Human infection with highly pathogenic H5N1 influenza virus

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Highly pathogenic H5N1 influenza A viruses have spread relentlessly across the globe since 2003, and they are associated with widespread death in poultry, substantial economic loss to farmers, and reported infections of more than 300 people with a mortality rate of 60%. The high pathogenicity of H5N1 influenza viruses and their capacity for transmission from birds to human beings has raised worldwide concern about an impending human influenza pandemic similar to the notorious H1N1 Spanish influenza of 1918. Since many aspects of H5N1 influenza research are rapidly evolving, we aim in this Seminar to provide an up-to-date discussion on select topics of interest to influenza clinicians and researchers. We summarise the clinical features and diagnosis of infection and present therapeutic options for H5N1 infection of people. We also discuss ideas relating to virus transmission, host restriction, and pathogenesis. Finally, we discuss vaccine development in view of the probable importance of vaccination in pandemic control.

Virus transmission

Most reported human infections with influenza H5N1 viruses have arisen because of direct handling of infected poultry, or close contact with live poultry.1–3 In poultry, infection with highly pathogenic avian influenza viruses results in systemic replication, the presence of infectious virus in many tissues and organs, and excretion of large amounts of virus in faeces and other secretions that could contaminate the direct environment.4 Natural transmission of virus between birds occurs directly or indirectly through contact with contaminated fomites (ie, inanimate objects or substances capable of carrying infectious organisms). Although bird-to-human transmission probably occurs via much the same routes, the few cases of reported human infections, despite large-scale poultry outbreaks, suggest inefficient transmission of present H5N1 viruses to human beings. Contact with the contaminated environment, such as water, has been suggested in individuals without apparent direct exposure to poultry. H5N1 virus infectivity is inactivated in chicken faecal manure at room temperature after 24 h;10 however, contact with contaminated bird faeces used as fertiliser exists as a theoretical route of transmission. Although most human infections probably result from transmission via respiratory routes, the gastrointestinal tract remains a possible means of entry. This notion is supported by natural and experimental infections in tigers, leopards, domestic cats, and poultry who fed on infected birds.10,11 Furthermore, laboratory experiments showed evidence of viral replication in gastrointestinal plexi of cats infected after feeding on infected chickens but not in those infected via the respiratory route.4 In human beings, the possibility of initial gastrointestinal infection is suggested by patients presenting with diarrhoea as the only initial symptom and by those reporting consumption of raw duck blood as the sole exposure to poultry. The presence of viral antigen or pathological changes in intestinal tissues has not been recorded in the few autopsy studies,15,16 but evidence of replicating virus or viral RNA in faecal material or intestines has frequently been reported.15,16 Whether actual replication takes place in intestinal epithelia is unclear, although replicating virus or viral RNA in faecal matter at least suggests that gastrointestinal secretions should be considered in the implementation of infection-control measures for patients infected with H5N1 influenza virus. A few possible human-to-human transmissions of H5N1 influenza virus have been reported, which all involved lengthy, close, and unprotected contact with infected patients.16–22 Reports of clustering of human H5N1 virus infections within families, usually without crossing blood lines, might suggest the presence of genetic factors which predispose to H5N1 virus infection or severe disease.17–28

Clinical features

In most cases of human H5N1 influenza virus infection, the first symptoms develop 2–4 days after the last exposure to sick poultry, but periods of up to 8 days have been reported (figure 1).4,5,7,26,29 Whether and to what extent virus shedding occurs during the incubation time is unknown. Most patients with H5N1 influenza present with an influenza syndrome that is characterised by symptoms of fever, cough and shortness of breath, and radiological evidence of pneumonia.17,26,28,29 Abnormalities on chest radiographs include extensive, often bilateral infiltration, lobar collapse, focal consolidation, and air bronchograms (figure 2). At presentation, the pneumonia usually seems to be of primary viral origin without evidence of bacterial superinfection. Beside respiratory symptoms, many patients infected with H5N1 virus complain of
patients,\textsuperscript{9,15,16,19} suggest the potential of H5N1 viruses to replicate virus in extrapulmonary tissues of some infections.\textsuperscript{33–35} No additional H5N1 virus infections were associated with seasonal human influenza A and B virus similar to the occasional reports of CNS manifestations in felids,\textsuperscript{11,30–32} this human case could represent a rare clinical manifestation of H5N1 influenza virus infection that is associated with prolonged shedding at high nasopharyngeal titres, and high concentrations of proinflammatory cytokines and chemokines. \textsuperscript{1} Neutralising antibody responses are detectable 14 or more days after the onset of symptoms.\textsuperscript{1} The cytotoxic CD8+ T-cell response peaks at 6–9 days but could potentially be extended because of prolonged exposure to viral antigens.

Gastrointestinal symptoms such as diarrhoea, vomiting, and abdominal pain.

