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REVIEW

Avian influenza A H5N1 virus: a continuous threat to humans

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We report the first case of severe pneumonia due to co-infection with the emerging avian influenza A (H5N1) virus subclade 2.3.2.1 and *Mycoplasma pneumoniae*. The patient was a returning traveller who had visited a poultry market in South China. We then review the epidemiology, virology, interspecies barrier limiting poultry-to-human transmission, clinical manifestation, laboratory diagnosis, treatment and control measures of H5N1 clades that can be transmitted to humans. The recent controversy regarding the experiments involving aerosol transmission of recombinant H5N1 virus between ferrets is discussed. We also review the relative contribution of the poor response to antiviral treatment and the virus-induced hyperinflammatory damage to the pathogenesis and the high mortality of this infection. The factors related to the host, virus or medical intervention leading to the difference in disease mortality of different countries remain unknown. Because most developing countries have difficulty in instituting effective biosecurity measures, poultry vaccination becomes an important control measure. The rapid evolution of the virus would adversely affect the efficacy of poultry vaccination unless a correctly matched vaccine was chosen, manufactured and administered in a timely manner. Vigilant surveillance must continue to allow better preparedness for another poultry or human pandemic due to new viral mutants.

CASE SUMMARY

A 59-year-old woman, who had no known underlying disease, first presented at the accident and emergency department on 12 November 2010, with 1 week of fever associated with haemoptysis, dyspnea, sore throat and rhinorrhea. She travelled to Shanghai, Nanjing and Hangzhou for 10 days and returned to Hong Kong on 1 November 2010. She visited a wet market while she was in Shanghai, but she denied any direct contacts with birds or poultry. Upon admission, her body temperature was 38.7 ℃, with a respiratory rate of 24 breaths per minute and oxygen saturation of 96% while breathing ambient air. Chest radiograph revealed left middle zone consolidation. The clinical diagnosis was acute community-acquired pneumonia, for which she was treated as an outpatient with 1 g of oral amoxicillin–clavulanate twice daily.

Two days later, her symptoms of dyspnea and haemoptysis worsened, and she was admitted to the hospital. Chest radiograph showed progression of consolidation, involving both left middle and lower zones. She required oxygen supplementation via a nasal cannula because her oxygen saturation was 88% while breathing ambient air. Blood tests upon admission showed a total leukocyte count of 4.2×10^9/L, an absolute neutrophil count of 3.8×10^9/L and lymphopenia of 0.3×10^9/L. Liver enzymes were elevated, with an alkaline phosphatase level of

223 U/L and an alanine transaminase level of 71 U/L. The values for haemoglobin, platelet count and renal function test were within the normal range. Oral azithromycin at a dose of 500 mg once daily was added to treat atypical agents of pneumonia. A nasopharyngeal swab collected on the day of admission tested negative for influenza A and B by enzyme immunochromatographic assay. On day 3 of hospitalisation, she developed type I respiratory failure, required 100% oxygen supplementation via a non-rebreathing mask and was admitted to the intensive care unit. Antibiotics were switched to 2 g of intravenous ceftriaxone every 24 h and 100 mg of oral doxycycline every 12 h for enhanced coverage of bacterial pathogens. Because of her clinical deterioration, a nasopharyngeal swab sample was tested by polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) for *Mycoplasma pneumoniae* and influenza A virus, respectively, which were both positive. Other tests for *Chlamydophila pneumoniae*, *Legionella* and human metapneumovirus were negative. The RT-PCR for influenza A virus subtype H5 was positive. The antibody titre against influenza A by complement fixation test increased from 80 on admission (7 days after onset of symptoms) to 2560 on day 5 after admission. Microbiological investigations on the sputum sample, including Gram smear and bacterial culture, Ziehl–Neelsen staining and PCR for *Mycobacterium tuberculosis*, were negative. Oral oseltamivir at a dose of 150 mg every 12 h

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and 100 mg of oral amantadine every 12 h were started on day 3 and day 4 of hospitalisation, respectively. Her condition gradually improved. Oxygen supplementation was weaned off 10 days after admission. A total of 15 days of oseltamivir and 6 days of amantadine therapy were administered.

Computed tomography of the thorax was performed on day 24 of hospitalisation and revealed left pleural effusion with a small loculated pleural effusion over the anterior aspect of left hemithorax, subsegmental collapse consolidation with bronchiectatic changes over the left lobe, and patchy ill-defined consolidation and ground glass opacities with adjacent interstitial thickening in both lungs. Ultrasound-guided aspiration of the left pleural fluid was performed. Analysis of the pleural fluid showed a pH of 7.51, glucose level of 5.2 mol/L, total protein level of 48 g/L, lactate dehydrogenase level of 256 U/L and adenosine deaminase level of 16 U/L. The cell count was not performed because the sample was blood stained. The Gram stain and Ziehl–Neelsen stain were negative. Bacterial and mycobacterial culture results were negative. She was discharged after 29 days of hospitalisation.

The avian influenza A (H5N1) virus was isolated using the Madin–Darby canine kidney cell line from the nasopharyngeal swab collected on the day of admission. Viral genome sequencing and analysis confirmed the features of highly pathogenic avian H5N1 virus. No unusual mutations associated with pathogenic properties, including known polymorphisms that affect receptor binding specificity, were identified. Phylogenetic analysis of haemagglutinin (HA) showed that the virus belongs to subclade 2.3.2.1 (Figure 1).

REVIEW OF LITERATURE AND DISCUSSION

Epidemiology

Avian influenza viruses H5N1,[1] H9N2,[2] H7N7,[3] H7N2,[4] H7N3[5] and H10N7[6] have jumped species barriers and caused human infection. Among these avian influenza viruses, H5N1 virus is the most virulent, with a crude mortality rate of 60%, which markedly surpasses the mortality rate of pandemic influenza viruses[7] (Table 1). The H5N1 virus was first documented to cause human infections in Hong Kong in 1997.[1] It reappeared again in 2003 in Hong Kong, China and Vietnam and has spread to other parts of Asia, Africa and Europe.[8,13] Since 2009, Egypt has had the highest incidence of human H5N1 virus infection, temporally associated with a high incidence of H5N1 virus infection in its poultry population.[14] According to the World Health Organization, there were more than 600 confirmed human cases up to May 2012.[8] However, the number of human cases is likely an underestimate, and a meta-analysis suggested an overall seropositive rate of 1.2% among the exposed population.[15]

Seasonal variations in the incidence of H5N1 virus infection have been observed in some countries. In Egypt, H5N1 virus infection peaks during late winter and early spring, which are associated with low precipitation and moderate humidity and temperature.[16] However, the seasonal pattern is less clear for Indonesia.

