Scientific barriers to developing vaccines against avian influenza viruses

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Abstract | The increasing number of reports of direct transmission of avian influenza viruses to humans underscores the need for control strategies to prevent an influenza pandemic. Vaccination is the key strategy to prevent severe illness and death from pandemic influenza. Despite long-term experience with vaccines against human influenza viruses, researchers face several additional challenges in developing human vaccines against avian influenza viruses. In this Review, we discuss the features of avian influenza viruses, the gaps in our understanding of infections caused by these viruses in humans and of the immune response to them that distinguishes them from human influenza viruses, and the current status of vaccine development.

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Emerging and re-emerging infectious diseases in humans and animals have been reported with increased frequency in recent years[1]. The growing demands on land use, intensified farming practices to feed a larger population and the increase in travel and transportation allow the emergence, re-emergence and rapid spread of infectious agents around the globe. The emergence of high-pathogenicity avian influenza (HPAI) viruses in domestic poultry and the increasing number of cases of direct transmission of avian influenza viruses of different subtypes to humans are a significant threat to public health because of the potential for pandemic spread of these viruses. The ongoing outbreak of HPAI H5N1 viruses in the bird population and the nearly 50% case-fatality rate among people who become infected with H5N1 viruses underscore the need for control strategies to prevent a potential influenza pandemic.

Research efforts to control emerging viral diseases are focused on improving surveillance and diagnostic methods, and on the development of antiviral drugs and effective vaccines. Vaccination is the cornerstone of prevention. Interest in the development of pandemic influenza vaccines intensified with the outbreak of H5N1 influenza virus infections of humans in Hong Kong in 1997 and has increased further as H5N1 viruses have spread in birds and humans since 2003. Despite extensive experience with vaccines against human influenza viruses, researchers face several additional obstacles in developing successful vaccines against avian influenza viruses with pandemic potential. In this Review, we discuss the challenges associated with generating and evaluating vaccines against avian influenza viruses and the current status of pandemic vaccine development.

Avian influenza viruses

Influenza viruses belong to the Influenza A genus of the family *Orthomyxoviridae*. Although the natural reservoir for all influenza A viruses is aquatic birds, these viruses also infect and cause disease of varying severity in domestic poultry and several species of mammal, including humans[2,3]. The genome of influenza A viruses consists of 8 single-stranded RNA segments that encode 11 proteins (FIG. 1a). Influenza A viruses can be divided into subtypes on the basis of genetic and antigenic differences in their main surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA)[3]. So far, 16 HA (H1–H16) and 9 NA (N1–N9) glycoprotein subtypes have been identified in influenza A viruses[3,4]. The HA and NA glycoproteins are the targets of the protective immune response and can vary as a result of antigenic drift and antigenic shift. The genes encoding the internal virus proteins are highly conserved between influenza A viruses. The matrix 2 (M2) protein is an integral membrane protein that functions as an ion channel that is important in the uncoating of the virus. Antibodies specific for the M2 protein cannot neutralize virus infectivity but are protective *in vivo*[5,6]. The cellular immune response is directed against the nucleoprotein (NP) and the RNA polymerase proteins PB2 (polymerase basic protein 2) and PA (polymerase acidic protein).

Aquatic birds do not usually show signs of disease when infected with avian influenza viruses[7], but infections of domestic poultry can be associated with disease. In addition to their division into subtypes on the basis of genetic and antigenic differences, avian influenza viruses can also be divided into two groups on the basis of their ability to cause severe disease in chickens — namely, HPAI viruses and low-pathogenicity avian influenza

a | Influenza A virus

b | Haemagglutinin

Figure 1 | **Schematic of an influenza A virus. a** | The influenza A virus particle has a lipid envelope that is derived from the host cell membrane. Three envelope proteins — haemagglutinin (HA), neuraminidase (NA) and an ion channel protein (matrix protein 2, M2) — are embedded in the lipid bilayer of the viral envelope. HA (rod shaped) and NA (mushroom shaped) are the main surface glycoproteins of influenza A viruses. The ratio of HA to NA molecules in the viral envelope usually ranges from 4:1 to 5:1. **b** | The HA glycoprotein is synthesized as an HA0 molecule that is post-translationally cleaved into HA1 and HA2 subunits; this cleavage is essential for virus infectivity. The HA glycoprotein is responsible for binding of the virus to sialic-acid residues on the host cell surface and for fusion of the viral envelope with the endosomal membrane during virus uncoating. The NA glycoprotein cleaves sialic-acid receptors from the cell membrane and thereby releases new virions from the cell surface. M2 functions as a pH-activated ion channel that enables acidification of the interior of the virion, leading to uncoating of the virion. Matrix protein 1 (M1), which is the most abundant protein in the virion, underlies the viral envelope and associates with the ribonucleoprotein (RNP) complex. Inside the M1 inner layer are eight single-stranded RNA molecules of negative sense that are encapsidated with nucleoprotein (NP) and associated with three RNA polymerase proteins — polymerase basic protein 1 (PB1), PB2 and polymerase acidic protein (PA) — to form the RNP complex. The PB1, PB2 and PA proteins are responsible for the transcription and replication of viral RNA. The virus also encodes a non-structural protein (NS) that is expressed in infected cells and a nuclear export protein (NEP). The location of NEP in the virion is not known.

(LPAI) viruses. HPAI viruses are restricted to the HA glycoprotein subtypes H5 and H7 and cause systemic infection, which can result in 100% mortality within a flock. Specific NA subtypes have not been associated with the pathogenicity of HPAI viruses. LPAI viruses, which include viruses of all HA subtypes, cause milder infection and do not usually cause mortality[7].

The molecular basis of the difference in pathogenicity between HPAI and LPAI viruses is mainly attributed to the cleavability of the precursor HA glycoprotein HA0 into HA1 and HA2 subunits. Cleavage of HA0 generates the carboxyl terminus of HA1 and the amino terminus of HA2, the latter being necessary for membrane fusion and for virus infectivity[8] (FIG. 1b). The HA0 glycoproteins of LPAI viruses and human influenza viruses are cleaved at a conserved arginine residue by trypsin-like proteases[9]. Therefore, infection with these viruses is restricted to tissues in which trypsin and trypsin-like proteases are present: the respiratory tract of humans and the intestinal tract of birds. By contrast, the HA0 glycoproteins of HPAI viruses are highly cleavable; they can have multiple basic amino acids at the cleavage site[9] that can be cleaved by ubiquitous intracellular proteases, such as furins[10], or by non-trypsin-like extracellular proteases. The HA0 glycoprotein can also have additional glycosylation sites[11] that are associated with virulence. Because the HA0 glycoproteins of HPAI viruses are easily cleaved in extrapulmonary sites, these viruses can replicate in extrapulmonary organs, including the brain, causing fatal disease and death of the infected birds[7]. However, the significance of the highly cleavable HA0 glycoprotein as a virulence determinant in human infections with HPAI viruses is not known. HPAI viruses are believed to emerge as a result of mutations that occur after LPAI viruses move into domestic poultry[12]. This has prompted the establishment of agricultural controls for LPAI virus H5 and H7 subtype infections in poultry in an effort to prevent the emergence of HPAI viruses[13,14].

HA glycoproteins bind to sialic-acid residues with terminal oligosaccharides on the host cell surface. An important difference between avian influenza viruses and human influenza viruses is their preference for specific sialic-acid linkages. Most avian influenza viruses preferentially bind to sialic acids that are linked to galactose with α-2,3 linkages, whereas human influenza viruses preferentially bind to sialic acids with α-2,6 linkages[15–17]. NA glycoproteins cleave sialic-acid residues from the surface of the infected cell to release progeny virions from that cell, thereby facilitating virus dissemination[3].
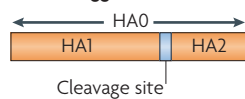
The transmission of avian influenza viruses to humans was thought to occur rarely because of the host-range restriction of the viruses. The observation that the 1957 H2N2 and 1968 H3N2 viruses that caused human pandemics were reassortant viruses, with gene segments derived from both avian and human influenza A viruses, indicated that the transmission of avian influenza viruses to humans might require reassortment between a human and avian influenza virus. Reassortment was proposed to occur in an intermediate host, such as the pig, which has both α-2,3-linked and α-2,6-linked

sialic-acid residues in the respiratory tract. However, the outbreak of human infections with H5N1 viruses in 1997 in Hong Kong showed that avian influenza viruses could be transmitted directly from domestic poultry to humans[18,19]. Since 1997, LPAI and HPAI viruses of several subtypes, including H9N2, H5N1, H7N7, H7N3 and H10N7, have been implicated in human infections by direct transmission from birds (TABLE 1). In each case, poultry in the region were infected with viruses that were genetically related to the viruses isolated from humans, which indicates that the human infections occurred as a result of direct transmission from birds to humans. Severe illness and death have not occurred following infection with LPAI viruses, whereas human infections with HPAI viruses have been more severe and fatal in many cases. However, human infections with H5 and H7 subtype viruses are not uniformly severe or fatal.