CNS involvement has been suggested by one reported patient who developed coma and from whom H5N1 virus was isolated from specimens of blood and cerebrospinal fluid.\textsuperscript{9} Although neurotropism of H5N1 influenza viruses has been noted in mammals such as mice, ferrets, and ferrets,\textsuperscript{11,30–32} this human case could represent a rare clinical manifestation of H5N1 influenza virus infection that is similar to the occasional reports of CNS manifestations associated with seasonal human influenza A and B virus infections.\textsuperscript{11–15} No additional H5N1 virus infections were identified in 200 Vietnamese children with clinical encephalitis admitted to a paediatric referral hospital in Ho Chi Minh City during the year 2004, when the H5N1 outbreaks were occurring in Vietnam (de Jong MD, unpublished).

In severe cases, the clinical course of H5N1 influenza virus infection is characterised by rapidly progressive bilateral pneumonia, requiring ventilatory support within days of onset.\textsuperscript{5,7,24,26,29} Complications include acute respiratory distress syndrome, renal dysfunction, and multiorgan failure. On the basis of reported cases, the mortality of human influenza H5N1 exceeds 60%, with most patients dying of progressive respiratory failure. Whether and to what extent disseminated virus infection occurs and contributes to disease pathogenesis in people is uncertain. The presence of viral RNA or replicating virus in the blood of many patients, especially from those who died from infection,\textsuperscript{5,7,24,29} and the presence of replicating virus in extrapulmonary tissues of some patients\textsuperscript{5,7,24,29} suggest the potential of H5N1 viruses to disseminate to other organs, similar to what occurs in animals. A few postmortem examinations suggest that actual replication in human non-respiratory tissues, such as liver, lymph nodes, brain and placenta, takes place.\textsuperscript{5,7,24,29} In addition to the direct damage caused by viral replication, an intense inflammatory reaction in response to the high amounts of virus, possibly enhanced by H5N1 virus-induced cytokine dysregulation,\textsuperscript{38–40} probably also plays an important part in disease pathogenesis (figure 1).\textsuperscript{11}

The occurrence of mildly symptomatic and asymptomatic infections in individuals exposed to patients or poultry infected with H5N1 virus has been suggested by seroepidemiological studies after the 1997 outbreak in Hong Kong.\textsuperscript{20,21,41} Serological studies during the H5N1 outbreaks in Vietnam showed no evidence of infection in exposed health-care workers.\textsuperscript{42,43} whereas sera studies in a village affected with H5N1 in Cambodia showed no infections despite frequent direct contact with poultry suspected of having H5N1 virus infection.\textsuperscript{44} These studies suggest inefficient transmission of H5N1 viruses to human beings, but additional studies in individuals exposed to infected birds are essential.

Characteristic laboratory results in patients infected with H5N1 influenza virus, especially those with severe virus, are an early onset of lymphopenia, with an inverted ratio of CD4+ T cells to CD8+ T cells. The depletion of lymphocytes might be secondary to virus-induced apoptosis as suggested by in-vitro and murine experiments with H5N1 influenza viruses.\textsuperscript{45,46} Thrombocytopenia and increased serum concentrations of hepatic transaminases are also often recorded.\textsuperscript{17,26,29} Increased plasma creatinine concentrations have been noted in some patients.

**Diagnosis**

In regions where influenza A viruses of avian (eg, H5N1) and human (ie, H3N2 and H1N1) subtypes circulate together, the availability of rapid diagnostic assays which distinguish influenza subtypes is essential. Isolation of H5N1 influenza viruses is time consuming and requires laboratory facilities of biosafety level 3 and therefore is not feasible for routine diagnostics. Furthermore, culturing influenza viruses without previous knowledge of the subtype of the viruses risks isolating viruses of human or

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![Figure 1: Course of human infection with highly pathogenic H5N1 influenza virus](image1)

URT=upper respiratory tract. LRT=lower respiratory tract. Presumed course of nasopharyngeal virus load (blue), cytokine response (red), antibody titre (black), and cytotoxic T-cell response (green) during infection with H5N1 influenza virus. H5N1 infection predominantly causes pneumonia, which is usually complicated by acute respiratory distress syndrome. Infection is associated with prolonged shedding at high nasopharyngeal titres, and high concentrations of proinflammatory cytokines and chemokines. \textsuperscript{1} Neutralising antibody responses are detectable 14 or more days after the onset of symptoms.\textsuperscript{1} The cytotoxic CD8+ T-cell response peaks at 6–9 days but could potentially be extended because of prolonged exposure to viral antigens.

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![Figure 2: H5N1 influenza pneumonia](image2)

Chest radiographs of a 24-year-old man with H5N1 influenza virus infection showing rapid progression from left-sided pneumonia at admission (A) to bilateral pneumonia 4 days later (B).
avian origin in the same laboratory, which should be prevented to keep the risk of laboratory contamination of the isolates to a minimum.