H5N1 virus infection affects individuals of all ages. Similar to pandemic influenza, adolescents and young adults are disproportionately affected.[13,17,18] Our patient was 59 years of age, which is older than the median age of 18 years for patients with H5N1 virus infection. However, the age of patients with H5N1 virus infection differs widely in different countries. In Egypt, the median age is 6 years, whereas in Asian countries, the 20- to 29-year age group is most affected.[13] The relatively lower incidence in the elderly population may be explained by prior exposure to various types of poultry or human influenza viruses, which may lead to cross-reactive immunity. In mice, prior seasonal influenza virus infection reduced the severity of H5N1 virus

infection.[19] Age may be associated with the severity of illness, but contrary findings have been reported. In the first outbreak of Hong Kong in 1997, all patients aged over 13 years had severe disease, while most children only had mild upper respiratory tract symptoms.[1] However, a study in Vietnam showed that patients 16 years of age or younger had a higher risk of death than older patients.[20]

Although migratory birds are often infected with H5N1 virus, live domestic poultry are thought to be the main source of human infections.[21] Our patient did not have direct contact with live poultry, although she did visit a wet market. A multivariate analysis showed that visiting a wet poultry market and being in indirect contact within 1 m of sick or dead poultry are independent risk factors for acquiring H5N1 virus infection.[22] Many environmental samples from wet markets have been contaminated with the H5N1 virus,[23] suggesting that indirect contact can be a mode of transmission of H5N1 virus. Although human clusters occur, human-to-human transmission is still limited and usually requires a prolonged period of contact with infected individuals.[24] Consumption of raw duck blood is suspected for some cases of human H5N1 virus infection,[25] and this mode of transmission is compatible with studies in mammals that intragastric inoculation of H5N1 virus can lead to systemic dissemination via the lymphatics and venous route.[26] H5N1 virus can also be detected in frozen poultry and contaminated eggs, but transmission to humans via these food items has not been documented.[27]

While H5N1 virus is highly pathogenic to human, the incidence of human infection is still low. Some identified factors may reduce the human susceptibility to this virus. First, most H5N1 viruses have a predilection to attach to the avian-like α2,3-linked sialic acid receptor (α2,3 SA) rather than the human-like α2,6-linked sialic acid receptor (α2,6 SA). All pandemic or seasonal epidemic human influenza viruses have preferential binding for α2,6 SA, which is abundant in the upper respiratory tract in human and may be a prerequisite for efficient human-to-human transmission.[28] Second, in addition to the host cell surface receptor requirement, the avian- and human-adapted influenza viruses also have different importin-α isoform requirements.[29] Importin-α is a cellular protein responsible for the entry of the viral ribonucleoprotein complex into the host cell nucleus. Efficient replication of avian-adapted influenza strains is dependent on importin-α3, while that of human-adapted strains is dependent on importin-α7. Furthermore, when mice are infected by human influenza viruses, viral dissemination is less prominent in mice lacking importin-α7 than wild-type mice. This specificity has been attributed to the D701N and N319K substitutions of the PB2 (polymerase subunit PB2) and NP (nucleoprotein) genes, respectively. Third, the H5N1 virus may be more susceptible to the human host defence system. Interferon(IFN)-induced Mx GTPases are important in the suppression of H5N1 but not the pandemic influenza A H1N1 2009 (A(H1N1)pdm09) virus due to differences in the NP, which partially explains the decreased susceptibility of human H5N1 virus infection.[30] Finally, mutations that are responsible for human adaptation seem to be unstable in H5N1 viruses.[31]

Virology and virulence factors

The influenza virus is an RNA virus belonging to the family of *Orthomyxoviridae*. Its genome consists of eight gene segments that potentially encode 12 proteins. Influenza viruses are typed by their matrix and NPs antigenically into types A, B and C. The influenza A virus is further subtyped by its surface HA and neuraminidase (NA) into many subtypes as various combinations of 17 different HAs and 10 different NAs.[32] The avian H5N1 virus can be classified into
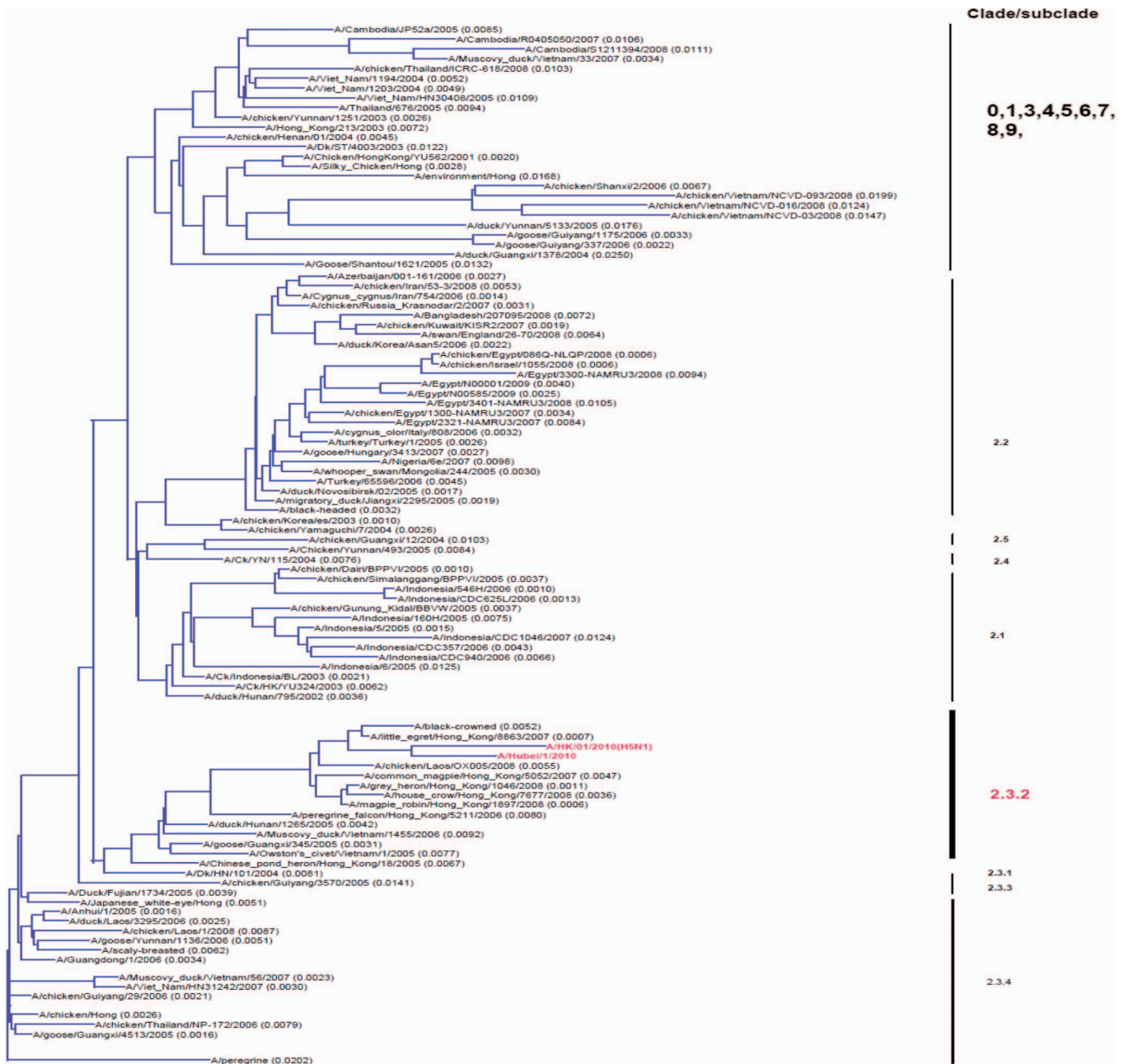
Clade/subclade