The goal of a pandemic influenza vaccine

The increasing number of reports of direct transmission of avian influenza viruses to humans in the past few years (reviewed in REFS 20–22) and the ongoing outbreak of H5N1 influenza virus infections in avian species and humans in several countries (reviewed in REFS 20,23,24) highlight the significant threat posed by HPAI and LPAI viruses to human health. Because it is not possible to predict which subtype of avian influenza virus will cause the next human pandemic, an ideal vaccine would elicit an immune response that protects the host from infection with a broad range of influenza viruses from the same or different subtypes. Lessons from the previous influenza pandemics guide our efforts to prepare for a future pandemic (BOX 1).

The HA and NA glycoproteins of influenza viruses (FIG. 1) undergo genetic and antigenic variation to escape the immune response[25,26]. The presence of neutralizing antibodies specific for the HA glycoprotein at systemic or mucosal sites of infection provides immediate protection against infection with influenza viruses, whereas the clearance of human influenza viruses depends mainly on cell-mediated immunity[27] (FIG. 2). Although antibodies specific for the NA glycoprotein do not neutralize infectivity, they restrict virus replication by preventing the release of new virus particles, a process that requires viral NA proteins. Therefore, antibodies specific for NA can decrease the severity of the disease[28,29]. Epitopes recognized by cytotoxic T lymphocytes (CTLs) are present on NP, PB2 and PA proteins of human influenza viruses. Therefore, if a virus with a new HA and/or NA glycoprotein emerges in the human population, cell-mediated immunity directed against the highly conserved internal proteins could have a role in protection at the time of a pandemic. The principle underlying the currently licensed vaccines against human influenza viruses is the induction of protective antibodies specific for the HA glycoprotein of the predicted epidemic strain. The concentration of HA glycoprotein in licensed, inactivated virus vaccines for seasonal influenza is standardized, but the concentration of NA glycoprotein is not standardized.

Table 1 | Laboratory-confirmed cases of human infection with avian influenza viruses

Virus subtype	Year; country	Number of cases (deaths)	Clinical illness	Characteristics of the virus isolated from human case(s)		Refs
				Genetically related avian influenza virus isolate(s)*	Sialic-acid linkage specificity‡	
H5N1	1997; Hong Kong	18 (6)	Fever, respiratory symptoms, pneumonia, ARDS, multi-organ dysfunction in fatal cases	HA: A/goose/Guangdong/1/96 (H5N1) NA: A/teal/Hong Kong/W312/97 (H6N1) Internal protein genes: A/chicken/Hong Kong/G1/97 (H9N2) or A/teal/Hong Kong/W312/97 (H6N1)	α-2,3	18,19, 114–117
	2003–present; Cambodia, China, Egypt, Indonesia, Iraq, Thailand, Turkey, Vietnam	256 (151)	Respiratory symptoms, ARDS, multi-organ dysfunction	ND	α-2,3	16,17, 118–121
H7N7	2003; Netherlands	89 (1)	Conjunctivitis, mild influenza-like illness, pneumonia followed by respiratory-distress syndrome in the fatal case	A/chicken/Netherlands/1/03 (H7N7)	ND	31,122
H7N3	2004; Canada	2	Conjunctivitis	A/chicken/Canada/AVFV1/04 (H7N3) A/chicken/Canada/AVFV2/04 (H7N3)	ND	32,123
H9N2	1999; Hong Kong	2	Mild, self-limiting febrile pharyngitis	A/quail/Hong Kong/G1/97 (H9N2)	α-2,6	116,124
	1999; China	5	Mild	A/chicken/Hong Kong/G9/97 (H9N2)	α-2,6	124,125
	2003; Hong Kong	1	Mild	A/chicken/Hong Kong/G9/97 (H9N2)	α-2,6	126
H10N7	2004; Egypt	2	Fever and cough	ND	ND	127

*Genetically related avian influenza viruses that were identified as the probable source of the indicated gene segment of the virus that caused human infection.
‡Denotes the binding preference of the viral HA glycoprotein for avian (α-2,3-linked) or human (α-2,6-linked) sialic-acid residues. ARDS, adult respiratory distress syndrome; HA, haemagglutinin; NA, neuraminidase; ND, not determined.

The 1918 Spanish influenza pandemic
Of the three influenza pandemics that occurred in the twentieth century, the Spanish influenza (H1N1 virus) pandemic of 1918–1919 was the most notable. More than 40 million people around the world died from influenza[101]. An important feature of this pandemic was the high mortality in the unusually young age group of 20–40-year olds[102]. Studies in a mouse model using viruses containing genes from a reconstructed 1918 virus generated by reverse genetics indicate that the haemagglutinin (HA) glycoprotein has an important role in the pathogenicity of this virus. The introduction of the HA glycoprotein of the 1918 virus into a non-pathogenic human influenza virus made it highly pathogenic in mice[103,104]. However, the fully reconstructed 1918 virus was even more virulent[104,105]. Structural analysis showed that the HA glycoprotein of the 1918 virus could bind to α-2,6-linked sialic-acid residues, despite the presence of amino-acid residues in the receptor-binding site that are characteristic of the HA glycoprotein of an avian influenza virus[106]. Further studies of host, as well as viral, factors that contributed to the virulence of the 1918 pandemic H1N1 virus might help in the development of strategies to combat future pandemics.

The 1957 and 1968 Asian influenza pandemics
The 1957 Asian influenza (H2N2 virus) pandemic and the 1968 Hong Kong influenza (H3N2 virus) pandemic were milder than the 1918 pandemic, but both still caused significant morbidity and mortality around the world. The 1957 pandemic was caused by a reassortant virus that was derived from the HA (H2), neuraminidase (N2) and PB1 (polymerase basic protein 1) genes from an avian influenza virus infecting ducks and the remaining gene segments from the previously circulating human H1N1 virus[107,108]. The H3N2 virus that caused the 1968 pandemic consisted of avian HA (H3) and PB1 genes in a background of other internal protein genes of the human H2N2 virus that was circulating at the time[107,108]. The presence of an avian HA H3 glycoprotein made the reassortant virus antigenically novel to humans and it spread in the susceptible human population causing a pandemic.

The role of cell-mediated immunity in the control of avian influenza virus infections is not known. A potential advantage of a vaccine that induces a cell-mediated immune response, over one that elicits a protective antibody response, is that the internal proteins of the virus that are targets of the cell-mediated immune response tend to be conserved and are less prone to genetic drift (see later) than are the antibody-binding sites on the HA and NA glycoproteins.

There are at least two scenarios in which a vaccine that elicits a cell-mediated immune response might be useful in the event of a pandemic. First, although a cell-mediated immune response might not prevent infection with the virus, it may prevent severe illness and death from influenza. Although this might not be an acceptable outcome for a seasonal influenza vaccine, it might be reasonable for a pandemic influenza vaccine. Second, if the incubation period and course of infection with avian influenza viruses are longer than those with human influenza viruses, as has been reported[23], vaccines that elicit protection through a cell-mediated immune response might be effective.

Diversity of avian viruses and vaccine design
Although most influenza vaccines are designed to induce HA-specific antibody responses to protect the host from infection, the biology of avian influenza viruses presents several unique challenges, compared with human influenza viruses. These challenges include the presence of different subtypes of HA and NA glycoproteins and the genetic and antigenic diversity within each subtype. The antigenic diversity has consequences for pandemic vaccines that must be considered in the design of a protective vaccine.

One of the first questions that must be considered is whether vaccines should be developed against all of the subtypes of HA and NA glycoproteins. The 16 HA and 9 NA subtypes of avian influenza viruses might not have similar pandemic potential (BOX 2). Although HPAI H5N1 viruses are the main focus of global attention, LPAI H9N2 viruses are also widespread in poultry in Asia[30] and HPAI H7 viruses have caused large outbreaks in poultry in Europe[31], North America[123] and South America[33]. Although HPAI viruses cause morbidity and mortality in poultry, HPAI viruses might not be intrinsically more likely to cause a human pandemic than LPAI viruses; interestingly, there are no known examples of a pandemic caused by an H5 or H7 HPAI virus, although virological data are limited to those from the three influenza pandemics that occurred in the last century.