Since H5N1-specific antibodies might not be detected until 14 days or longer after the onset of symptoms (figure 1), serological assays need paired acute and convalescent sera and are therefore not useful for rapid diagnostics. However, they remain important for diagnostic confirmation and epidemiological studies. For reasons of sensitivity, neutralisation assays are the method of choice for detection of antibodies against H5N1 viruses in people, but these also require biosafety level 3 facilities. Haemagglutination-inhibition assays using red-blood cells from horses have shown promising sensitivity for detection of H5N1 antibodies in people and have the advantage of not necessitating biosafety level 3 containment. Rapid antigen detection tests are widely used for diagnosis of seasonal human influenza but do not seem useful for diagnosis of H5N1 influenza viruses because of low sensitivity and the inability of validated tests to distinguish between influenza subtypes. With nasopharyngeal or throat swabs—the most commonly used diagnostic specimens in countries affected by H5N1 influenza virus—reported sensitivity of rapid antigen testing in patients ranged from 0% to 36%.

Reverse transcriptase-PCR (RT-PCR) assays are the methods of choice for rapid subtype-specific diagnostics. Several commercially available RT-PCR methods have been formulated, and an H5-specific assay developed by the US Centers for Disease Control has been approved by the US Food and Drug Administration for diagnostic use in human beings. However, the presence of several distinct genetic sublineages of H5N1 viruses and the changing nature of the H5 gene pose challenges to the design of RT-PCR assays that are reliable. Genetic sequence information of the most recent human and especially avian H5N1 virus isolates is essential to design new assays and update existing ones. Improving accessibility of databases within the WHO influenza networks that are restricted and in which such information is mostly stored, would help with and improve the establishment and maintenance of reliable diagnostics in many laboratories in countries affected by H5N1 influenza virus. Viral RNA has been detected in respiratory specimens, rectal swabs, cerebrospinal fluid, and blood of patients infected with H5N1 virus. In one study, viral RNA was present in the blood from nine of 11 patients who died and from none of five who survived, which could suggest that the detection of virus in the peripheral blood is prognostic of a poor clinical outcome. Although pharyngeal swabs, rather than nasal swabs, seem to represent the specimens of choice for diagnosis of H5N1 influenza virus infection, specimens of the lower respiratory tract such as tracheal aspirates or bronchoalveolar washes probably provide the highest diagnostic yield in patients with pneumonia.

Therapy
The two available classes of drugs with antiviral activity against influenza viruses are the adamantanes (amantadine, rimantadine), which act by inhibiting the M2 ion channel, and the neuraminidase inhibitors (oseltamivir, zanamivir, peramivir). Primary resistance against amantadine in the sublineage of H5N1 viruses prevalent in Vietnam, Cambodia, and Thailand has prevented the use of this drug in these countries, but adamantane-sensitive viruses, belonging to different distinct sublineages, have been isolated from birds and human beings in other regions of Asia and Europe. H5N1 viruses isolated from untreated patients are susceptible to oseltamivir and zanamivir in vitro.

WHO advises the use of oseltamivir for treatment of human H5N1 influenza virus infections, and either oseltamivir phosphate or zanamivir for prophylaxis. Although treatment with oseltamivir phosphate has been given to several patients with H5N1 influenza virus in recent years, no definitive conclusions about its efficacy can be made. However, clinical experience does not suggest a substantial decrease in mortality by antiviral treatment with oseltamivir phosphate; when combining reported cases and case series, the survival rate in treated patients was 30% (13 of 34) compared with 25% (four of 16) in untreated patients. The apparent little efficacy could be partly explained by late initiation of treatment in many patients and the possibilities of irreversible pulmonary damage or an irrevocable chain of events leading to pathological changes that are immune mediated at that time; survival rates in the above patients were 53% for those who were treated within 5 days after illness onset, compared with 26% in those treated after 6 days of illness or longer. Nevertheless, benefits might still be noted in patients affected with H5N1 virus when started late in the course of illness since replicating virus can still be detected at that time. Such benefits are suggested by small case series showing sharp decreases of pharyngeal viral loads to undetectable concentrations in surviving patients, but not in those who died, despite late start of treatment. Although optimum treatment efficacy might be expected when treatment is started early in the course of illness, these observations suggest that antiviral treatment should not be withheld when patients are presenting late.

Besides the timing of treatment, the possibility of inadequate drug concentrations might also affect therapeutic efficacy. The recommended dose of oseltamivir phosphate, which is based on clinical studies in patients with uncomplicated human influenza, could be insufficient because of reduced drug absorption in severely ill patients with H5N1 influenza virus who often have symptoms of diarrhoea. Additionally, studies in animals suggest that increased doses or extended administration of oseltamivir phosphate are needed to protect mice and ferrets against fatal disease caused by recent H5N1 strains that are highly virulent. Finally, the emergence of drug-resistant viral variants might result in therapeutic failure. A minor
Influenza can be high. In at least one of these patients the clinical course suggested that resistance development contributed to disease progression and death. Recently, oseltamivir-resistant H5N1 viruses were also isolated from two patients with fatal H5N1 virus infections in Egypt.