Figure 1 Phylogenetic relationships of the haemagglutinin genes from the virus strain of the 2010 Hong Kong patient and other avian H5N1 influenza virus strains. The phylogenetic tree was constructed using the neighbour-joining method. The numbers of clades and subclades are based on the World Health Organization nomenclature on H5N1 virus and are indicated on the right panel. Two human H5N1 isolates identified in 2010 in Hubei province and Hong Kong are indicated in red.

genotypes or clades. Based on the sequences of internal genes, the H5N1 virus was classified into genotypes designated A, B, C, D, E, P, V, W, X0-3, Y, Z and Z+, which are seldom used in most recent studies.[33] Reassortments between different genotypes occur frequently in avian species.[34] More commonly, the H5N1 virus can be classified into clades/subclades based on the phylogeny of HA. The World Health Organization/World Organization for Animal Health/Food and Agriculture Organization H5N1 Evolution Working Group proposed a unified nomenclature system using clades.[35] A clade is further subdivided into second-order clades when it evolves and satisfies the criteria for discrete clades. The H5N1 progenitors closest to Gs/

Guangdong/1/96 and closely related to the 1997 human isolates from Hong Kong are designated as Clade 0. Avian H5N1 viruses have evolved rapidly since 2003, when mainly clade 1 viruses were identified. Since 2004, clade 2 viruses emerged and subsequently evolved into different subclades in different geographical regions. Human infections are caused by H5N1 viruses in clades 0, 1, 2 and 7 (Table 1).

Clade 1 originated from Yunnan in 2002.[36] The clade has further diverged into subclades 1.1 and 1.2. Human cases due to clade 1 viruses have been found in Hong Kong, Vietnam, Thailand and Cambodia.[12,37–39] Mutation 701N of PB2 is critical for the virulence of this clade.[40] The N66S mutation in PB1-F2 protein, which has been

Table 1 Human cases of H5N1 virus infection*

	Total NO. of cases (NO. of fatal cases) [Clade]									
Year	Hong Kong SAR	China	Vietnam, Laos, Myanmar, Thailand	Cambodia	Indonesia	Egypt	Pakistan, Bangladesh	Turkey, Azerbaijan, Iraq	Nigeria, Djibouti	All areas
1997	18 (6) [0]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	18 (6)
2003	2 (1) [1]	1 (1) [7]	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (2)
2004	0 (0)	0 (0)	46 (32) [1]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	46 (32)
2005	0 (0)	8 (5) [2.3.4, 2.3.4.2]	66 (21) [1, 2.3.4]	4 (4) [1]	20 (13) [2.1, 2.1.3, 2.1.3.2]	0 (0)	0 (0)	0 (0)	0 (0)	98 (43)
2006	0 (0)	13 (8) [2.2, 2.3.4, 7]	3 (3) [1]	2 (2) [1]	55 (45) [2.1.2, 2.1.3, 2.1.3.2]	18 (10) [2.2, 2.2.1]	0 (0)	23 (11) [2.2]	1 (0) [2.2]	115 (79)
2007	0 (0)	5 (3) [2.3.4]	11 (7) [2.3.4, 2.3.4.3]	1 (1) [1.1]	42 (37) [2.1.3.2]	25 (9) [2.2.1]	3 (1) [2.2]	0 (0)	1 (1) [2.2.1]	88 (59)
2008	0 (0)	4 (4) [2.3.4]	6 (5) [2.3.4.2, 2.3.4.3]	1 (0) [1.1]	24 (20) [2.1.3.2]	8 (4) [2.2.1, 2.2.1.1]	1 (0) [2.2.2]	0 (0)	0 (0)	44 (33)
2009	0 (0)	7 (4) [2.3.2.1, 2.3.4, 2.3.4.1]	5 (5) [2.3.4.2]	1 (0)	21 (19) [unknown]	39 (4) [2.2.1]	0 (0)	0 (0)	0 (0)	73 (32)
2010	1 (0) [2.3.2.1]	2 (1) [2.3.2.1]	7 (2) [2.3.4.1, 2.3.4.2]	1 (1) [1.1]	9 (7) [unknown]	29 (13) [2.2.1]	0 (0)	0 (0)	0 (0)	49 (24)
2011	0 (0)	1 (1) [2.3.2.1]	0 (0)	8 (8) [1.1]	12 (10) [2.1.3.2]	39 (15) [2.2.1]	2 (0) [2.2.2]	0 (0)	0 (0)	62 (34)
2012 (up to 29 May)	0 (0)	1 (1) [2.3.4.2]	4 (2) [1.1]	3 (3) [1.1]	6 (6) [2.1.3.2]	9 (5) [2.2.1]	3 (0)	0 (0)	0 (0)	11 (8)
Total	21 (7)	42 (28)	151 (80)	21 (19)	189 (157)	167 (60)	9 (1)	23 (11)	2 (1)	625 (364)

*Total number of cases and mortality based on data from the World Health Organization[8] and data from Hong Kong.[1] Clade information based on Refs. 9–12.

associated with virulence in mammals,[41] has been identified in two strains in subclade 1.1 in Cambodia in 2010 and 2011.[42] Clade 1 viruses are more virulent than clade 2 viruses in a ferret model with a limited number of isolates.[43]

