it is recommended to use the specimens as soon as possible (Loeffelholz M J, 2011).

Diagnosis of H5N1 influenza infection requires a systematic analysis. A history of recent of travel and personal contacts as well as clinical symptoms and many other factors should be considered (Loeffelholz M J, 2011). Many rapid diagnostic techniques can detect seasonal influenza in clinical specimens, but the rapid antigen tests designed to detect seasonal strains of influenza are less effective for H5N1 (Beigel J H, et al., 2005). In addition, since the clinical presentation of numerous illnesses may resemble influenza, and the many different influenza virus share a number of clinical features, diagnosis of a particular subtype can only be accurately identified by laboratory tests (Shaw M, et al., 1992). Sensitive and specific tests are needed, but remain to be developed. At this time, laboratory diagnosis for H5N1 includes virus culture, rapid antigen detection, and viral nucleic acid amplification methods in addition to serology (Loeffelholz M J, 2011). All these methods have advantages and disadvantages. For effective surveillance, the proper time to apply a particular diagnostic methods or combination of methods must be carefully considered, particularly at the pre-pandemic period of H5N1 spread.

Viral nucleic acid amplification, for example reverse-transcription polymerase chain reaction (RT-PCR), is highly sensitive. The human H5N1 virus outbreaks in Hong Kong were the first time that the RT-PCR method was used for specific detection of the H5 gene segment. It proved to be a valuable diagnostic tool when used in association with identification of clinical features (Yuen K Y, et al., 1998). The more recently developed real-time RT-PCR (RRT-PCR) is more efficient and accurate than the standard RT-PCR method (Das A, et al., 2006). Other assays such as nucleic acid sequence-based amplification, TaqMan, and fluorescence resonance energy transfer have also been studied. These molecular assays are highly specific for H5N1, and can be used to detect H5N1 from a wide range of hosts (Chantratita W, et al., 2008). The disadvantage of all nucleic acid-based methods is the need for sequence information for specific genes such as HA and NA, as well as NP. The existence of several distinct sub-lineages and the high mutability of H5N1 viruses may then lead to increasing difficulties in identifying the virus in patient isolates (Cattoli G, et al., 2009; Loeffelholz M J, 2010).

Hemagglutination inhibition and micro-neutralization are sensitive serological assays for virus detection, and are considered the gold standards for detection of anti-H5-specific antibodies in humans (Noah D L, et al., 2009; Ohnishi K, et al., 2012; Stelzer-Braid S, et al., 2008). These assays are essential to providing a retrospective diagnosis to avoid potential false-negatives and are essential for epidemiological investigations. The major disadvantage of these methods is that paired specimens and H5-specific reagents for the subtype-specific antibodies are always not widely available and/or are difficult to detect (Ohnishi K, et al., 2012). As such, results from these assays may only serve as additional, but not necessarily preferential, methods for laboratory diagnosis. Other methods such as rapid antigen detection tests (e.g., immunofluorescence) are simple and rapid, but suffer from a lack of sensitivity (Clementi N, et al., 2011). The rapid antigen test usually fails to distinguish between type A and type B viruses, and cannot distinguish among AIV subtypes (Clementi N, et al., 2011).

Treatment and prevention

Two classes of antiviral drugs that target influenza virus M2 or NA are commonly used for prophylaxis and treatment of influenza virus infection (Sambhara S, et al., 2010). Both amantadine and rimantadine block the ion channel formed by the M2 protein, thus inhibiting viral entry and replication (Couch R B, 2000; Sambhara S, et al., 2010). The second class of antiviral drug, including zanamivir and oseltamivir, acts on NA to prevent virus release from infected cells (de Jong M D, et al., 2006). However, there appear to be several virus clades that are resistant to these drugs (Le Q M, et al., 2005). The emergence of oseltamivir- or amantadine-resistant strains

