INFLUENZA: LESSONS FROM PAST PANDEMICS, WARNINGS FROM CURRENT INCIDENTS

Taisuke Horimoto[*§] *and Yoshihiro Kawaoka*[*‡§‖]

Abstract | Recent outbreaks of highly pathogenic avian influenza A virus infections (H5 and H7 subtypes) in poultry and in humans (through direct contact with infected birds) have had important economic repercussions and have raised concerns that a new influenza pandemic will occur in the near future. The eradication of pathogenic avian influenza viruses seems to be the most effective way to prevent influenza pandemics, although this strategy has not proven successful so far. Here, we review the molecular factors that contribute to the emergence of pandemic strains.

*Division of Virology, Department of Microbiology and Immunology, and ‡International Research Centre for Infectious Diseases, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan.
§Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan.
‖Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA.
Correspondence to Y.K.
e-mail: kawaoka@ims.u-tokyo.ac.jp
doi:10.1038/nrmicro1208

Influenza viruses belong to the Orthomyxoviridae family. Antigenic differences in their nucleoprotein (NP) and matrix protein (M1) allow influenza viruses to be classified as types A, B or C (FIG. 1). For type A viruses, further subtyping is based on the antigenicity of the HAEMAGGLUTININ (HA) and NEURAMINIDASE (NA) surface glycoproteins[1]. Currently, 16 HA (H1–H16) and 9 NA (N1–N9) subtypes have been identified in type A viruses[2,3].

Type A influenza viruses have been isolated from various animals, including humans, pigs, horses, sea mammals and birds. Phylogenetic studies of type A isolates have revealed that the viral genes form species-specific lineages, and aquatic birds are thought to be the source of all influenza A viruses in other animal species[4]. Influenza viruses usually do not produce disease in wild aquatic birds, indicating that they have achieved an optimal level of adaptation in this natural reservoir. All 16 HA and 9 NA subtypes of type A viruses are maintained in aquatic bird populations, especially ducks, shorebirds and gulls[4].

Past influenza pandemics

Spanish influenza (1918–1919). The so-called Spanish influenza was caused by an H1N1 virus and was responsible for the deaths of at least 40 million people in 1918–1919 (REF. 5). Although its clinical symptoms and pathological manifestations were largely confined to the respiratory tract[6], almost 50% of deaths (the case mortality rate in the USA averaged 2.5%) occurred in an unusually young age group, 20–40-year-olds[7]. The biological properties of the virus that was responsible for the Spanish influenza still remain obscure, owing to the lack of viral isolates available for study. However, the available data on gene sequences of the 1918 virus, obtained from lung tissue samples, suggest 'an unusual avian precursor'[8] (FIG. 2). The three-dimensional structure of the HA of the 1918 virus indicates that, despite retaining the residues in the host-receptor-binding site that are characteristic of an avian precursor HA[9,10], the 1918 virus HA could bind human cell-surface receptors, an observation that is consistent with receptor-binding assays carried out with a recombinant virus carrying the HA from the 1918 virus[11]. Recent studies with viruses generated by REVERSE GENETICS that carry genes derived from the 1918 virus indicate that the HA of this virus had a role in its increased virulence in a mouse model[11–13]. Indeed, the lung infiltration by inflammatory cells and the haemorrhagic pneumonia seen in these mice were hallmarks of the illness in humans in the original PANDEMIC[11,13].

Other pandemics. Other, less serious pandemics occurred in 1957 (Asian influenza, H2N2), 1968 (Hong Kong influenza, H3N2), and 1977 (Russian influenza, H1N1)[14]. The 1957 virus consisted of HA (H2),

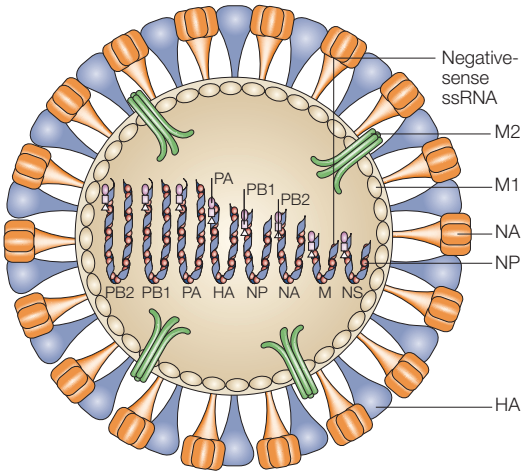

Figure 1 | **Schematic diagram of an influenza A virus virion.** Two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), and the M2 ion-channel protein are embedded in the viral envelope, which is derived from the host plasma membrane. The ribonucleoprotein complex comprises a viral RNA segment associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1 and PB2). The matrix (M1) protein is associated with both ribonucleoprotein and the viral envelope. A small amount of non-structural protein 2 is also present, but its location within the virion is unknown.