Viruses in clade 2 or its subclades have become the predominant clade in human infections since 2005. Subclade 2.1, with the majority of these viruses in the 2.1.3 subclade, is the only clade found in Indonesia, which has the highest cumulative number of H5N1 human cases and deaths in the world.[44] Subclade 2.2 virus (also called Qinghai-like virus) caused a large outbreak among wild birds around Qinghai Lake in 2005 and has since spread to other parts of Asia, Europe and Africa.[45] Human H5N1 cases due to subclade 2.2 viruses are mainly found in the Middle East (particularly in Egypt), South Asia and China.[46] This clade has diverged sufficiently such that subclades are designated. Subclade 2.2.1 viruses emerged in Egypt in 2008 and are associated with increased binding avidity to α2,6 SA, which may explain the increased incidence of human cases in the affected countries.[47] Another distinct feature of subclade 2.2 viruses is the E627K substitution of the PB2 protein, which is only found in some human cases infected by other subclades of H5N1 viruses.[48] Subclade 2.3 represents another major variant besides 2.1 and 2.2 described above. Variants of subclade 2.3.4 viruses first emerged in humans in 2005 in Anhui, became the predominant variant found in poultry in southern China and are responsible for most human infections in China.[49] In Vietnam, subclade 2.3.4 first appeared in humans in 2005 and has been

the predominant clade since 2007.[50] In addition, this subclade is also found in other parts of Southeast Asia.[38] Two strains in the subclade 2.3.4 have increased binding affinity to α2,6 SA.[51] 627K of PB2 in subclade 2.3.4 viruses may also contribute to its virulence.[40] Subclade 2.3.4.2 has also been found in Bangladesh and Myanmar.[11] Subclade 2.3.2 was first found in China and Vietnam in 2005.[52] A new subclade, 2.3.2.1, has been found in migratory birds and poultries from China, Japan and Bangladesh since 2009.[53,54] Subsequently, the first human case caused by a 2.3.2.1 virus was found in Guangxi in 2009, and additional cases, including our presently reported case, have been infected by this new variant.[10,55] The dead wild bird and poultry surveillance in Hong Kong showed that the dominant isolate before 2007 belonged to subclade 2.3.4, but most of the isolates found after 2007 belonged to subclade 2.3.2.1 (personal communication with Dr Thomas Sit of Agriculture Fishery and Conservation Department of Hong Kong Special Administrative Region). However, the detection of a human case in this study suggests that subclade 2.3.2.1 has emerged as the dominant variant in some regions of China.

Clade 7 viruses were first isolated from human in 2003 and subsequently found in Anhui in 2006. Viruses from this clade were also detected in chickens in Vietnam.[56] This clade has not been detected in humans since 2007. Data on the genetic evolution of this clade are scarce.

It is notable that among human cases, the fatality rate was 83.1% in Indonesia but only 35.9% in Egypt (Table 1). Because subclades 2.1

and 2.2 are exclusively circulating in Indonesia and Egypt, respectively, certain subclades may be more virulent than others. Sequence comparisons revealed some variations between different geographical subclades (Table 2). When comparing the known polymorphisms responsible for virulence or mammalian adaptation between the human strains from different countries, most mutations enriched in the Egypt strains may have enabled the strains to bind better to the α2,6 SA. For example, deletion of position 129 together with I151T substitution of HA is found in 40% of strains from Egypt but not from Asia (H5 numbering). This double mutation increases the binding to α2,6 SA, and the virus can attach mainly to type I and type II pneumocytes but minimally to the larynx in human.[47] T156A of HA is also present in most strains from Egypt but only in a few strains in China and none from Southeast Asia. This mutation results in loss of glycosylation at positions 154–156 and is associated with increased binding to α2,6 SA and increased transmission efficiency among guinea pigs.[60] Another major difference is the S235P mutation, which is present in most strains from Asia but only in 10% of strains from Egypt. However, this mutation does not change the affinity for α2,6 SA in direct binding assays.[47] Polymorphisms in PB2 are also important in mammalian adaptation, but these polymorphisms between strains from Egypt and other countries cannot be compared because the PB2 amino-acid sequences for only two Egyptian strains from humans have been deposited into GenBank. However, the 627K of PB2 is more common among isolates from Vietnam/Thailand/Cambodia/Laos (50%) than from Indonesia (11%) and China/Hong Kong (20%). Only further investigations can ascertain the relevance of these mutations to their effect on virulence in humans. Besides viral genetic polymorphisms, the difference in fatality may also be related to the availability and standard of medical care in these countries. H5N1 viruses circulating in Cambodia are similar to those in Thailand, Vietnam and China, but human cases from Cambodia exhibited the highest mortality rate (90.5%; Table 1). Close monitoring and examination of genetic variations in different geographical areas are necessary for a better understanding of the virulence and molecular evolution of the H5N1 viruses.

Clinical and laboratory features

The usual incubation period is 2 to 9 days.[24,64] The incubation period was usually longer in patients who acquired the infection from the wet market than those patients who were exposed to sick or dead poultry.[65] Our patient had a typical course of the disease, which manifested with upper respiratory tract symptoms and was followed by rapid deterioration into pulmonary disease. Haemoptysis is an alarming symptom that may signify pulmonary haemorrhage. Acute respiratory distress syndrome (ARDS) is a frequent pulmonary complication.[66] H5N1-induced ARDS is associated with poor outcomes even with prompt medical care. In contrast to human seasonal influenza viruses, H5N1 viruses are more likely to cause severe pneumonia. This finding is consistent with the fact that H5N1 viruses preferentially bind to α2,3 SA, which is abundant on the surface of type II pneumocytes, alveolar macrophages and non-ciliated bronchiolar cells of the lower respiratory tract.[67] Although H5N1 virus can replicate in ex vivo upper respiratory tract cell lines,[68] binding to upper respiratory tract tissue is much less efficient when compared with the seasonal influenza viruses.[69] Some H5N1 viruses isolated from humans can recognize both α2,3 SA and α2,6 SA, such as A/Hong Kong/213/03 (H5N1) and some sublineages of the subclade 2.2.1 viruses from Egypt and subclade 2.3.4 viruses from China.[47,51,70] The dual-binding capability may indicate a wider range of cellular tropism and, therefore, more

efficient spread of this virus in the respiratory tract. However, the ability to bind α2,6 SA in vitro may not equate to binding of α2,6 SA in human. In the human respiratory tract, α2,6 SAs have long oligosaccharide branches with multiple lactosamine repeats, which is different from the short α2,6 SA used in previous studies.[71] This study also demonstrated that influenza viruses that transmit efficiently between humans have high affinity for the long α2,6 SA, while those viruses with poor transmissibility only bind the short α2,6 SA. The difference in binding specificity between the long and short α2,6 SA may be related to their structures. The long glycans exhibit an umbrella-like topology that binds onto a wider region of the receptor-binding pocket of HA, while the short glycans that bind a smaller region of HA have a cone-like topology.