Because of this uncertainty, it will be prudent to develop vaccines against each of the subtypes of avian influenza virus, although the order of development can be prioritized on the basis of epidemiological data. It is probable that avian influenza virus subtypes that are widely prevalent in nature and those that have been shown to infect humans will be of greater concern than subtypes that are not circulating widely in nature. A comparison of the predicted protein structures of HA glycoprotein subtypes 1 to 15 has led to the classification of these subtypes into four different clades — namely, clade 1 (H1, H2, H5, H6, H11 and H13), clade 2 (H8, H9 and H12), clade 3 (H3, H4 and H14) and clade 4 (H7, H10 and H15)[34]. The implications for vaccine development of the phylogenetic relationships between the HA glycoproteins of different subtypes are not yet known. Can HA glycoproteins that are grouped together in the same clade elicit crossreactive immunity against other subtypes in the same clade?
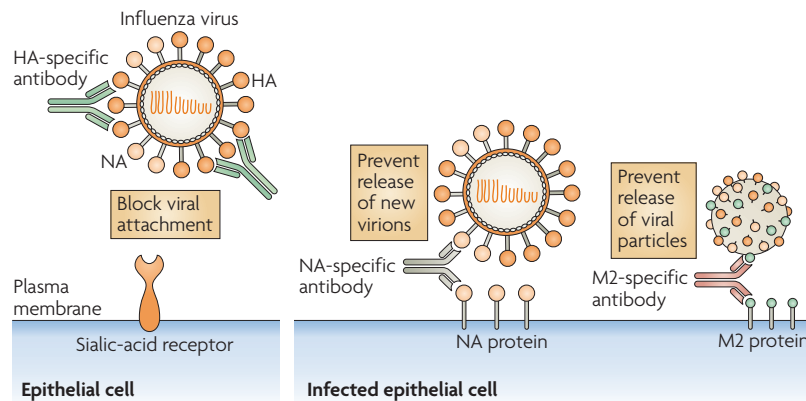
Phylogenetic analysis of the genes encoding certain subtypes of HA glycoprotein reveals a separation into lineages that correspond to the geographical separation of the birds that they infect. These genetic lineages are referred to as the Eurasian and North American lineages and they generally correspond to the flight paths of migratory birds[7,12,35]. Viruses from these two lineages might also be antigenically distinguishable, but the consequences of these genetic and antigenic differences for vaccine development are not known. Are the antigenic differences that have been identified between avian influenza viruses of significance for human infections? Do viruses of the two lineages have similar pandemic potential? If the genetic differences between viruses are the result of co-evolution of an avian influenza virus with its avian host, and if the antigenic differences are driven by evolution in an avian host rather than by positive selection by an immune response to infection, then a vaccine generated against a virus of the North American lineage might protect against a virus of the Eurasian lineage. Unfortunately, this information is not known yet.

Circulating human influenza viruses undergo rapid mutation owing to the low fidelity of the viral RNA-dependent RNA polymerase[36]. Antigenic drift occurs when the genes encoding the HA and/or NA glycoproteins undergo stepwise mutations, resulting in variant viruses with amino-acid changes at one or more antibody-binding sites of HA and/or NA[37] that allow the viruses to evade neutralization by antibodies generated as a result of previous natural infection or vaccination. The internal protein genes of avian influenza viruses are not under positive immune selection in waterfowl and shorebirds. However, the use of veterinary vaccines to protect poultry from infection with avian influenza viruses might drive evolution of the HA glycoprotein if such vaccines do not induce sterilizing immunity. This was particularly evident when a widespread vaccination programme was launched in 1995 in Mexico to control the outbreak of a LPAI H5N2 virus infection that started in 1993. Phylogenetic and serological analyses of viruses that were isolated a few years after the introduction of the vaccine indicated the presence of multiple sublineages of HA glycoproteins with marked antigenic differences from the HA glycoproteins used in the vaccine[38]. Several countries in Asia are using veterinary vaccines to control H5N1 virus infections in poultry. If avian influenza viruses undergo antigenic drift in poultry as a consequence of vaccine use, then the pandemic influenza vaccines that are stockpiled for human use might need to be updated. However, an effective animal vaccination programme would reduce the burden of disease in poultry substantially, thereby reducing the risk of a human pandemic. Also, changes in the virus that are driven by positive immune selection in poultry might not be significant in humans. It will be important to assess the potential advantages and disadvantages of animal vaccination and to determine the significance of antigenic drift in avian influenza viruses for humans.

The viral determinants of pathogenicity of avian influenza viruses in humans are multigenic. Further studies are required to understand how the pathogenicity of avian influenza viruses affects the infectivity and transmissibility of these viruses in humans and to establish whether these factors have implications for vaccine design.

Types of vaccine

Inactivated virus vaccines and live attenuated virus vaccines that are being developed for pandemic influenza are based on technologies that are licensed for the existing seasonal human influenza vaccines. Vaccines based on various other platforms, such as live virus vectors expressing influenza virus proteins and DNA vaccines, are also being developed and have shown promise in preclinical studies (TABLE 2).

The currently licensed vaccines against human influenza viruses are produced in embryonated chicken eggs and the manufacturing process can take 6–9 months. Therefore, for vaccines that are based on the currently licensed technologies, the availability of embryonated eggs is a crucial factor and if the pandemic virus causes widespread morbidity and mortality in poultry, the supply of embryonated eggs might be compromised. Therefore, alternative substrates, including mammalian cell lines such as Madin–Darby canine kidney (MDCK) cells and Vero cells, must be developed for the production of influenza viruses for use in vaccines.

a Antibody-mediated immunity
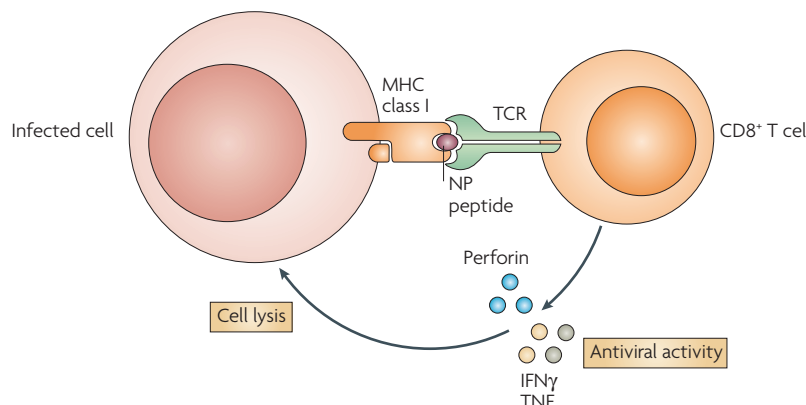
b Cell-mediated immunity

Figure 2 | **The adaptive immune response during infection with influenza virus.** Influenza viruses attach to the epithelial cell surface of host cells through binding of the viral haemagglutinin (HA) glycoprotein to cell-surface sialic-acid residues. The virion is internalized through endocytosis and fusion between host and viral membranes occurs in acidic vacuoles. Opening of the ion channel formed by matrix protein 2 (M2) triggers this fusion and the release of viral genes into the cytoplasm, through which they travel to the nucleus. Viral mRNAs are transported from the nucleus to the cytoplasm, where viral proteins are translated and progeny virions assemble and bud from the cell membrane. The release of progeny virions requires the action of the viral neuraminidase (NA) glycoprotein, which cleaves sialic-acid receptors from the host cell membrane. **a** | Antibodies specific for HA block virus attachment, thereby preventing infection of cells, or they can prevent fusion. Antibodies specific for NA bind virus to the cell, thereby preventing the release of virions. Antibodies specific for M2 bind virus to the cell and prevent the release of viral particles into the extracellular fluid. **b** | Cell-mediated immunity contributes to host resistance when CD8+ T cells specific for viral proteins such as nucleoprotein (NP) or the RNA polymerase proteins polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) recognize viral peptides presented by MHC class I molecules, resulting in the release of cytokines with antiviral activity — such as interferon-γ (IFNγ) and tumour-necrosis factor (TNF) — and perforins that mediate cytolysis of the infected cell. Lysis of the infected cell decreases the amount of virus released by the cell. The latter three mechanisms, NA-specific antibodies, M2-specific antibodies and CD8+ T cells, operate after a cell becomes infected. Only antibodies specific for HA can prevent infection; this is probably why they are the most effective mediators of immunity *in vivo*. TCR, T-cell receptor.

Positive immune selection
This is usually defined as a significant excess of non-silent over silent nucleotide substitutions in a gene, and occurs when natural selection favours a particular genetic variation and therefore the frequency of the genetic variation shifts.

Considerable progress has been made in the development of vaccines based on inactivated influenza viruses and live cold-adapted influenza viruses grown in these cell lines in microcarrier fermentors[39–41].

The virulence of HPAI viruses for chickens, embryonated eggs and humans, as well as safety concerns for agriculture and humans, has limited the use of conventional methods for the production of vaccines from wild-type HPAI viruses. Two strategies have been used to address this issue. One strategy is the use of anti-genically related surrogate avian influenza viruses that are not pathogenic for poultry or humans. Such viruses can be handled safely in the laboratory or a vaccine-manufacturing plant, and a vaccine manufactured from the surrogate virus should elicit an immune response that crossreacts well with the antigenically related HPAI virus. An example of this approach is the use of a LPAI H5N3 virus (A/duck/Singapore/97) to generate a vaccine to protect against HPAI H5N1 viruses[42,43].

The other strategy is to modify the virulence of the HPAI viruses by genetic engineering[44,45]. This involves the use of plasmid-based reverse genetics, whereby infectious influenza viruses can be recovered from cells transfected with plasmids encoding each of the eight gene segments of the virus[46–48] (FIG. 3). The cells that are transfected with plasmids for the generation of a vaccine seed virus must be approved for use in humans. However, another major challenge in the field of vaccine development is the choice of appropriate animal models for preclinical studies.