NA (N2) and a viral RNA polymerase gene segment, PB1 (polymerase basic 1), from an avian virus, with the other gene segments derived from a previously circulating human virus[15,16]. The 1968 virus had avian HA (H3) and PB1 segments in a background of human viral genes[15,16]. The acquisition of avian surface antigens allowed these viruses to circumvent the human immune response. Why both of these pandemic strains also carried the gene that encodes PB1 from an avian virus remains unclear. In view of a recently identified North American swine virus, a triple REASSORTANT that combined swine, human and avian viral genes and possessed the genes that encode PB1 and HA from the same human virus[17], one could argue that the genes that encode HA and PB1 interact functionally, either at a protein or nucleic acid level, in ways that enhance the replicative ability of hybrid viruses.

The Russian influenza H1N1 strain was essentially identical to those H1N1 strains that had circulated in humans in the 1950s (FIG. 2) (REF. 18), and it is thought likely that the virus was maintained in a freezer until it was somehow reintroduced into the general population. The magnitude of this outbreak was limited, as most individuals over 27 years of age had some immunological memory to the virus.

Properties of avian influenza viruses

Influenza pandemics are caused by viruses that possess an HA molecule to which most of the population lacks immunity. Recently, purely avian influenza viruses, including the H5N1, H9N2 and H7N7 subtypes, have been directly transmitted to humans, raising concern over the possibility of a new influenza pandemic among the world's immunologically naive populations. As the viruses responsible for pandemics in the past century were all characterized by the acquisition of HAs from avian viruses, with the exception of the Russian influenza virus, clues to the molecular changes that give rise to human pandemic strains can be found in the avian reservoir.

Avian influenza viruses can be sorted on the basis of virulence: highly pathogenic avian influenza (HPAI) viruses cause systemic lethal infection, killing birds as soon as 24 hours post-infection, and usually within one week, whereas low-pathogenic avian influenza (LPAI) viruses rarely generate outbreaks of severe disease in the field, and their associated morbidity and mortality rates are lower than those of HPAI viruses[4]. Phylogenetic studies of avian influenza viruses have revealed two geographically separate sublineages (Eurasian and American), which probably reflects the separation of viruses owing to the distinct migratory patterns of the host birds in these two regions. Both HPAI and LPAI viruses are found within these two sublineages, indicating that viral pathogenicity is not determined by geographical distribution. Without exception, all of the HPAI viruses belong to the H5 or H7 subtype, for reasons that are still unclear. There do not seem to be any associations of specific NA subtypes with HPAI viruses.

Susceptibility and pathology. Many domestic and wild avian species are susceptible to influenza virus infection, although viruses that are highly pathogenic in one avian species might not be pathogenic in another[19]. For example, ducks tend not to succumb to viruses that are lethal in chickens, even though virus can be detected in various internal organs and in the blood of infected ducks. Among domestic avian species, chickens and turkeys are the most frequently involved in outbreaks of HPAI-virus-related disease. The host factors that determine differences in susceptibility to avian influenza viruses in different avian species are unknown.

LPAI viruses replicate mainly in intestinal and respiratory organs, and are shed in the faeces of infected birds. Therefore, transmission of viruses through the faecal-contaminated-water–oral route is an important mechanism of LPAI-virus dissemination among aquatic birds. High concentrations of HPAI viruses, which replicate systemically in poultry, are also shed in faeces. However, these viruses are more readily transmitted among birds in densely populated flocks by the nasal and oral routes through contact with virus-contaminated materials. LPAI viruses cause localized infections in the respiratory and/or intestinal tract, resulting in mild or asymptomatic infection. In chickens infected with HPAI viruses, common symptoms include swelling of the microvascular endothelium, multifocal haemorrhages and thrombosis[19,20]. HPAI viruses can replicate efficiently in vascular endothelial and perivascular parenchymatous cells, which aids viral dissemination and systemic infection. Additionally, involvement of the cardiovascular system is indicated, as HPAI antigens have been found in necrotic cardiac myocytes[21].