restricts the ability to prevent influenza infections and treat patients effectively (Cheung C L, et al., 2006; de Jong M D, et al., 2005; Le Q M, et al., 2005; Malaisree M, et al., 2009). Following analysis of the three- dimensional structure of NA from oseltamivir-resistant influenza strains, six additional drug analogs have been proposed to fight H5N1 (Du Q S, et al., 2007). Including inhibitors of inflammation, such as the combination of celecoxib or mesalazine to zanamivir treatment has been shown to greatly reduce cell death and host tissue damage with a similar viral load compared to zanamivir alone (Zheng B J, et al., 2008). In addition to antiviral drugs that target influenza virus M2 or NA, various molecules with pharmacological activity, such as isopentenyl pyrophosphate and aminobisphosphonates like pamidronate (PAM), were able to activate and expand human γδ T cells (Qin G, et al., 2009; Tu W, et al., 2011), suggesting activation of γδ T-cell-based immunotherapy as an alternative strategy for treating influenza A infection (Qin G, et al., 2011). Recent studies have also identified several additional candidate therapeutic agents, such as a RIG-I pathway activator and IFNs, that inhibit influenza viral replication, DAS181 and its analogs that down-regulate receptor expression on the surface of the host cell, and antiviral siRNAs that target viral genes that inhibit H5N1 infection *in vitro* and in animal models (Chan R W, et al., 2009; Luke J M, et al., 2011; Sambhara S, et al., 2010; Stewart C R, et al., 2011; Szretter K J, et al., 2009). Other candidate drugs such as saponin derivatives or other active compounds that have a chacotriosyl residue and chlorogenin moiety may also serve as host cell entry inhibitors for highly pathogenic H5N1 influenza virus (Ding N, et al., 2012).

Development of an effective H5N1 vaccine poses a number of substantial challenges. Newly emerging virus strains make the choice of vaccine strains problematic. As a result, production and deployment of vaccine are of vital importance. To date, there are at least 23 candidate vaccine viruses that can be used in clinical trials and in model systems of pandemic vaccine production (Nduati R, et al., 2001). Based on specific properties of the H5N1 viruses in particular geographic areas, using one or more of these candidate viruses for vaccine production should be considered.

Unlike seasonal influenza virus vaccine production, using the wild-type H5N1 virus for vaccine production in eggs is not practical due to its high virulence. Recent data also showed that the prototype H5N1 vaccine, which used a plasmid-based reverse genetics system, was poorly immunogenic (Cheung C L, et al., 2006; Harvey R, et al., 2008). Luckily, it was discovered that the addition of an adjuvant (e.g., $AlPO_4$ or Iscomatrix) to this prototype vaccine could induce a clinically significant protection in animal models (Nolan T, et al., 2008; Rockman S, et al., 2010). Beyond recombinant or attenuated vaccines, whole inactivated virus influenza vaccine can also provide heterosubtypic cross-protection against influenza infection (Budimir N, et al., 2012). Other options that use newly established recombinant adenoviral-vector-based vaccines to express H5 HA or a polyvalent DNA vaccine expressing HA antigens from H5N1 viruses have achieved protective antibody responses in animals (Hoelscher M A, et al., 2006; Wang S, et al., 2011). Nevertheless, the high mortality and pathogenicity of H5N1 viruses, additional development of antiviral drugs and vaccines, and novel preventive and therapeutic strategies are needed to protect the world from a potential pandemic.

CONCLUSION

History witnessed three major influenza pandemics in the 20th century: The 1918 H1N1 Spanish Flu, 1957 H2N2 Asian Flu, and 1968 H3N2 Hong Kong Flu. Collectively, these pandemics caused millions of deaths and countless more infections. The highly pathogenic influenza H5N1, despite its low morbidity (i.e., low case incidence rate) and inefficient human-to-human transmission, remains an important public health threat because of its high mortality. Control efforts should be coordinated before this potentially dangerous virus becomes the next pandemic. The last 16 years have resulted in a huge influx of research into the replication and pathogenicity of H5N1, but our understanding of H5N1 remains incomplete. The recent emergence of the 2009 swine-origin H1N1 influenza underlines the challenges remaining to establish a control network to contain virus pandemics. Controlling the spread of the influenza virus requires a modern, global, and interlinked cooperative effort.

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