Influenza A viruses replicate in several experimental animals, including chickens, mice, cotton rats, ferrets, hamsters, guinea pigs and non-human primates. The use of mouse models for the study of influenza is limited because intranasally administered influenza A viruses do

Table 2 | **Current status of the development of vaccines against avian influenza viruses**

Virus subtype	Type of vaccine	Vaccines evaluated in preclinical studies based on indicated virus	Vaccines evaluated in clinical studies based on indicated virus
H5N1	Inactivated virus	A/Hong Kong/156/97 (H5N1) virus[43] A/duck/Singapore/97 (H5N3) virus[42] A/duck/Hokkaido/67/96 (H5N4) virus[54] Recombinant H5N1–PR8 virus[45] Recombinant A/Hong Kong/213/03 (H5N1)–PR8 virus[55–58] Recombinant A/duck/Singapore/97 (H5N3)–PR8 virus[56]	A/duck/Singapore/97 (H5N3) virus[59,60,128] Recombinant A/Vietnam/1203/04 (H5N1)–PR8 virus[70] Recombinant A/Vietnam/1194/04 (H5N1)–PR8 virus[62,64]
	Live attenuated virus	H5N1–AA cold-adapted reassortant virus (H5N1 genes from A/Vietnam/1203/04, A/Hong Kong/213/2003 and A/Hong Kong/491/97 viruses)[75]	In progress
	Subunit	HA and NA proteins of A/Hong Kong/156/97 (H5N1) virus[80] M2 protein of A/Hong Kong/483/97 (H5N1) virus[79]	Baculovirus-expressed HA proteins of A/Hong Kong/156/97 and A/Hong Kong/483/97 (H5N1) viruses[69]
	DNA	HA gene of A/Hong Kong/156/97 and A/Hong Kong/483/97 (H5N1) viruses[81] Matrix and NP genes of PR8 virus[82,85]	–
	Vectored	Adenovirus expressing HA protein of A/Hong Kong/156/97 and A/Vietnam/1203/04 (H5N1) viruses[86,87]	–
H7	Subunit	HA protein of A/mallard/Netherlands/12/00 (H7N3) virus and NA protein of A/Netherlands/33/03 (H7N7) virus[78]	–
H9	Inactivated virus	A/Hong Kong/1073/99 (H9N2) G1-like virus[53] Recombinant A/chicken/Hong Kong/G9/97 (H9N2)–PR8 virus[52]	A/Hong Kong/1073/99 (H9N2) G1-like virus[63,66] A/chicken/Hong Kong/G9/97 (H9N2) virus[61]
	Live attenuated virus	H9N2–AA cold-adapted reassortant virus (H9N2 genes from A/chicken/Hong Kong/G9/97 virus)[74]	In progress
	Subunit	M2 protein of H9N2 virus[79]	–
	Virus-like particles	Expressing HA, NA and matrix proteins of A/Hong Kong/1073/99 (H9N2) virus[129]	–
	DNA	HA and NA genes of A/chicken/Jiangsu/7/02 (H9N2) virus[84]	–

AA, A/Ann Arbor/6/60 influenza virus; HA, haemagglutinin; M2, matrix protein 2; NA, neuraminidase; NP, nucleoprotein; PR8, A/Puerto Rico/8/34 (H1N1) influenza virus.

not cause symptoms of respiratory-tract disease in mice, although some influenza A viruses are lethal in some other animal models. In addition, in general, human influenza viruses require an adaptation to replicate to high titres in mice. However, it is probable that mice will continue to be used for research into influenza viruses because reagents for immunological studies in mice are widely available.

Ferrets are generally thought to be the best available model for influenza research. Unlike mice, ferrets develop fever, rhinorrhea and sneezing after infection with intranasally administered human influenza viruses and the virus replicates in the respiratory tract of these animals. Seronegative ferrets develop a strain-specific

immune response to human influenza viruses. However, less is known about the replication of avian influenza viruses in mice and ferrets or the relevance of observed morbidity and mortality to humans, so the choice of either animal as an ideal model for the development of pandemic influenza vaccines requires further research. Golden Syrian hamsters, cotton rats[49,50], guinea pigs[51] and non-human primates support the replication of influenza A viruses, but experience with these models is not as extensive as with mice and ferrets for human or avian influenza viruses. Currently, preclinical studies of pandemic influenza vaccines are carried out in mice and ferrets.
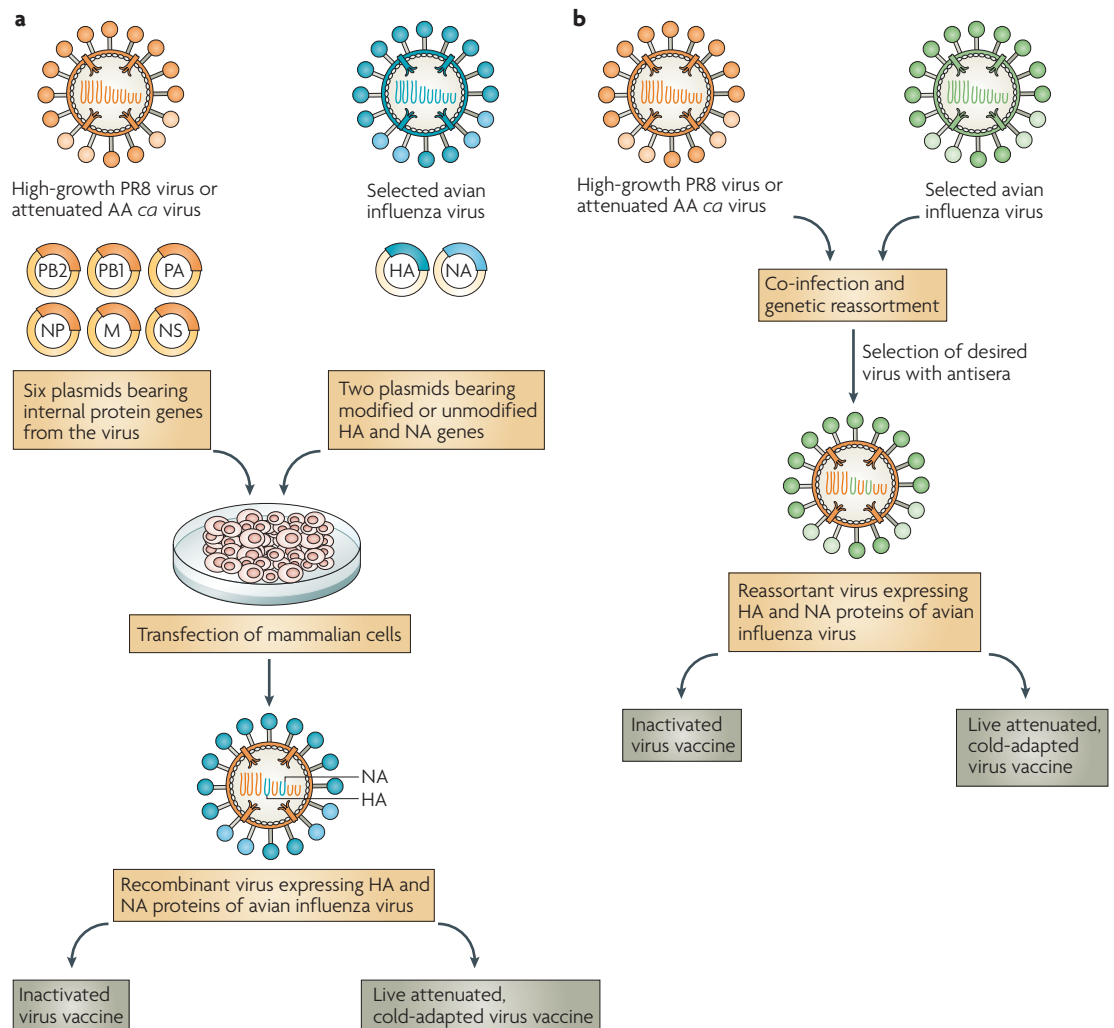
Figure 3 | **The eight-plasmid reverse-genetics system.** Generation of recombinant vaccines for pandemic influenza. **a** | Six plasmids encoding the internal proteins of the high-growth influenza A/Puerto Rico/8/34 (PR8) donor virus or the attenuated, cold-adapted (*ca*) H2N2 A/Ann Arbor/6/60 (AA) donor virus are co-transfected with two plasmids encoding the avian influenza virus haemagglutinin (HA; modified to remove virulence motifs, if necessary) and neuraminidase (NA) glycoproteins into qualified mammalian cells and the recombinant virus is then isolated. Recombinant viruses containing internal protein genes from the PR8 virus are used to prepare inactivated influenza virus vaccines. Recombinant viruses containing internal protein genes from the attenuated, cold-adapted AA virus are used to prepare live attenuated influenza virus vaccines. **b** | The generation of pandemic influenza vaccine viruses by classical reassortment. The reassortant viruses derive six internal protein genes from the vaccine donor virus and the HA and NA genes from the circulating avian influenza virus. The reassortant virus is selected using antisera specific for the HA and NA glycoproteins of the donor virus. M, matrix protein; NP, nucleoprotein; NS, non-structural protein; PA, polymerase acidic protein; PB, polymerase basic protein.