HAEMAGGLUTININ
Type I integral membrane glycoprotein that binds to cell-surface receptors and facilitates fusion between the viral envelope and endosomal membrane. It is the main target antigen of the humoral immune response in host animals.

NEURAMINIDASE
Type II integral membrane glycoprotein, which facilitates viral release from cells by removing sialic acid from sialyloligosaccharides on the cell and viral surface.

REVERSE GENETICS
A method that allows the production of viruses that possess genes derived from cloned cDNA.

PANDEMIC
Global influenza infection caused by the emergence of a virus with an HA subtype to which most of the human population lacks immunity.

REASSORTANT
A virus with gene segments derived from more than one virus, achieved by co-infection of a single cell by these viruses.

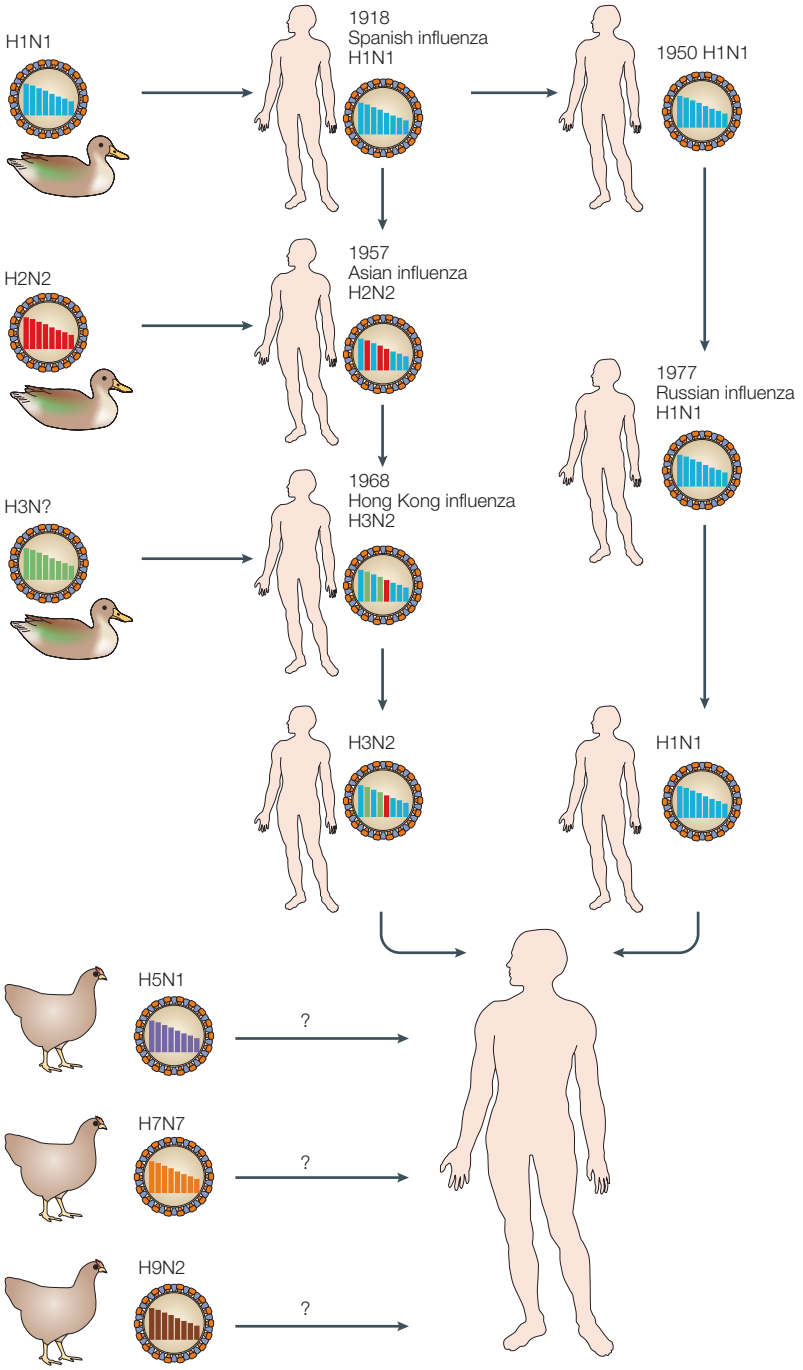

Figure 2 | **Past and potential future pandemic influenza viruses.** Evidence indicates that the 1918 Spanish influenza pandemic was caused by an H1N1 avian influenza virus that was transmitted to humans. A reassortant H1N1 virus possessing three avian gene segments (PB1, haemagglutinin (HA) and neuraminidase) caused the 1957 pandemic (Asian influenza; H2N2 subtype). The H1N1 virus subsequently disappeared from human circulation. In 1968, another reassortant, carrying the genes that encode PB1 and HA from an H3 avian virus and the remaining gene segments from an H2N2 human virus, emerged as a new pandemic strain (Hong Kong influenza; H3N2 subtype); the H2N2 virus then disappeared from circulation. In 1977, an H1N1 virus that was genetically almost identical to the H1N1 virus from the 1950s spread rapidly among immunologically naive younger people (Russian influenza). The H1N1 and H3N2 viruses that have evolved from the strains responsible for the Russian and Hong Kong influenzas, respectively, continue to circulate in humans and produce annual epidemics. Since 1997, purely avian influenza viruses, including H5N1, H7N7 and H9N2 subtypes, have been directly transmitted to humans, raising concern over the possibility of a new influenza pandemic equal in impact to, or greater than, the Spanish influenza.