Efforts to diagnose influenza H5N1 early in the course of infection, and treat by effective antiviral strategies which keep to a minimum the risk of resistance development, such as combinations of antiviral drugs that have non-overlapping resistance patterns, deserve attention. Such combinations can include neuraminidase inhibitors and amantadine in case of adamantane-susceptible H5N1 viruses. Parenteral formulations of neuraminidase inhibitors are needed to ensure adequate drug concentrations, and these formulations are in clinical development for zanamivir and the novel neuraminidase inhibitor peramivir. Furthermore, agents with targets other than neuraminidase, such as the viral polymerase, could be a valuable antiviral treatment. In view of the probable role of inflammatory responses in H5N1 disease pathogenesis, treatment with immunomodulating agents, such as corticosteroids or statins, could have theoretical benefits. However, a safe and rational immunomodulatory intervention will need improved insight in the inflammatory processes during H5N1 disease and the exact role of the virus herein. In reported case series, 16 of 19 patients infected with H5N1 who received treatment with corticosteroids did not survive the infection, suggesting no substantial benefits of adjunctive steroid treatment.

Host restriction and pathogenesis

Influenza A viruses possess eight negative-sense single-stranded RNA segments as their genome, encoding 10–11 proteins. However, with respect to host range restriction and pathogenicity, direct experimental evidence for these properties exists for only four proteins—haemagglutinin, PB2 (polymerase basic 2), NS1 (non-structural 1), and neuraminidase. We describe how these four proteins are associated with these two properties.

Haemagglutinin protein

Haemagglutinin protein is responsible for virus attachment and the subsequent fusion of the viral and cellular membranes, and it has a major role in establishing the pathogenicity of avian influenza viruses. Haemagglutinin is synthesised as a single polypeptide (HA0), which is then cleaved into HA1 and HA2 by cellular proteases. This cleavage is essential for viral infectivity because it exposes the hydrophobic amino terminus of HA2 (ie, fusion peptide), which mediates fusion between the viral envelope and the endosomal membrane.

Haemagglutinin proteins of highly pathogenic H5 and H7 viruses contain several basic amino acids at the cleavage site, which are recognised by ubiquitous proteases, furin, and proprotein convertase 6, leading to systemic viral infections. By contrast, haemagglutinin proteins of avirulent avian viruses contain a single arginine at this site and are cleaved in only respiratory and intestinal organs. These viruses, therefore, cause only localised infection that is usually asymptomatic or mild. The tissue tropism of the viruses is thus partly identified by the availability of host proteases that recognise and cleave the two types of amino-acid sequences found at the haemagglutinin cleavage site.

Receptor specificity of haemagglutinin

The receptor specificity of haemagglutinin is responsible for the host-range restriction of influenza virus. Human influenza viruses preferentially bind to sialic acid linked to galactose by α2,6 linkages (SAα2,6Gal), whereas most avian viruses preferentially bind to SAα2,3Gal. Epithelial cells in human trachea contain SAα2,6Gal molecules on their surface, but they do not contain SAα2,3Gal molecules (figure 3). However, studies of human respiratory tissues...
have shown that although SAα2,6Gal oligosaccharides are dominant on epithelial cells of the nasal mucosa, paranasal sinuses, pharynx, trachea, and bronchi, SAα2,3Gal oligosaccharides are located on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus, and on cells lining the alveolar wall (figure 3). Attachment and infection data for viruses of known receptor specificity are consistent with this distribution of SAα2,3Gal and SAα2,6Gal oligosaccharides. Receptor specificity is established by the nature of the amino acids that form the receptor binding pocket of haemagglutinin, with glutamine or leucine at position 226 and glycine or serine at position 228 conferring specificity for SAα2,3Gal or SAα2,6Gal, respectively, in H2 and H3 viruses. For an H5N1 virus, introduction of the human-type residues at position 226 and 228 of the haemagglutinin conferred the ability to recognise SAα2,6Gal oligosaccharides in addition to SAα2,3Gal oligosaccharides. Collectively, these findings offer an explanation for the ability of H5N1 avian viruses to transmit to human beings and cause severe pulmonary disease (figure 3).

PB2 protein
PB2 protein, a component of the viral RNA replication complex, recognises and binds to type I cap structures of cellular mRNAs. Evidence suggests that the PB2 segment is involved in host range restriction of pathogenic avian influenza viruses in cell lines. The replication in mammalian but not avian cells of a human virus containing an avian virus PB2 gene was shown to be dependent on lysine (identified in human viruses), but not glutamic acid (found in avian species), at position 627 of PB2. The significance of this finding in the context of interspecies transmission was recognised when reverse genetics showed that a Glu-to-Lys mutation at this position converts H5N1 viruses that are non-pathogenic for mice to ones that are pathogenic. This mutation is also associated with the severity of disease in human beings. When avian viruses are transmitted to mammals, those with Glu-to-Lys mutations appear to be selected. Such selection leads to efficient viral replication and potentially lethal outcomes in mammals, including human beings, since the replicating virus overwhelms the host immune response. Recent H5N1 isolates from avian species, including those isolated during the Qinhai Lake outbreak in 2005, already possess Lys at position 627 in PB2, suggesting that these H5N1 viruses are possibly one step closer to becoming adapted for transmission to mammals.