Inactivated virus vaccines. In preclinical studies, parenterally administered, inactivated whole-virus H9 and H5 subtype vaccines have been shown to be effective in mice against challenge with homologous and heterologous viruses[42,43,52–54]. Recombinant H5 influenza viruses — which contain a modified HA glycoprotein, a wild-type NA glycoprotein from the 1997 or 2003 H5N1 viruses or from an LPAI H5N3 virus, and internal protein genes from the PR8 H1N1 influenza virus (A/Puerto Rico/8/34) that confer high yield in eggs — have been generated by reverse genetics[45,55–58]. The removal of the multibasic amino-acid motif in HA that makes the HA0 precursor of HPAI viruses highly cleavable attenuated the virus for infection of chickens, mice and ferrets without altering the antigenicity of the HA glycoprotein. Two doses of these inactivated virus vaccines provided complete protection from lethal challenge with homologous and heterologous H5N1 viruses in mice and ferrets[45,55–58].

Data from phase I clinical trials of inactivated virus vaccines against H9N2, H5N3, H5N1 and H2N2 viruses have been reported and other vaccines are still under evaluation (TABLE 2). Studies that have been carried out so far indicate that inactivated split-virion vaccines against avian influenza viruses — in which the virions are disrupted or split by detergent treatment and the surface glycoproteins are then partially purified — are not optimally immunogenic[59] and require multiple doses[60] or the inclusion of an adjuvant[61–64] to induce a protective immune response. Whole-virus vaccines are more immunogenic than split-virion vaccines, but they are likely to be more reactogenic[65]. Adjuvants are required to increase the immunogenicity of inactivated virus vaccines and to decrease the concentration of viral proteins that is required to induce protective immunity, and several adjuvants for this purpose are under investigation, including aluminium salts, the squalene–oil–water emulsion (MF59) and other proprietary compounds that cannot be discussed in detail.

An inactivated whole-virus H9N2 vaccine was shown to be immunogenic in individuals who had circulating antibodies induced by prior exposure to H2N2 viruses that crossreacted with H9N2 viruses, but the vaccine was not immunogenic in individuals who were born after 1968, when H2N2 viruses stopped circulating in humans[66]. This observation is consistent with findings from studies of an H1N1 vaccine in 1976–1977, when prior exposure to H1N1 viruses that had circulated in the population earlier ('priming') was found to be a determinant of the response to vaccination[65,67]. These studies also showed the need for two doses of vaccine in 'unprimed' individuals. In other studies of vaccines against H9N2 viruses, aluminium hydroxide and MF59 adjuvants improved immunogenicity[61,63].

Inactivated virus vaccines prepared from recombinant PR8 viruses that contain a modified HA glycoprotein and wild-type NA glycoproteins from H5N1 viruses isolated in 2004 have been evaluated as subvirion vaccines or whole-virus vaccines, with or without adjuvants[58,62,64,68,70]. The subvirion vaccines were safe and well-tolerated in healthy adults, and the antibody response that was induced could be enhanced by increasing the dose of antigen used or by the addition of an adjuvant[62,70]. A whole-virus vaccine was also well-tolerated by humans, and when administered with an adjuvant this vaccine was immunogenic at a lower dose than the subvirion vaccines[64]. However, the available data indicate that inactivated H5 influenza virus vaccines are poorly immunogenic and require a large concentration of HA glycoprotein or co-administration with an adjuvant to achieve the desired antibody response.

Live attenuated virus vaccines. Live attenuated, cold-adapted influenza virus vaccines against human influenza viruses elicit both systemic immunity and mucosal immunity at the primary portal of infection. The vaccine strains are generated by the reassortment of a wild-type influenza virus carrying the HA and NA genes of interest with a cold-adapted donor AA (H2N2) influenza virus (A/Ann Arbor/6/60), which was generated by serial passage of the wild-type AA virus at successively lower temperatures[71]. The temperature-sensitive, attenuated, cold-adapted donor AA virus has five mutations in three gene segments that contribute to the temperature-sensitive or attenuation phenotype[72] and the virus has a high degree of phenotypic and genotypic stability[73]. Candidate live attenuated virus vaccines against H9N2 and H5N1 avian influenza viruses generated on this cold-adapted donor backbone using reassortment and plasmid-based reverse genetics, respectively (FIG. 3), were safe and effective in mice and ferrets[44,74,75]. Phase I clinical evaluation of these vaccines is currently in progress.

Generally, live attenuated virus vaccines must retain some infectivity to be immunogenic. Therefore, virus shedding during clinical testing of these vaccines must be closely monitored. The potential challenges in the development of live attenuated virus vaccines for pandemic influenza are: first, to generate reassortant viruses that are sufficiently infectious when the HA glycoprotein is derived from an avian influenza virus, in particular if the HA used has a preference for α-2,3-linked oligosaccharides; second, to reproducibly achieve the desired level of viral attenuation with different combinations of HA and NA genes; and third, to minimize the risk of reassortment with circulating human influenza viruses. The evaluation of live attenuated virus vaccines of different subtypes in preclinical studies in appropriate animal models and in clinical studies will address the first two challenges. The standard approach of preclinical evaluation that is applied to vaccines against human influenza viruses might not be uniformly applicable to avian influenza viruses, because the infectivity, immunogenicity and protective efficacy of avian influenza viruses of different subtypes have not been studied extensively[76]. The risk of reassortment of the live attenuated vaccine virus with human influenza viruses during clinical trials can be minimized by conducting vaccine studies in isolation units when human influenza viruses are not circulating in the community. In the event of an influenza pandemic, the potential benefits of a live attenuated virus vaccine will have to balanced against

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PR8 H1N1 influenza virus (A/Puerto Rico/8/34)
A well-characterized laboratory strain of influenza virus that confers high growth in eggs and is used as the genetic backbone for viruses from which inactivated influenza virus vaccines are generated.

Adjuvant
An agent mixed with an antigen that increases the immune response to that antigen after immunization.

Subvirion vaccine
In a subvirion vaccine, the virions are disrupted or split by detergent treatment and the surface glycoproteins are then partially purified.

Cold-adapted virus
A virus that replicates efficiently at low temperatures, which can be generated by serial passage of a wild-type virus at successively lower temperatures.

the risks associated with it, and this type of vaccine will only be introduced judiciously when a pandemic is imminent.

Recombinant subunit, DNA and vectored vaccines. The use of recombinant or expressed proteins of the influenza virus in a vaccine is an attractive option for vaccine development because these approaches do not require handling of HPAI or infectious viruses for vaccine production.

Preclinical studies of recombinant HA, NA and M2 proteins as vaccine antigens (TABLE 2) showed that the proteins were poorly immunogenic and required multiple doses[77] or the inclusion of adjuvants[78,80] for improved immunogenicity and efficacy. DNA vaccines encoding the HA and NA glycoproteins of avian influenza viruses or conserved internal virus proteins, such as matrix proteins and nucleoproteins, induced protective immunity in mice and chickens[81–84]. The protective efficacy of a nucleoprotein-encoding DNA vaccine was increased by a booster vaccination in the form of a recombinant replication-defective adenovirus (rADV) expressing the nucleoprotein[85]. In two recent studies, intramuscular or intranasal immunization of mice with a human rADV vaccine expressing the influenza virus HA glycoprotein induced both humoral and cell-mediated immune responses and conferred protection against challenge with the wild-type virus in mice and chickens[86,87]. A recombinant baculovirus-expressed H5 glycoprotein subunit vaccine was well-tolerated but was poorly immunogenic in humans, indicating the need for an adjuvant[69]. The production of recombinant proteins and DNA vaccines is safe and economical, but clinical studies of their safety and immunogenicity in humans are awaited.

Universal influenza virus vaccines. An ideal influenza vaccine would be effective against a range of virus subtypes and could be useful during pandemic and interpandemic periods. One approach to creating a universal vaccine would be to target an antigenically stable protein or an antigenically stable part of a variable protein that is essential for virus replication. The high degree of conservation of the M2 protein makes it a prime candidate for a universal influenza vaccine. The M2 protein induced crossreactive immunity that decreased the severity of disease in animal models after challenge with wild-type virus[79,88]. However, the emergence of immune-escape mutants of the M2 protein in mice in the presence of specific antibodies raises concerns regarding the usefulness of the M2 protein as a target for a universal vaccine[89]. Clinical studies are required to evaluate the immunogenicity of the M2 protein in humans.

It has been suggested that the use of the NA glycoprotein, which is less variable than the HA glycoprotein, to induce cross-protective immunity might be worth exploring[90]. NA-specific immunity in mice provides significant cross-protection against antigenically distinct viruses of the same subtype[91,92]. Although NA-specific antibodies do not prevent infection with influenza viruses, they decrease the severity and duration of ill-

ness in humans by limiting the release and spread of the virus[28,29].