In addition to pulmonary disease, H5N1 virus infection also leads to extrapulmonary manifestations more often than infections caused by pandemic influenza viruses.[72] Diarrhea occurred in more than 50% of patients in a series from Vietnam[9] but less than 10% in other series.[20] Liver impairment can occur, and our patient had elevated alkaline phosphatase and alanine transaminase. Renal impairment is common. Elevated creatine kinase is also common, but true rhabdomyolysis has not been described. In addition, encephalitis may occur. Many of these features are attributable to the direct invasion of the virus, and the multibasic cleavage site of HA, which allows cleavage by proteases that are ubiquitously expressed in most cells, is critical for the systemic spread of the virus.[73] The higher virulence of the H5N1 virus, which leads to a high pulmonary and extrapulmonary viral load, together with its intrinsic pro-inflammatory property, often causes a cytokine storm in these patients. Other common laboratory abnormalities included leucopoenia, lymphopenia, thrombocytopenia and impaired coagulation profiles.[1] Reactive haemophagocytic syndrome can occur and may be related to prolonged IFN-γ production.[74] Encephalopathy has been reported.[74] Reye syndrome can occur after aspirin is administered.[1]

Viral antigens can be detected in small and large intestinal epithelial cells, bone marrow and the brain, but viral cultures from these organs are negative.[74,75] In addition, H5N1 RNA can be detected in the spleen,[76] and the H5N1 virus can be cultured in the cerebrospinal fluid of infected humans.[77] In vitro, the H5N1 virus can replicate in cell lines originating from different anatomical sites.[78] In ferrets, H5N1 can cause encephalitis and non-supportive vasculitis with haemorrhage via infection of the olfactory system.[79] In a mouse model, H5N1 infection has also led to aggregated alpha-synuclein, prolonged microgliosis and loss of dopaminergic neurons, which is compatible with neurodegenerative diseases.[80]

In addition to virus-induced cytolysis driving this pathogenesis, the pulmonary and systemic manifestations of H5N1 virus infection may be related to the cytokine storm. The H5N1 virus can induce strong cytokine responses in vitro and in vivo.[81,82] Infection of pulmonary microvascular endothelial cells has been associated with a marked inflammatory response.[83] Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signalling is essential for the expression of IFN-β in H5N1 virus-infected endothelial cells.[84] Upregulation of genes associated with keratin filaments and keratinisation in human bronchial epithelial cells may be related to hypercytokinaemia.[85] Viruses that have high affinity for α2,3 SA may induce a stronger inflammatory response in human dendritic, macrophages and respiratory epithelial cells than viruses with high affinity for α2,6 SA.[86] In addition to the pro-inflammatory cytokine response, the H5N1 virus also inhibits a lipoxin-mediated anti-inflammatory response.[87]

Table 2 Natural mutations in human isolates of the H5N1 virus that are associated with virulence or adaptation to humans. Gene information obtained from the NCBI Influenza Virus Reource,[57] sequences with duplicate strain names but different sequences were excluded from the analysis.

Amino acid mutations	Indonesia[a]	Egypt[b]	Vietnam, Thailand, Cambodia, Laos[c]	Hong Kong, China[d]
Haemagglutinin (H5 numbering)				
L129V[58]	S=89 (86%)	S=82 (59%)	S=12 (14%)	S=41 (89%)
	L=15 (14%)	L=2 (1%)	L=69 (81%)	L=5 (11%)
	Δ=0 (0%)	Δ=56 (40%)	Δ=0 (0%)	Δ=0 (0%)
	V=0 (0%)	V=0 (0%)	V=2 (2%)	V=0 (0%)
			I=1 (1%)	
129 deletion+I151T[47]	I=102 (98%)	I=84 (60%)	I=85 (100%)	I=45 (98%)
	T=2[e] (2%)	T=56[f] (40%)	T=0 (0%)	T=1[a] (2%)
A134V[58]	A=104 (100%)	A=135 (96%)	A=75 (88%)	A=41 (89%)
	S=0 (0%)	S=1 (<1%)	V=6 (7%)	S=0 (0%)
	V=0 (0%)	V=1 (<1%)	X=3 (4%)	V=3 (7%)
	X=0 (0%)	X=3 (2%)		X=1 (2%)
	T=0 (0%)	T=0 (0%)		T=1 (2%)
139G[59]	G=103 (99%)	G=140 (100%)	G=84 (99%)	G=46 (100%)
	R=1 (1%)	R=0 (0%)	R=1 (1%)	R=0 (0%)
T156A (absence of glycosylation at 154–156)[60,61]	T=104 (0%)	T=2 (1%)	T=85 (100%)	T=33 (72%)
	A=0 (%)	A=138 (99%)	A=0 (%)	A=9 (20%)
	S=0 (0%)	S=0 (0%)	S=0 (0%)	S=4 (9%)
N182K[62]	N=104 (100%)	N=135 (96%)	N=83 (98%)	N=46 (100%)
	K=0 (0%)	K=2 (1%)	K=0 (0%)	K=0 (0%)
	X=0 (0%)	X=3 (2%)	X=0 (0%)	X=0 (0%)
	D=0 (0%)	D=0 (0%)	D=1 (1%)	D=0 (0%)
	S=0 (0%)	S=0 (0%)	S=1 (1%)	S=0 (0%)
K189R[61]	K=1 (1%)	K=0 (0%)	K=79 (93%)	K=41 (89%)
	R=104 (99%)	R=138 (99%)	R=5 (6%)	R=5 (11%)
	G=0 (0%)	G=1 (<1%)	G=0 (0%)	G=0 (0%)
	S=0 (0%)	S=1 (<1%)	S=0 (0%)	S=0 (0%)
	N=0 (0%)	N=0 (0%)	N=1 (1%)	N=0 (0%)
Q192H, Q192R[47,62]	Q=105 (100%)	Q=134 (95%)	Q=84 (99%)	Q=46 (100%)
	H=0(0%)	H=4 (3%)	H=0 (0%)	H=0 (0%)
	R=0 (0%)	R=0 (0%)	R=0 (0%)	K=0 (0%)
	K=0 (0%)	K=2 (2%)	K=0 (0%)	
			X=1 (1%)	
Q222L[59]	Q=104 (99%)	Q=140 (100%)	Q=85 (100%)	Q=46 (100%)
	L=0 (0%)	L=0 (0%)	L=0 (0%)	L=0 (0%)
	R=1 (1%)	R=0 (0%)	R=0 (0%)	R=0 (0%)
G224S[59]	G=105 (100%)	G=140 (100%)	G=85 (100%)	G=46 (100%)
	S=0 (0%)	S=0 (0%)	S=0 (0%)	S=0 (0%)
S235P[47]	S=0 (0%)	S=126 (90%)	S=0 (0%)	P=43 (93%)
	P=105 (100%)	P=14 (10%)	P=84 (99%)	S=3 (7%)
			Q=1 (1%)	
PB2[g]				
Q591K[63]	Q=89 (99%)	Q=2 (100%)	Q=54 (93%)	Q=39 (100%)
	K=1 (1%)	K=0 (0%)	K=0 (0%)	K=0 (0%)
	R=0 (0%)		R=4 (7%)	R=0 (0%)
E627K[63]	E=80 (89%)	E=0 (0%)	E=29 (50%)	E=35 (80%)
	K=10 (11%)	K=2 (100%)	K=29 (50%)	K=9 (20%)
D701N[63]	D=88 (100%)	D=2 (100%)	D=50 (86%)	D=35 (85%)
	N=0 (0%)	N=0 (0%)	N=8 (14%)	N=6 (15%)

[a] *n* = 105 for haemagglutinin and *n* = 90 for PB2. For haemagglutinin, nucleotide information for position 129, 134, 139, 156 and 189 is available for only 104 strains; for PB2, nucleotide information for position 701 is available for only 88 strains.
[b] *n* = 140 for haemagglutinin and *n* = 2 for PB2.
[c] *n* = 85 for haemagglutinin and *n* = 58 for PB2. For haemagglutinin, nucleotide information for position 129 and 134 is available for only 84 strains.
[d] *n* = 46 for haemagglutinin. For PB2, nucleotide information is available for 39 strains at position 591, 44 strains at position 627, and 41 strains at position 701.
[e] S at position 129.
[f] Deletion at position 129.
[g] All strains had glutamic acid at position 158 of the PB2 gene.