NS1 protein and cytokine dysregulation
NS1 functions as an interferon-antagonist, targeting both interferon-β production and the activation of interferon-induced antiviral genes, thus allowing efficient virus replication in interferon-competent hosts. NS1 protein of the H5N1 viruses isolated in Hong Kong in 1997, confers resistance to the antiviral effects of interferon while inducing high concentrations of proinflammatory cytokines, such as tumour necrosis factor α (TNFα). Recombinant viruses containing the 1997 H5N1 NS1 gene were most pathogenic in pigs and enhanced the transcription of TNFα and interferon β in primary monocyte-derived macrophages in human beings. Moreover, the NS1 gene of the 1918 Spanish influenza virus, interfered with the expression of interferon-regulated genes more efficiently than did its counterpart in a control A/PR/8/34 (H1N1) virus. The activation of cytokines and chemokines is regulated through cellular signalling pathways, such as the mitogen-activated protein kinase (MAPK) pathway. H5N1, but not H1N1, viruses strongly activate p38 MAPK. Collectively, these findings suggest that NS1 of highly pathogenic viruses might cause the cytokine imbalance that was noted in victims of H5N1 infection in Hong Kong in 1997 and in more recent outbreaks in Hong Kong (figure 1). A primary role for cytokine dysregulation in the multiorgan manifestations of human influenza H5N1 infection is supported by findings from a few postmortem examinations showing an absence of viral replication in organs other than lungs and intestines. Further evidence has been provided by pathology examinations showing reactive haemopagocytosis, which is believed to be a cytokine-driven disorder, as the most prominent feature. Although cytokine dysregulation is at least partly responsible for the severe outcome of H5N1 virus infections in human beings, the exact mechanism by which these viruses cause such dysregulation remains unknown.

Neuraminidase protein
The sialidase activity of neuraminidase removes sialic acid from sialyloligosaccharides of haemagglutinin, neuraminidase, and the cell surface, easing virus release and the removal of sialic acid from the mucin layer and allowing the virus to reach the surface of the epithelial cells. Neuraminidase protein of A/WSN/33 (H1N1) influenza virus has been associated with pathogenicity since it is crucial for neurovirulence. The absence of a carbohydrate chain at position 146 of neuraminidase (N2 numbering) and the presence lysine at the C-terminus allow the neuraminidase protein to bind to and sequester plasminogen, which is a plasmin precursor. This function helps haemagglutinin cleavage and, thereby, virus pathogenicity in mice. This neuraminidase modification has been identified only in the A/WSN/33 (H1N1) strain so far. Neuraminidase is also associated with adaptation of waterfowl H5N1 viruses to poultry that are land based. Almost all neuraminidases isolated from land-based poultry possess a deletion in the neuraminidase stalk which is known to reduce its functionality. The haemagglutinins of viruses from land-based poultry have reduced affinity for receptors. This mechanism of reduction of neuraminidase function by a stalk deletion is thought to balance the reduced affinity of haemagglutinin towards the receptor.
**Vaccine development**

Before we discuss specific vaccine approaches, the role of the different aspects of the immune response that could be important in vaccine-induced control of H5N1 infection is worth addressing. In view of the rapid viral replication and onset of disease of H1, H2, and H3 epidemic influenza virus infections, the tenet in influenza immunology has been that neutralising antibodies are the only component of immunity that contribute to protection. Moreover, within humoral immunity, antibody responses in mucosal tissues are believed to be the most effective since the respiratory tract is the portal of entry. However, several studies in animals suggest that cell-mediated immunity might be important in protection from influenza virus infection, including lethal strains such as those of the H5N1 and H7N7 subtypes, and preventative vaccines that generate cell-mediated protection deserve consideration.

The onset of disease caused by primary H5N1 influenza virus infection seems to be delayed, providing time for cell-mediated responses to develop (figure 1). In a pre-exposed or immunised individual, secondary influenza-specific cytotoxic T-cell responses arise roughly 2 days faster than does the primary response, with a greatly increased amount of activity. A further important consideration is that cell-mediated immunity can be raised against conserved, internal viral proteins such as the matrix or nucleoprotein that can provide more universal protection against divergent influenza virus strains than can neutralising antibodies. In the absence of an influenza H5N1 clade-specific humoral response, one can hypothesise that a strong cross-reactive T-cell response induced through vaccination might change the course of disease by lowering viral burden and reducing disease severity. Although no studies have been done so far to specifically document the role of cellular immunity in protection against H5N1 infection in human beings, studies in animals accord with the notion that generation of robust and sustained memory T-cell responses that can respond early during H5N1 infection could be key to the success of vaccines designed to stimulate heterosubtypic protection. Another strategy to induce heterosubtypic protection might be to include in the vaccine preparation the conserved ectodomain of M2 protein derived from H5N1 virus, since this strategy elicits potent neutralising antibodies that may cross-react with different subtypes.