Also, if common immunogenic epitopes are identified within the four clades of HA glycoprotein subtypes, HA-based immunogens could induce widely crossreactive immunity[93]. Alternatively, genetically engineered viruses that have several conserved immunogenic epitopes on the viral envelope could be developed and evaluated for use as a universal influenza vaccine[90]. Recombinant viruses expressing chimeric HA glycoproteins have been described recently[94]. Although universal influenza vaccines are still in preclinical development, the potential benefits of such vaccines are so great that strategies to develop them must be encouraged.

Immunogenicity of pandemic influenza vaccines

Although principles derived from decades of experience with seasonal vaccines against human influenza viruses form the basis for the development of pandemic influenza vaccines, our knowledge of the human immune response to avian influenza viruses is incomplete. Investigators rely on extrapolation from experience with vaccines against human influenza viruses, where the immune correlates of protection were elucidated in challenge studies in humans. However, the evaluation of vaccines against potential pandemic strains of avian influenza viruses presents a unique challenge: vaccines developed against these viruses can only be evaluated for safety and immunogenicity, and not for protection, in clinical trials, because challenge studies to assess the efficacy of the vaccines cannot be undertaken in humans. When the immunogenicity of candidate pandemic vaccines is assessed, the data can be difficult to interpret because specific information on the nature and magnitude of the antibody response that correlates with protection is lacking. If a vaccine is immunogenic, it might be possible to assess its efficacy by testing the vaccine in a large group of people who are at high risk from infection with avian influenza virus, such as poultry farmers in areas with severe epizootics.

Serum and mucosal antibodies can independently mediate immunity to influenza viruses. Live viruses and inactivated virus vaccines differ in the induction of protective antibodies, but there are no standardized methods for evaluating the mucosal antibody response. The conventional assay for assessing the immunogenicity of a human influenza vaccine is the haemagglutination-inhibition assay. The standard haemagglutination-inhibition assay, which uses chicken or turkey erythrocytes, is relatively insensitive for the detection of antibodies specific for H5N1 viruses, but a modified assay using horse erythrocytes was found to be more sensitive[95,96] because horse erythrocytes exclusively express the α-2,3-linked oligosaccharide side chains that are preferred for binding by avian influenza viruses. Most studies have shown that serum haemagglutination-inhibition antibody titres of approximately 1:32 to 1:40 protected 50% of the study subjects from infection after immunization with inactivated human influenza virus vaccines[25,26,97]. However, in other studies of experimental infection in adult humans, serum haemagglutination-inhibition antibody titres as low as

Haemagglutination-inhibition assay
An assay used to measure the concentration of antibodies that inhibit the agglutination of erythrocytes by a standard amount of influenza virus. Serum haemagglutination-inhibition antibody titres correlate with protection from infection with human influenza viruses.

1:8 provided resistance to infection with human influenza viruses, which indicates that the levels of antibody required for protection are fairly low[98]. The horse erythrocyte haemagglutination-inhibition assay has not been well standardized and the antibody titres determined by this assay that correlate with protection are not known. Therefore, the choice of assays by which the immune response is assessed poses a practical challenge for the evaluation of pandemic influenza vaccines.

An alternative to the haemagglutination-inhibition assay that might be more biologically relevant is a neutralization assay, in which the ability of antibodies to neutralize the infectivity of the avian influenza virus is assessed. Using paired sera from individuals infected with H5N1 influenza virus in 1997 in Hong Kong collected at the acute and convalescent stages of infection, a neutralizing antibody titre of 1:80 was shown to be indicative of infection with an H5N1 virus[99]. However, it is not known whether this antibody titre correlates with protection from re-infection. Other barriers to the use of the neutralization assay are the requirement for appropriate biosafety containment measures, as the assay requires handling of the infectious virus, and the fact that the test has not yet been standardized[100].

Another consideration in the assessment of the immune response to pandemic influenza vaccines is the lack of reagents for quality control. Post-infection or hyperimmune post-vaccination animal sera are used as controls at present because human sera containing antibodies specific for avian influenza viruses are not available.

Careful analysis of the immune responses in individuals who have recovered from natural infections with avian influenza viruses and in volunteers who participate in clinical trials of pandemic influenza vaccine candidates using standardized assays will increase our understanding of the human immune response to infection with avian influenza viruses. The efficacy of pandemic influenza vaccine candidates cannot be assessed directly in humans; therefore, this information will have to be extrapolated from studies in experimental animals.

Future prospects

The circulation of influenza A viruses of all subtypes in aquatic and wild birds, and the apparent expansion of the host range of avian influenza viruses increase the probability of a pandemic in humans, either by the direct introduction of an avian influenza virus into humans or by the generation of an avian–human reassortant virus that has an increased ability to replicate and spread in the human population. Because it is difficult to predict which avian influenza virus will cross the species barrier and cause a future pandemic, it is crucial that a library of candidate vaccines of different influenza virus subtypes is generated and evaluated in animal models and humans. Animal models that allow comparison with the corresponding wild-type avian influenza virus should be used.

The immune correlates of protection against influenza are not well understood and assays for different aspects of the immune response to avian influenza viruses, in particular cell-mediated immune responses, are not available. The contribution of viral determinants of infectivity, transmissibility and virulence of avian influenza viruses to infection of humans and their implications for vaccine design require further study. The relevance of adaptive genetic changes in avian influenza viruses as they replicate in poultry and other avian species to their infectivity for humans, and the effect of veterinary vaccine use on the evolution of avian influenza viruses are both areas of research that could have a direct impact on vaccines developed for human use. The biological basis for the poor immunogenicity of avian HA glycoproteins should be explored. Until this is understood, there is a need for adjuvants to increase the immunogenicity of avian influenza vaccines. Although an ideal vaccine would prevent infection, a more realistic goal for a pandemic influenza vaccine might be to prevent severe illness and death. Efforts to develop cell-culture-based vaccines are under way. The development of a safe, immunogenic universal vaccine that could be used to control both seasonal and pandemic influenza would be a great achievement.

1. Morens, D. M., Folkers, G. K. & Fauci, A. S. The challenge of emerging and re-emerging infectious diseases. *Nature* **430**, 242–249 (2004).
2. Crawford, P. C. *et al.* Transmission of equine influenza virus to dogs. *Science* **310**, 482–485 (2005).
3. Wright, P. F. & Webster, R. G. in *Fields Virology* 4th edn (eds Knipe, D. M. & Howley, P. M.) 1533–1579 (Lippincott Williams & Wilkins, Philadelphia, 2001).
4. Fouchier, R. A. *et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* **79**, 2814–2822 (2005).
5. Treanor, J. J., Tierney, E. L., Zebedee, S. L., Lamb, R. A. & Murphy, B. R. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. *J. Virol.* **64**, 1375–1377 (1990).
6. Zebedee, S. L. & Lamb, R. A. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J. Virol.* **62**, 2762–2772 (1988).
7. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179 (1992).
8. Skehel, J. J. & Wiley, D. C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* **69**, 531–569 (2000).
9. Perdue, M. L., Garcia, M., Senne, D. & Fraire, M. Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. *Virus Res.* **49**, 173–186 (1997).
10. Klenk, H. D. & Garten, W. Host cell proteases controlling virus pathogenicity. *Trends Microbiol.* **2**, 39–43 (1994).
11. Kawaoka, Y., Naeve, C. W. & Webster, R. G. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? *Virology* **139**, 303–316 (1984).
12. Banks, J., Speidel, E. C., McCauley, J. W. & Alexander, D. J. Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses. *Arch. Virol.* **145**, 1047–1058 (2000).
13. Akey, B. L. Low-pathogenicity H7N2 avian influenza outbreak in Virginia during 2002. *Avian Dis.* **47**, 1099–1103 (2003).
14. Marangon, S. & Capua, I. Control of avian influenza in Italy: from stamping out to emergency and prophylactic vaccination. *Dev. Biol. (Basel)* **124**, 109–115 (2006).
15. Connor, R. J., Kawaoka, Y., Webster, R. G. & Paulson, J. C. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* **205**, 17–23 (1994).
16. Shinya, K. *et al.* Avian flu: influenza virus receptors in the human airway. *Nature* **440**, 435–436 (2006).
17. van Riel, D. *et al.* H5N1 virus attachment to lower respiratory tract. *Science* **312**, 399 (2006).
18. Claas, E. C. *et al.* Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* **351**, 472–477 (1998).
19. Subbarao, K. *et al.* Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279**, 393–396 (1998). **This paper reported the characterization of the H5N1 virus isolated from the index case of human infection with H5N1 avian influenza virus in 1997 in Hong Kong, and showed that an avian H5N1 virus could transmit directly to humans.**
20. Gillim-Ross, L. & Subbarao, K. Emerging respiratory viruses: challenges and vaccine strategies. *Clin. Microbiol. Rev.* **19**, 614–636 (2006).
21. Lewis, D. B. Avian flu to human influenza. *Annu. Rev. Med.* **57**, 139–154 (2006).