Poor prognostic factors include older age, delay in hospitalisation, diarrhoea, mucosal bleeding, chest X-ray showing extensive lung involvement, desaturation on admission, a delay in receiving antiviral therapy, neutropaenia, thrombocytopaenia, and increased levels of d-dimer, serum glucose, urea, alanine aminotransferase and lactate dehydrogenase.[1,20,65,88] Secondary community-acquired bacterial infection is a well-established poor prognostic factor for influenza virus infection,[89] but its role has not been demonstrated conclusively for H5N1 virus infection because most of the co-infections that occurred after H5N1 infection were due to hospital-acquired bacteria or fungi.[1] For our patient, M. pneumoniae was detected in the naso-pharyngeal swab sample collected on the day of admission and is therefore acquired from the community. Our case is the first report of H5N1 virus infection with community-acquired co-infection by M. pneumoniae. Although our patient started taking azithromycin, the progression of pneumonia may have been related to a macrolide-resistant strain of M. pneumoniae.[90] Therefore, it is difficult to delineate the relative contribution of M. pneumoniae in our patient's clinical presentation. Treatment with corticosteroids is associated with increased mortality.[20] Higher viral load in the respiratory tract is associated with poor outcome in humans.[91] The higher viral load may be related to the poor innate immune control of the virus. Viraemia is also associated with a poor outcome of H5N1 infection in a ferret model, although this association has not been systematically analysed in human H5N1 cases. Viral genetic polymorphisms are associated with increased virulence in animal models.[59] However, none of these polymorphisms has been associated with more severe outcome in human H5N1 infection, unlike the D222G substitution in A(H1N1)pdm09 virus, which is clearly correlated with more severe disease in humans.[92,93] As expected, genetic polymorphisms such as the polymorphisms of CD55, are associated with severe A(H1N1)pdm09 infection.[94]

Although H5N1 infection is usually associated with severe disease, some patients, especially children, only suffer from mild upper respiratory tract symptoms without pneumonia.[1,9,88] The disease severity also varies between patients from different countries. For example, Egypt has a particularly low rate of pneumonia, which is still unexplained but may be attributed to the higher predilection of the Egyptian virus for the upper airway.[9]

Diagnosis

Currently, antigen detection by rapid immunochromatographic assays or direct immunofluorescence and nucleic acid detection by RT-PCR provides a rapid diagnosis, which guides immediate management; in contrast, viral culture and serology allow for retrospective diagnosis, which is essential for epidemiological studies. Respiratory tract specimens are the best samples for detecting the virus, although the virus can also be found in blood or rectal swabs.[91] Unlike human seasonal influenza viruses, a throat swab has a higher yield than a nasopharyngeal swab for the H5N1 virus due to the general predilection of the H5N1 virus for the lower respiratory tract. Although technically simple, immunochromatographic assays have a low sensitivity, and their detection limits are at least 3 $\log_{10}$ TCID$_{50}$ per 100 μl.[95] Therefore, further diagnostic tests were performed for our patient even though the immunochromatographic assay showed a negative result for influenza A and B. Direct immunofluorescence assays also have poor sensitivity.[1] Another limitation of antigen detection assays is their failure to differentiate H5N1 from other influenza A viruses. RT-PCR is the most sensitive detection method,[96] but mutations in the HA gene can cause false negatives due to mismatches between the viral gene sequence and the primers or the probes.

Serology is especially useful in documenting asymptomatic infections, but is not useful for patient management because a ⩾ 4-fold rise in complement fixation antibody titre in serum samples collected 2 weeks apart is necessary to make the diagnosis of influenza A infection. Another problem is that complement fixation antibody titre is not specific for H5N1 virus infection. For a more specific diagnosis of H5N1 virus infection, the viral microneutralisation assay is more specific than the haemagglutination inhibition assay. Unlike the human H1N1 or H3N2 infections, the viral microneutralisation assay, preferably with confirmation by Western blotting with a baculovirus-expressed H5 protein, is the preferred gold standard for serological assay and is presently accepted as the more specific way of serodiagnosis.[97] Most individuals develop a positive titre 3 weeks after the onset of disease.[98]

Treatment

Adamantanes were used in the initial outbreak of H5N1 in Hong Kong in 1997.[1] However, adamantane resistance is now widespread,[99] and susceptible strains are limited to those strains in clade 2 from Eurasia and Africa[9] and subclade 2.1 from Indonesia.[100] Resistance to adamantanes is due to L26I or S31N mutations of the M2 protein, and the latter mutation reduces drug binding.[101]

The neuraminidase inhibitors oseltamivir or zanamivir are the mainstay of treatment for H5N1 infection. The survival benefit is greatest if oseltamivir is started within 2 days of symptom onset. It is doubtful if oseltamivir is useful if initiated later, although there is a report of benefit even if given within 8 days after symptom onset.[102] Our patient received oseltamivir >8 days after symptom onset, which may explain why our patient developed severe disease. Zanamivir is usually administered by oral inhalation. Intravenous zanamivir, which was successfully used in humans during the 2009 H1N1 pandemic, is effective against H5N1 virus infection in a macaque model.[103] Other neuraminidase inhibitors, including peramivir and CS-8958, are also active against the H5N1 virus.[104,105] Strains with reduced susceptibility to oseltamivir are present in clade 2 viruses from Indonesia[106] and subclade 2.3.4 viruses from Vietnam.[50] Studies reporting the oseltamivir susceptibility of clade 1 viruses from Cambodia have been contradictory. An earlier study showed that these strains had reduced susceptibility to oseltamivir, while a more recent study of the same strains reported full susceptibility.[107] Oseltamivir resistance can emerge during treatment, leading to treatment failure.[108] The mutations responsible for oseltamivir resistance are most commonly due to H274Y and N294S (N2 numbering) substitutions within the neuraminidase. H5N1 viruses with these mutations are still highly pathogenic.[109] Other mutations associated with reduced susceptibility to oseltamivir, including V116A, I222L, K150N and S246N, have also been reported.[110] However, oseltamivir-resistant strains are usually susceptible to zanamivir. The discrepancy between oseltamivir and zanamivir susceptibility is due to differences in how oseltamivir and zanamivir bind to the virus. The binding of oseltamivir to the viral neuraminidase requires a conformational change in the side chain of 276E, and thus, the carboxyl group of 276E is oriented away from the hydrophobic pentyloxy group of oseltamivir. In contrast, the binding of zanamivir involves hydrogen bond formation without a side chain conformational change.[111] True zanamivir resistance has not been reported, but a clade 1 strain from Cambodia isolated from a human patient had an half maximal