A further consideration in vaccine development is the use of veterinary vaccines that target populations of both poultry and wild birds. Immunisation of birds has obvious benefits through reduction of the spread of disease and keeping the economic effect of an outbreak to a minimum. Furthermore, in view of the present restrictions in manufacturing capacity for influenza vaccines, veterinary manufacturing facilities might serve to supplement production of human vaccines. Alternatively, increased standards of quality could be implemented for veterinary vaccines that might be redirected to human populations in the event of pandemic outbreaks. Veterinary vaccines need to be economically viable because of the fairly low cost of individual chickens. Widespread vaccination of poultry and wildfowl raise some additional concerns, since difficulties in distinguishing vaccinated from naturally-immunised animals could complicate epidemiological investigations of pandemic outbreaks. Additionally, veterinary vaccines could provide immunological pressure on the circulating strains that might lead to the emergence of drifted or shifted variants with raised potential for pathogenicity in human beings.

**Candidate vaccines**

Many H5N1 influenza vaccines have been experimentally tested so far (table). We have categorised these vaccines as either protein-based or gene-based, and they differ in their ability to induce humoral, cellular, or both types of immune responses. We will discuss only platforms that have the greatest potential as pandemic influenza virus vaccines.

**Protein-based vaccines**

The common feature of vaccine platforms in this category is the presentation of so-called preformed proteins to the immune system that preferentially stimulate humoral immune responses and neutralising antibodies.

**Inactivated influenza virus vaccines**

This vaccine platform accounts for 98% of the licensed products for epidemic influenza vaccination used in human beings. There are three types of inactivated influenza vaccines: whole-virus, split-product or subvirion, and surface-antigen. Whole-virus vaccines are associated with adverse reactions, especially in children. Nearly all inactivated epidemic influenza vaccines are split-product vaccines, which are produced from highly purified influenza virus, or surface-antigen vaccines containing purified neuraminidase and haemagglutinin. Inactivated epidemic influenza vaccines generally are reassortant viruses containing two influenza A subtypes and one influenza B subtype, and are propagated in embryonated chicken eggs. Unadjuvanted subvirion H5N1 influenza vaccine has been tested in 451 healthy adults with two intramuscular doses of 90, 45, 15, or 7.5 µg haemagglutinin. Even at the highest doses only 54% of participants seroconverted by microneutralisation assay, suggesting that improvements are needed, such as formulation with adjuvants. This H5N1 influenza vaccine product has recently received approval from the US Food and Drug Administration (FDA). Alum and MF59 have been tested in human clinical studies for H5N1 influenza vaccines. Adjuvanted H5N1 whole virus and subvirion vaccine are more immunogenic than are unadjuvanted vaccines. Both preclinical and clinical testing have shown that whole virus vaccines are more potent than are subvirion vaccines. A low pathogenicity H5N3 subvirion influenza vaccine has been shown to be highly effective in preventing and controlling H5N3 poultry influenza in a number of Asian countries.

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immunogenic when formulated with adjuvant MF59 in human clinical studies,127,131 and it has recently received the approval of the European Medicines Agency (EMEA).

A substantial limitation of the production of the inactivated influenza virus vaccines is the need for infection of fertilised chicken eggs for virus production. This drawback was emphasised in 1997–98 during production of vaccine against A/Hong Kong/97 (H5N1), since the virus killed chicken eggs.24 Lethality has been overcome with use of reverse genetics to generate H5N1 influenza vaccine strains with reduced pathogenicity, because the resulting viruses can readily be propagated in embryonated chicken eggs.25 Another weakness of this technology (which is still the one of choice for an H5N1 influenza vaccine) is the poor induction of cellular immune responses that might be beneficial to a pandemic influenza vaccine, as discussed above.

**Baculovirus-based vaccines**

The influenza haemagglutinin protein can be produced in insect cells by recombinant baculovirus and used as a vaccine either alone or in combination with an adjuvant. Recombinant haemagglutinin vaccines that are baculovirus produced protect chickens against lethal virus challenge.112 When tested in human beings, a recombinant baculovirus-expressed H5 vaccine was less efficacious than it was in chickens, but still comparable with a subvirion H5N1 inactivated vaccine that is egg grown.113 Potential disadvantages of this technology include the possible alteration of haemagglutinin structure because of different glycosylation patterns in insect cells that can impair immunogenicity. However, similar antibody responses to those induced by approved vaccines have been noted in people.114–116 The main advantage of baculovirus-based H5 influenza vaccine over inactivated products is the capacity for large scale production that this technology offers, and efforts aimed at increasing immunogenicity should be pursued.