22. Stephenson, I., Nicholson, K. G., Wood, J. M., Zambon, M. C. & Katz, J. M. Confronting the avian influenza threat: vaccine development for a potential pandemic. *Lancet Infect. Dis.* **4**, 499–509 (2004).
23. Beigel, J. H. *et al.* Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.* **353**, 1374–1385 (2005).
    A Review summarizing the clinical and laboratory aspects of H5N1 infections in humans in several Asian countries.
24. de Jong, M. D. & Hien, T. T. Avian influenza A (H5N1). *J. Clin. Virol.* **35**, 2–13 (2006).
25. Couch, R. B. & Kasel, J. A. Immunity to influenza in man. *Annu. Rev. Microbiol.* **37**, 529–549 (1983).
    This Review summarizes the importance of antibodies specific for HA and NA glycoproteins in providing protection against influenza virus infections in humans.
26. Potter, C. W. & Oxford, J. S. Determinants of immunity to influenza infection in man. *Br. Med. Bull.* **35**, 69–75 (1979).
27. Gerhard, W. The role of the antibody response in influenza virus infection. *Curr. Top. Microbiol. Immunol.* **260**, 171–190 (2001).
28. Kilbourne, E. D., Laver, W. G., Schulman, J. L. & Webster, R. G. Antiviral activity of antiserum specific for an influenza virus neuraminidase. *J. Virol.* **2**, 281–288 (1968).
29. Murphy, B. R., Kasel, J. A. & Chanock, R. M. Association of serum anti-neuraminidase antibody with resistance to influenza in man. *N. Engl. J. Med.* **286**, 1329–1332 (1972).
30. Choi, Y. K. *et al.* Continuing evolution of H9N2 influenza viruses in Southeastern China. *J. Virol.* **78**, 8609–8614 (2004).
31. Fouchier, R. A. *et al.* Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl Acad. Sci. USA* **101**, 1356–1361 (2004).
32. Tweed, S. A. *et al.* Human illness from avian influenza H7N3, British Columbia. *Emerg. Infect. Dis.* **10**, 2196–2199 (2004).
33. Suarez, D. L. *et al.* Recombination resulting in virulence shift in avian influenza outbreak, Chile. *Emerg. Infect. Dis.* **10**, 693–699 (2004).
34. Russell, R. J. *et al.* H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. *Virology* **325**, 287–296 (2004).
    This study describes the genetic basis for the classification of the 15 HA glycoprotein subtypes into four clades.
35. Makarova, N. V., Kaverin, N. V., Krauss, S., Senne, D. & Webster, R. G. Transmission of Eurasian avian H2 influenza virus to shorebirds in North America. *J. Gen. Virol.* **80**, 3167–3171 (1999).
36. Cox, N. J. & Bender, C. A. The molecular epidemiology of influenza virus. *Semin. Virol.* **6**, 359–370 (1995).
37. Wilson, I. A. & Cox, N. J. Structural basis of immune recognition of influenza virus hemagglutinin. *Annu. Rev. Immunol.* **8**, 737–771 (1990).
38. Lee, C. W., Senne, D. A. & Suarez, D. L. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J. Virol.* **78**, 8372–8381 (2004).
    This study provides evidence for the evolution of the HA glycoprotein of avian influenza viruses when veterinary vaccines are used to prevent avian influenza outbreaks in poultry.
39. Ghendon, Y. Z. *et al.* Development of cell culture (MDCK) live cold-adapted (CA) attenuated influenza vaccine. *Vaccine* **23**, 4678–4684 (2005).
40. Oxford, J. S. *et al.* A new European perspective of influenza pandemic planning with a particular focus on the role of mammalian cell culture vaccines. *Vaccine* **23**, 5440–5449 (2005).
41. Palker, T. *et al.* Protective efficacy of intranasal cold-adapted influenza A/New Caledonia/20/99 (H1N1) vaccines comprised of egg- or cell culture-derived reassortants. *Virus Res.* **105**, 183–194 (2004).
42. Lu, X. *et al.* A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J. Virol.* **73**, 5903–5911 (1999).
43. Wood, J. M. *et al.* Vaccines against H5N1 influenza. *Vaccine* **18**, 579–580 (1999).
44. Li, S. *et al.* Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. *J. Infect. Dis.* **179**, 1132–1138 (1999).
45. Subbarao, K. *et al.* Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* **305**, 192–200 (2003).
46. Fodor, E. *et al.* Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73**, 9679–9682 (1999).
47. Hoffmann, E., Krauss, S., Perez, D., Webby, R. & Webster, R. G. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* **20**, 3165–3170 (2002).
48. Neumann, G. *et al.* Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl Acad. Sci. USA* **96**, 9345–9350 (1999).
    References 46–48 describe plasmid-based reverse-genetic systems for the generation of recombinant influenza viruses.
49. Ottolini, M. G. *et al.* The cotton rat provides a useful small-animal model for the study of influenza virus pathogenesis. *J. Gen. Virol.* **86**, 2823–2830 (2005).
50. Straight, T. M., Ottolini, M. G., Prince, G. A. & Eichelberger, M. C. Evidence of a cross-protective immune response to influenza A in the cotton rat model. *Vaccine* **24**, 6264–6271 (2006).
51. Lowen, A. C., Mubareka, S., Tumpey, T. M., Garcia-Sastre, A. & Palese, P. The guinea pig as a transmission model for human influenza viruses. *Proc. Natl Acad. Sci. USA* **103**, 9988–9992 (2006).
52. Chen, H. *et al.* Generation and evaluation of a high-growth reassortant H9N2 influenza A virus as a pandemic vaccine candidate. *Vaccine* **21**, 1974–1979 (2003).
53. Lu, X. *et al.* Immunity to influenza A H9N2 viruses induced by infection and vaccination. *J. Virol.* **75**, 4896–4901 (2001).
54. Takada, A. *et al.* Avirulent avian influenza virus as a vaccine strain against a potential human pandemic. *J. Virol.* **73**, 8303–8307 (1999).
55. Govorkova, E. A., Webby, R. J., Humberd, J., Seiler, J. P. & Webster, R. G. Immunization with reverse-genetics-produced H5N1 influenza vaccine protects ferrets against homologous and heterologous challenge. *J. Infect. Dis.* **194**, 159–167 (2006).
56. Lipatov, A. S., Hoffmann, E., Salomon, R., Yen, H. L. & Webster, R. G. Cross-protectiveness and immunogenicity of influenza A/Duck/Singapore/3/97 (H5) vaccines against infection with A/Vietnam/1203/04 (H5N1) virus in ferrets. *J. Infect. Dis.* **194**, 1040–1043 (2006).
57. Nicolson, C., Major, D., Wood, J. M. & Robertson, J. S. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. *Vaccine* **23**, 2943–2952 (2005).
58. Webby, R. J. *et al.* Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet* **363**, 1099–1103 (2004).
59. Nicholson, K. G. *et al.* Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet* **357**, 1937–1943 (2001).
60. Stephenson, I. *et al.* Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine* **21**, 1687–1693 (2003).
61. Atmar, R. L. *et al.* Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. *Clin. Infect. Dis.* **43**, 1135–1142 (2006).
62. Bresson, J. L. *et al.* Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. *Lancet* **367**, 1657–1664 (2006).
63. Hehme, N., Engelmann, H., Kunzel, W., Neumeier, E. & Sanger, R. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. *Med. Microbiol. Immunol. (Berlin)* **191**, 203–208 (2002).
64. Lin, J. *et al.* Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. *Lancet* **368**, 991–997 (2006).
65. Wright, P. F. *et al.* Trials of influenza A/New Jersey/76 virus vaccine in normal children: an overview of age-related antigenicity and reactogenicity. *J. Infect. Dis.* **136**, S731–S741 (1977).
66. Stephenson, I. *et al.* Safety and antigenicity of whole virus and subunit influenza A/Hong Kong/1073/99 (H9N2) vaccine in healthy adults: phase I randomised trial. *Lancet* **362**, 1959–1966 (2003).
67. Parkman, P. D., Hopps, H. E., Rastogi, S. C. & Meyer, H. M. Jr. Summary of clinical trials of influenza virus vaccines in adults. *J. Infect. Dis.* **136**, S722–S730 (1977).
68. Ozaki, H. *et al.* Generation of high-yielding influenza A viruses in African green monkey kidney (Vero) cells by reverse genetics. *J. Virol.* **78**, 1851–1857 (2004).
69. Treanor, J. J. *et al.* Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. *Vaccine* **19**, 1732–1737 (2001).
70. Treanor, J. J., Campbell, J. D., Zangwill, K. M., Rowe, T. & Wolff, M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N. Engl. J. Med.* **354**, 1343–1351 (2006).
71. Maassab, H. F. & Bryant, M. L. The development of live attenuated cold-adapted influenza virus vaccine for humans. *Rev. Med. Virol.* **9**, 237–244 (1999).
72. Jin, H. *et al.* Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology* **306**, 18–24 (2003).
73. Cha, T. A. *et al.* Genotypic stability of cold-adapted influenza virus vaccine in an efficacy clinical trial. *J. Clin. Microbiol.* **38**, 839–845 (2000).
74. Chen, H. *et al.* Generation and characterization of a cold-adapted influenza A H9N2 reassortant as a live pandemic influenza virus vaccine candidate. *Vaccine* **21**, 4430–4436 (2003).
75. Suguitan, A. L. *et al.* Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. *PLoS Med.* **3**, e360 (2006).
76. Luke, C. J. & Subbarao, K. Vaccines for pandemic influenza. *Emerg. Infect. Dis.* **12**, 66–72 (2006).
77. Nwe, N. *et al.* Expression of hemagglutinin protein from the avian influenza virus H5N1 in a baculovirus/insect cell system significantly enhanced by suspension culture. *BMC Microbiol.* **6**, 16 (2006).
78. de Wit, E. *et al.* Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. *J. Virol.* **79**, 12401–12407 (2005).
79. Ernst, W. A. *et al.* Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. *Vaccine* **24**, 5158–5168 (2006).
80. Rimmelzwaan, G. F., Claas, E. C., van Amerongen, G., de Jong, J. C. & Osterhaus, A. D. ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus. *Vaccine* **17**, 1355–1358 (1999).
81. Bright, R. A., Ross, T. M., Subbarao, K., Robinson, H. L. & Katz, J. M. Impact of glycosylation on the immunogenicity of a DNA-based influenza H5 HA glycoprotein vaccine. *Virology* **308**, 270–278 (2003).
82. Epstein, S. L. *et al.* DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. *Emerg. Infect. Dis.* **8**, 796–801 (2002).
83. Kodihalli, S., Kobasa, D. L. & Webster, R. G. Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. *Vaccine* **18**, 2592–2599 (2000).
84. Qiu, M. *et al.* Protection against avian influenza H9N2 virus challenge by immunization with hemagglutinin- or neuraminidase-expressing DNA in BALB/c mice. *Biochem. Biophys. Res. Commun.* **343**, 1124–1131 (2006).
85. Epstein, S. L. *et al.* Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine* **23**, 5404–5410 (2005).
86. Gao, W. *et al.* Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *J. Virol.* **80**, 1959–1964 (2006).
87. Hoelscher, M. A. *et al.* Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet* **367**, 475–481 (2006).
88. Gerhard, W., Mozdzanowska, K. & Zharikova, D. Prospects for universal influenza virus vaccine. *Emerg. Infect. Dis.* **12**, 569–574 (2006).
89. Zharikova, D., Mozdzanowska, K., Feng, J., Zhang, M. & Gerhard, W. Influenza type A virus escape mutants emerge *in vivo* in the presence of antibodies to the ectodomain of matrix protein 2. *J. Virol.* **79**, 6644–6654 (2005).
90. Palese, P. Making better influenza virus vaccines? *Emerg. Infect. Dis.* **12**, 61–65 (2006).
91. Chen, J., Fang, F., Li, X., Chang, H. & Chen, Z. Protection against influenza virus infection in BALB/c mice immunized with a single dose of neuraminidase-