inhibitory concentration (IC$_{50}$) of 3.35 nM, which is eightfold higher than the mean IC$_{50}$ of other strains.[107] Eight amino-acid substitutions were found in the NA of this virus, and the V149A mutation was postulated to be the important substitution responsible for the reduced susceptibility. A/Swan/Shanghai/10/09, an H5N1 virus belonging to the epidemic subclade 2.3.2, contains an S31N mutation in the M2 protein and an H274Y mutation in the NA protein; these mutations are responsible for resistance to adamantanes and neuraminidase inhibitors, respectively.[112]

The clinical efficacy of oseltamivir for the much milder A(H1N1)pdm09 infection was recently questioned because its clinical benefit appears minimal even if instituted within 48 h of symptom onset.[113] Several antivirals in development have targeted other parts of the viral life cycle. Nucleozin, a NP inhibitor, has potent *in vitro* and *in vivo* activity against H5N1 virus in a mouse model.[114] Antivirals that affect viral RNA synthesis, including 2'-deoxy-2'-fluorocytidine and favipiravir (T-705), also have good *in vitro* activity.[115,116] The sialidase fusion protein DAS181 can inhibit the H5N1 virus, including oseltamivir-resistant strains.[117,118] Antiviral peptides that bind to HA, therefore preventing viral entry, are also effective *in vitro*.[119]

Immunomodulatory therapy is an attractive treatment option because the currently available antivirals have only modest potency, and antiviral resistance may appear during treatment. COX-2 inhibitors, such as celecoxib, together with zanamivir, improved the survival of H5N1-infected mice.[120] Cytotoxic therapy has been proposed as an adjunctive treatment for H5N1, but real-life treatment experience has not been reported.[121] Etoposide and betamethasone were beneficial in a patient with A(H1N1)pdm09 infection complicated by haemophagocytic lymphohistiocytosis.[122] In guinea pigs, α-IFN treatment reduced lung viral titre,[123] but few would consider its use in a highly inflammatory condition such as H5N1 infection. A proteasome inhibitor that prevents NF-κB activation in infected cells reduces cytokine release.[124] Convalescent plasma and hyperimmune serum may be useful.[125] Monoclonal antibodies against HA of H5N1 appear effective in mouse models.[126]

Prevention

Most human H5N1 infections have been acquired via direct transmission from infected poultry, and human infections are associated with poultry outbreaks.[127] Hence, controlling the infection in poultry is crucial to prevent human infections. Several risk factors have been identified for H5N1 outbreaks in chickens. First, the isolation of H5N1 virus from ducks and geese precedes disease outbreaks and virus isolation in chickens.[128] Because infected ducks and geese are often asymptomatic, the amplification and spread of the virus among these poultry often goes unnoticed.[129] Therefore, segregating chickens from other poultry is important. In farms, chickens should not be reared with other poultry. In the retail markets, live ducks, geese and quails are now banned in Hong Kong.[128] Second, human-bird segregation via a central slaughtering has been proposed in Hong Kong but yet to be implemented due to resistance from the public. Third, the spread of H5N1 virus is associated with the transport of infected chickens.[130] Cross-border spread may be prevented by tighter control over illegal poultry trafficking. Fourth, biosecurity measures must be in place for the transport of chickens between farms. Visiting farms is associated with increased risk of poultry outbreak; hence, biosecurity measures should include policies for visitors.[131] Fifth, vaccination and disinfection can decrease the risk of poultry outbreaks.[130] Vaccination in the poultry population is now practiced in many countries. However, a vaccine against one clade of virus may not protect against other clades

or subclades due to antigenic differences.[132] Currently, there are four main groups of HA: clade 1 viruses are similar to clade 4, 5, 7 and 9 viruses; subclade 2.1 viruses are antigenically similar to subclade 2.4 viruses; subclade 2.2 viruses are similar to some viruses in subclade 2.3; and subclade 2.3.4 viruses represents a unique group. Further divergence of a particular clade/subclade can lead to vaccine failure, such as the recent emergence of subclade 2.3.2.1 viruses in southern China.[133] Due to the rapid mutation of the HA gene, poultry vaccine components must be regularly updated.[10]

Surveillance of poultry and wild birds has allowed for the early detection of the H5N1virus and has provided important information regarding the spread of H5N1 viruses. For each farm, unvaccinated poultry are also reared because these poultry will be more symptomatic if infected and therefore serve as a sentinel of H5N1 virus infection. Wild bird surveillance allows for the understanding of the virus activity and evolution in the natural reservoir. Because migratory birds can travel long distances, they can introduce or reintroduce H5N1 virus to a disease-free area and may pose a threat to humans.[134] In Hong Kong, wetland parks are temporarily closed when the H5N1 virus is detected in the park. Education regarding the safe handling and disposing of wild birds is regularly provided to the public. However, even though pigs are well-known mixing vessels for influenza viruses, pig surveillance is relatively less systematic. Besides early detection of new reassortants or clades of viruses in animals, rapid genetic characterisation of the virus is important for identifying important pathogenetic or drug-resistant genomic signatures. Early detection of human cases is also important. Increased awareness among frontline clinicians, together with sensitive and specific molecular testing, will allow for prompt recognition of an infected case for early administration of antiviral treatment, infection control measures, and tracing the source of infection.

Culling of chicken has been used in Hong Kong in 1997 as a means to terminate the outbreak of human H5N1 infection. It was used again in 2003 and 2011 following the identification of H5N1 virus in poultry in the market. To minimize the expensive mass culling, precautionary measures have been put in place. Twice monthly rest days in the wet markets in Hong Kong allow for a thorough cleansing of the environment which prevents the mixing of old and new chickens and disrupts the chain of virus transmission. During outbreaks, the wholesale market is thoroughly disinfected, and live poultry from farms cannot be sold in the markets for 21 days until there are no additional cases throughout Hong Kong. When the poultry vaccine cannot catch up with the rapid evolutionary of the H5N1 virus, no live poultry is allowed to stay overnight in wet markets. They are either sold out or culled in the evening. Such severe biosecurity measure is necessary to stop transmission in the poultry market which is basically a minifarm in an overcrowded urban area.