**Influenza virus-like particles**

Virus-like particles are being tested as vaccines against several different virus infections and have the advantage of generating robust mucosal immune responses when applied to a mucosal surface, especially when combined with a mucosal adjuvant.117 Recombinant expression of four influenza virus structural proteins (haemagglutinin, neuraminidase, M1, and M2) is sufficient for assembly and budding of influenza virus-like particles from the surface of Sf9 insect cells, with matrix protein M1 playing a central part in virus assembly and release.118 By contrast, neither haemagglutinin, neuraminidase, nor nucleocapsid expression leads to formation or virus-like particles or release, or both.118 An H9N2 influenza vaccine for virus-like particles consisted of only three influenza virus structural proteins (haemagglutinin, neuraminidase, and M1), was shown to elicit protective immune responses in BALB/c mice.119 A vaccine of virus-like particles from both clade 1 and clade 2 H5N1 isolates with pandemic potential was generated and tested in mice in either a one-dose or two-dose regimen. Mice vaccinated with virus-like particles were protected against challenge irrespective of whether the H5N1 challenge was homologous or heterologous to the vaccine. Conversely, animals immunised with recombinant HA protein had statistically significant weight loss and death after challenge with the heterologous H5N1 virus.120 Vaccines that are based on virus-like particles are fairly easy to produce in large quantities and hold promise in generation of broad, cross-protective immune responses to avian influenza viruses with pandemic potential.

**Genetic vaccines**

In the context of this Seminar, genetic vaccines refer to products that actively encode viral antigens from DNA or RNA at the time of administration. The shared trait of the genetic vaccines is that viral proteins are produced in vivo in host cells. Depending on the antigen selection, such protein synthesis will induce cellular or humoral immunity, or both. The following vaccines represent the best characterised genetic platforms under investigation for highly pathogenic avian influenza.

**Live attenuated influenza virus vaccines**

Cold-adapted epidemic influenza vaccines that are live attenuated elicit systemic and mucosal humoral and cellular immune responses and show protective efficacy.121 These vaccines are delivered intranasally and are licensed as FluMist (MedImmune Vaccines, Inc, Mountain View, CA, USA). Attenuated cold-adapted strains are generated by reassortment between a wild-type virus expressing

### Table: Candidate influenza virus vaccines

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<td>Flavivirus</td>
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target haemagglutinin and neuraminidase, and a cold-adapted master strain (A/Ann Arbor/6/60 [H2N2] or B/Ann Arbor/1/66, or both), which are obtained by repeated passage on chicken eggs under decreasing temperatures. The final cold-adapted reassortant replicates only in the upper respiratory tract in which temperatures do not exceed 32–33°C. Cold-adapted viruses have mutations in several of the viral genes that code for internal proteins of the virus particle. These live attenuated viruses are not transmissible to close seronegative contacts.141 Attenuated cold-adapted H5N1 influenza viruses have been generated and are safe in ferrets and protect chickens from wild-type H5N1 influenza virus challenge.142 Live attenuated influenza vaccines can stimulate robust secretory IgA responses in the upper respiratory tract143,144 that exhibit potential heterotypic reactivity to epidemic influenza virus strains.145,146 The ability of live attenuated vaccines to induce mucosal IgA and virus-specific T-cell responses suggest that these vaccines might provide broader protection against antigenic drift variants than can inactivated virus vaccines. However, because of the potential generation of a reassortant between a live vaccine virus containing the H5 haemagglutinin gene and a co-infecting human epidemic strain, this vaccine will probably not be used before a pandemic.

Adenovirus-based vaccines

Recombinant adenovirus serotype 5 vectors are highly immunogenic vectored vaccine platforms in comparative preclinical immunogenicity studies using several genetic inserts.147,148 Recombinant adenovirus serotype 5-based vaccines are highly effective at inducing both humoral and cellular immunity, and show promise in the prevention of human infectious disease in several studies of animals. Moreover, recombinant adenovirus serotype 5 vectors can be produced in large quantities and are therefore attractive choices for vaccine applications for human and veterinarian pandemic influenza. Studies have shown the efficacy and immunogenicity of adenovirus-vectored H3N2 HA influenza vaccines in pigs and mice, and have reported that cross-protection from heterotypic challenge can arise in the absence of neutralising humoral immunity.147 DNA-prime adenoviral-boosting with an H1N1 nucleocapsid epidemic influenza vaccine induces a T-cell response protective against heterosubtypic challenge in mice.148 Mice and chickens vaccinated with H5 haemagglutinin were fully protected from challenge with homologous and heterosubtypic H5N1 virus.149,150 Moreover, heterosubtypic H5N1 protection through vaccination with adenovirus-based vaccine to haemagglutinin 2, which fails to induce neutralising antibody responses,150 further accords with the role for T-cell recall responses in protection against different influenza subtypes, at least in these animal studies. Natural vector-specific immunity of some human populations against adenovirus 5151 could potentially reduce vaccine efficacy in the event that worldwide vaccination is implemented. In a recent report, however, adenovirus 5-based vaccines against laboratory influenza strain A/PR/8 (H1N1) were tested in human beings and were effective in inducing anti-influenza neutralising antibodies despite the presence of pre-existing adenovirus 5-specific antibodies, suggesting that vector-specific immunity can be overcome.152 However, the impairment due to neutralising vector antibodies on the efficacy of several immunisations needs to be established, and adenovirus-based vaccines can preferentially be used only to control a potential pandemic event.