expressing DNAs by electroporation. *Vaccine* **23**, 4322–4328 (2005).

92. Kilbourne, E. D. *et al.* Protection of mice with recombinant influenza virus neuraminidase. *J. Infect. Dis.* **189**, 459–461 (2004).
93. Subbarao, K., Murphy, B. R. & Fauci, A. S. Development of effective vaccines against pandemic influenza. *Immunity* **24**, 5–9 (2006).
94. Li, Z. N. *et al.* Chimeric influenza virus hemagglutinin proteins containing large domains of the *Bacillus anthracis* protective antigen: protein characterization, incorporation into infectious influenza viruses, and antigenicity. *J. Virol.* **79**, 10003–10012 (2005).
95. Stephenson, I., Wood, J. M., Nicholson, K. G., Charlett, A. & Zambon, M. C. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. *Virus Res.* **103**, 91–95 (2004).
This study describes the use of horse erythrocytes in a haemagglutination-inhibition assay to improve the sensitivity of detection of antibodies specific for H5N1 avian influenza viruses.
96. Stephenson, I., Wood, J. M., Nicholson, K. G. & Zambon, M. C. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J. Med. Virol.* **70**, 391–398 (2003).
97. Hobson, D., Curry, R. L., Beare, A. S. & Ward-Gardner, A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J. Hyg. (London)* **70**, 767–777 (1972).
This study indicates that serum haemagglutination-inhibition antibody titres are a correlate of protection in influenza virus infection of humans.
98. Treanor, J. & Wright, P. F. Immune correlates of protection against influenza in the human challenge model. *Dev. Biol. (Basel)* **115**, 97–104 (2003).
99. Rowe, T. *et al.* Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J. Clin. Microbiol.* **37**, 937–943 (1999).
This paper describes the development of a neutralization assay to detect antibodies specific for H5N1 avian influenza virus and records the titre of antibody in convalescent patients.
100. Stephenson, I. H5N1 vaccines: how prepared are we for a pandemic? *Lancet* **368**, 965–966 (2006).
101. Johnson, N. P. & Mueller, J. Updating the accounts: global mortality of the 1918–1920 'Spanish' influenza pandemic. *Bull. Hist. Med.* **76**, 105–115 (2002).
102. Reid, A. H. & Taubenberger, J. K. The origin of the 1918 pandemic influenza virus: a continuing enigma. *J. Gen. Virol.* **84**, 2285–2292 (2003).

103. Kobasa, D. *et al.* Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature* **431**, 703–707 (2004).
104. Tumpey, T. M. *et al.* Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. *Proc. Natl Acad. Sci. USA* **101**, 3166–3171 (2004).
105. Tumpey, T. M. *et al.* Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* **310**, 77–80 (2005).
106. Gamblin, S. J. *et al.* The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science* **303**, 1838–1842 (2004).
107. Kawaoka, Y., Krauss, S. & Webster, R. G. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* **63**, 4603–4608 (1989).
108. Scholtissek, C., Rohde, W., Von Hoyningen, V. & Rott, R. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* **87**, 13–20 (1978).
109. Proceedings of the 98th Annual Meeting of the United States Animal Health Association, Grand Rapids, Michigan (1994).
110. Hatta, M., Gao, P., Halfmann, P. & Kawaoka, Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* **293**, 1840–1842 (2001).
111. Castrucci, M. R. & Kawaoka, Y. Biologic importance of neuraminidase stalk length in influenza A virus. *J. Virol.* **67**, 759–764 (1993).
112. Matrosovich, M., Zhou, N., Kawaoka, Y. & Webster, R. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* **73**, 1146–1155 (1999).
113. Subbarao, E. K., London, W. & Murphy, B. R. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J. Virol.* **67**, 1761–1764 (1993).
114. Guan, Y., Shortridge, K. F., Krauss, S. & Webster, R. G. Molecular characterization of H9N2 influenza viruses: were they the donors of the 'internal' genes of H5N1 viruses in Hong Kong? *Proc. Natl Acad. Sci. USA* **96**, 9363–9367 (1999).
115. Hoffmann, E. *et al.* Characterization of the influenza A virus gene pool in avian species in southern China: was H6N1 a derivative or a precursor of H5N1? *J. Virol.* **74**, 6309–6315 (2000).
116. Lin, Y. P. *et al.* Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. *Proc. Natl Acad. Sci. USA* **97**, 9654–9658 (2000).
117. Xu, X., Subbarao, K., Cox, N. J. & Guo, Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreak in Hong Kong. *Virology* **261**, 15–19 (1999).

118. Apisarnthanarak, A. *et al.* Atypical avian influenza (H5N1). *Emerg. Infect. Dis.* **10**, 1321–1324 (2004).
119. de Jong, M. D. *et al.* Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N. Engl. J. Med.* **352**, 686–691 (2005).
120. Tran, T. H. *et al.* Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med.* **350**, 1179–1188 (2004).
121. Epidemic and Pandemic Alert and Response (EPR): Avian influenza. *World Health Organisation* [online], http://www.who.int/csr/disease/avian_influenza/en/
122. Koopmans, M. *et al.* Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* **363**, 587–593 (2004).
123. Hirst, M. *et al.* Novel avian influenza H7N3 strain outbreak, British Columbia. *Emerg. Infect. Dis.* **10**, 2192–2195 (2004).
124. Matrosovich, M. N., Krauss, S. & Webster, R. G. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology* **281**, 156–162 (2001).
125. Guo, Y. J. *et al.* Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology* **267**, 279–288 (2000).
126. Butt, K. M. *et al.* Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *J. Clin. Microbiol.* **43**, 5760–5767 (2005).
127. Avian influenza virus A (H10N7) circulating among humans in Egypt. *EID Weekly Updates* [online], http://www.paho.org/English/AD/DPC/CD/eid-eer-07-may-2004.htm#birdflu (2004).
128. Stephenson, I. *et al.* Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. *J. Infect. Dis.* **191**, 1210–1215 (2005).
129. Pushko, P. *et al.* Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. *Vaccine* **23**, 5751–5759 (2005).

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