Vaccination in human is still at the experimental stage. Human studies have shown that antibodies against HA and NA can be elicited by vero cell- and insect cell-derived vaccines.[135,136] Using a low-dose, adjuvanted vaccine with 3.75 μg of HA has been successful.[137] Two doses of adjuvanted whole virus vaccine can elicit high titres of cross-neutralising antibodies that are effective against clade 0, clade 1 and clade 2 viruses.[138] A low-dose intradermal H5N1 vaccine has been evaluated in a phase I clinical trial, and the elicited immune response was similar to that with the regular dose of intramuscular vaccine.[139] Patients previously primed with an H5 vaccine developed a rapid and strong neutralising antibody response upon a second challenge with an antigenically distinct H5 vaccine.[140] Recently, Lyall *et al.*[141] proposed that genetically modified chickens expressing a short-hairpin RNA

that prevents viral replication are resistant to H5N1 virus infection and may stop transmission of the H5N1 virus.

Controversy on the study of airborne transmission of H5N1

The current strain of H5N1 virus is highly virulent but poorly transmissible between humans.[24] Scientists have been examining factors that may give rise to a highly transmissible H5N1 virus and, hence, enhance the preparedness for the arrival of such a virus. Previous studies demonstrated that T156A of HA and 701N of PB2 are critical in the transmission of H5N1 virus in a mammalian host.[60] In that study, the virus can be transmitted via direct contact because the guinea pigs were put in the same cage. In another experiment, the K627E substitution of PB2 decreased contact transmission between guinea pigs, while 701N compensated for the lack of 627K.[142] While guinea pigs are similar to humans in the distribution of α2,3 SA and α2,6 SA in their respiratory tract, they do not develop the clinical signs observed in humans.[143] However, ferrets have a similar sialic acid distribution in the respiratory tract, susceptibility and clinical manifestations to humans; therefore, they are the preferred animal model.[144] A recent transmission study in ferrets demonstrated that a reassortant virus containing a mutant haemagglutinin (Q192R, Q222L and G224S), a human H3N2 neuraminidase and other genes from H5N1 virus, was transmitted to one out of two ferrets via the droplet route.[145]

Recently, mutant viruses capable of more efficient non-contact transmission arising from the passage of the H5N1 virus in ferrets and reverse genetics were found by two groups of scientists.[146,147] Whether the results of the research should be published has been controversial because of biosecurity concerns. In December 2011, the National Science Advisory Board for Biosecurity (NSABB) of the USA recommended that the journals not publish key details, and in February 2012, the World Health Organization also decided that the results should be withheld to allow more discussion on the impact of publishing the results.[148] Proponents for publishing the results suggest that air transmissibility between ferrets does not mean air transmissibility between humans. Moreover, the findings are important for understanding of the virus, which is a prerequisite for designing better treatment and prevention strategies.[149] Knowledge on the genomic signatures that are critical for efficient transmission identified via the ferret studies will allow us to have more targeted surveillance and research for viruses harbouring such signatures, so that early and specific preventive measures can be implemented. The proponents also raised the issue that previous studies on the rescue of the rather virulent 1918 pandemic influenza virus were allowed to be published in full. However, opponents suggested that the results will allow terrorists to replicate the experiment and produce an H5N1 virus capable of aerosol transmission that could be used in biological warfare. Furthermore, there have been suggestions that such research should be stopped because of the possibility of accidental or deliberate release of these viruses into the community. The first act of censoring scientific data may set the precedence of interfering with academic freedom in science.

Further analysis of the unpublished data suggested that the mutant virus is not as lethal as the wild type and that the mutant was not so readily air transmissible as initially perceived. Based on these findings, the NSABB has changed its position and recommended that the studies should be published in full.[150] In the study by Imai et al.,[146] reassortant influenza viruses containing the HA from an H5N1 virus and seven gene segments from an A(H1N1)pdm09 virus were created. One of the mutant viruses, which contained four mutations in HA (N154D, N220K, Q222L and T315I), was able to transmit more efficiently via the droplet route between ferrets. The mutations N154D,

N220K and Q222L were thought to be responsible for enhanced binding to α2,6 SA, while T315I was thought to improve fusion between the viral envelope and the intracellular membranes. In the study by Herfst et al.,[147] the mutant H5N1 virus was generated by site-directed mutagenesis and serial passage in ferrets. H103Y, T156A, Q222L, G224S of HA and E627K of PB2 were present in all viruses that were isolated from ferrets that acquired the H5N1 virus via the non-contact route. It is clear from these two studies that some of the initial fears were not justified. First, many commentaries have used the term 'airborne'.[151] However, both studies did not demonstrate that the viruses were transmitted via the airborne route but rather via non-contact routes (airborne or droplet) because the inoculated and naive ferrets were placed in cages that were ≤10 cm apart. Airborne transmission has been defined as transmission via droplet nuclei ≤5 μm, which may be transmitted over long distances >1 m.[152] Second, the mutant viruses were not highly pathogenic. No deaths occurred among the ferrets that were infected via the non-contact route.

CONCLUSION

In this review, we have reported a case of human infection due to H5N1 virus subclade 2.3.2.1 in South China and provided an update on the H5N1 virus related to human infection. Despite aggressive control measures in this area, sporadic human H5N1 infections still occur, highlighting the need for high vigilance, especially when encountering patients who have poultry contact or have visited a poultry market. Indonesia still has the highest number of cumulative human cases worldwide, although Egypt has the highest number of human H5N1 cases reported since 2009. Differential binding affinity to α2,3 SA and α2,6 SA by the H5N1 virus may explain the rapid spread of H5N1 virus in Egypt. Despite worldwide surveillance and aggressive strategies to eliminate the H5N1 virus, the virus continues to cause fatal outbreaks in both the avian and human population. The viruses have rapidly evolved, generating many subclades with potentially enhanced virulence or transmissibility. This evolution may have been driven by increased infection among non-chicken poultry, which often have mild manifestations,[153] increased mixing of viruses from different areas facilitated by migratory birds,[154] and mass H5N1 virus vaccination among poultries.[155] Tremendous progress has been made towards understanding the human H5N1 virus infection, but the mortality rate in human H5N1 cases has remained near 60% since 1997,[1,8] although the mortality rate is as low as 30% in Egypt. Due to the lack of resources, early detection is limited in developing countries, and even in affluent countries, detection may be delayed due to the lack of awareness. Analysis of the viral genome allows the scientific community to identify virulence determinants, but such knowledge has had little clinical impact yet. Despite antiviral treatment, many patients still succumbed to viral disease. Since the introduction of neuraminidase inhibitors more than 10 years ago, no new antivirals that are active against the influenza virus have been approved. Many preventive strategies, especially vaccinations, are effective in limiting H5N1 virus transmission in poultry, but the benefit of vaccination can be impaired by the low uptake rate in backyard farms and the lack of cross-protection between different clades or subclades.

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