Paramyxoviruses

Newcastle disease virus causes an economically important disease of poultry, and infection is controlled by routine vaccination in many parts of the world with live lentogenic vaccine strains added to drinking water or administered by spraying. A recombinant vaccine for Newcastle disease virus based on a commercially available lentogenic live vaccine strain was recently engineered to express H5 or H7 HA gene from high pathogenic strain of avian influenza.153,155 90–100% protection against both avian influenza and Newcastle disease was observed in chicken immunised with these vaccines. Since most chickens worldwide are vaccinated with a live vaccine for Newcastle disease virus, an H5N1 influenza vaccine based on recombinant Newcastle disease virus that has dual specificity and allow a single immunisation against both avian influenza and this virus is ideal. An influenza vaccine based on Newcastle disease virus can potentially also be developed against infection in human beings in view of the efficacy noted in non-human primates after intranasal immunisation.154

Plasmid DNA-based vaccines

DNA vaccines offer a simple, yet effective means of inducing broad-based immunity. Because antigens are expressed in situ after DNA vaccination, both humoral and cell-mediated immune responses, including cytotoxic T cells, are induced.151 What distinguishes DNA vaccines from other expression vaccines is their physical nature, since DNA vaccines consist simply of plasmid DNA and encode only the proteins of interest without additional viral or bacterial proteins. The absence of a protein component ensures that an immune response against the vector itself will not be induced. Several studies have shown that plasmid DNA vaccine encoding HA of the influenza virus elicited specific immune responses and provided protection against the influenza virus in mice, ferrets, and chickens when given via bioballistic gene gun, liposome, or intramuscular injection.120,121 Furthermore, when DNA vaccines against nucleocapsid and M1 are given to mice along with haemagglutinin, the plasmid DNA vaccines improve efficacy against drifted strains of virus compared with inactivated viruses or subvirion vaccines.122 Plasmid DNA influenza vaccines expressing influenza antigens in combination with DNA encoding various cytokines have also been tested in

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animals. A phase I clinical trial was undertaken to assess an influenza DNA vaccine containing the H3 gene from the epidemic A/Panama/2007/99 influenza strain given by gene gun. The influenza DNA vaccine elicited serum haemagglutination-inhibition antibody responses with a seroconversion rate of 33% at day 21 when 4 μg of DNA was used. Although the potency shown with plasmid DNA influenza vaccine is so far not satisfactory, in view of the great potential of this technology in terms of speed of vaccine generation, ease of manufacturing, large production capacity, and low cost, efforts into this vaccine platform are essential.

Conclusions
Although the capacity for circulating H5N1 influenza viruses to be transmitted from birds to man is inefficient, the clinical results of human infection are usually severe—e.g., pneumonia, frequent multiorgan disease, and high mortality. Several key molecular determinants of human infection with H5N1 influenza viruses have been identified, and the likely minimum requirements for human-to-human transmission are being defined. If H5N1 influenza viruses acquire the capacity for effective human-to-human transmission while retaining their characteristically high pathogenicity, the ensuing pandemic will be devastating. Therapeutic approaches for control of disease can be restricted, leaving widespread vaccination as the probable cornerstone of public-health measures for pandemic control. Continued research into influenza pathogenesis and development of broadly-protective vaccines that can be rapidly produced is needed in anticipation of an H5N1 influenza virus pandemic.

Conflict of interest statement
AG and SBB are listed as co-inventors on the following patent application: 11/288,102, which has been licensed to PazVax. MJD has no conflict of interest. GN is named as co-inventor on several patents regarding influenza virus reverse genetics and/or the development of influenza virus vaccines or antivirals. GN is also a co-founder of FluGen, Inc. (Madison, WI, USA). YK named as inventor/co-inventor on several patents regarding influenza virus reverse genetics and/or the development of influenza virus vaccines or antivirals. GN and YK did not hold shares in the company. It is the company’s intent that AG and SBB are listed as co-inventors on the following patent application: 11/288,102. YK is named as inventor/co-inventor on several patents regarding influenza virus reverse genetics and/or the development of influenza virus vaccines or antivirals. GN is named as co-inventor on several patents regarding influenza virus reverse genetics and/or the development of influenza virus vaccines or antivirals. AG and SBB are listed as co-inventors on the following patent application: 11/288,102.

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