HA and pathogenicity. The HA surface glycoprotein mediates virus binding to host-cell receptors and promotes the release of the viral RNA that is complexed with polymerase and NP (ribonucleoprotein, RNP) through membrane fusion. A precursor HA molecule undergoes post-translational cleavage into HA1 and HA2 subunits by host proteases, with the generation of a fusogenic domain at the amino terminus of HA2 that mediates fusion between the viral envelope and the endosomal membrane[22]. Proteolytic activation is therefore essential for viral infectivity and dissemination[23], and the HA glycoprotein has a key role in influenza virus pathogenicity[24].

The HAs of LPAI viruses possess a single arginine at the cleavage site (FIGS 3,4) and are usually cleaved in only a limited number of organs, resulting in mild or asymptomatic infection. Proteases capable of cleaving the HAs of LPAI and HPAI viruses are often called 'trypsin-like' enzymes, and *in vitro* include blood-clotting factor Xa, tryptases, mini-plasmin and bacterial proteases[25–27], although the enzymes responsible for HA cleavage *in vivo* are still unidentified. By contrast, the HAs of HPAI viruses possess a series of basic amino acids at the cleavage site, which are cleaved by ubiquitous proteases, such as furin and PC6 (proprotein convertase 6), which are present in a broad range of different host cells, supporting lethal systemic infection in poultry[28,29]. A carbohydrate side chain near the cleavage site can affect HA cleavability by interfering with the accessibility of the host proteases to the cleavage site[30,31]. Therefore, HA cleavability is considered the main determinant of the tissue tropism of avian influenza viruses[32], and differences in the tissue distribution of proteases and HA susceptibility to these enzymes can determine the outcome of virus infection.

The acquisition of enhanced HA cleavability is an essential event in the conversion of avirulent avian influenza viruses to virulent strains, as was found in Pennsylvania (H5N2) in 1983 (REF. 30), in Mexico (H5N2) in 1994 (REFS 33,34), in Italy (H7N1) in 1997 (REF. 35), in Chile (H7N3) in 2002 (REF. 36) and in Canada (H7N3) in 2004 (REF. 37). Sequence conversion could be caused by polymerase slippage or non-homologous recombination between the gene that encodes HA and other genes at the cleavage site.

Direct transmission to humans

In general, as avian influenza viruses do not replicate efficiently in humans, direct transmission of avian viruses to humans is probably an extremely rare event, and high doses of avian virus are required to produce a quantifiable level of replication in human volunteers[38]. The restricted growth of avian influenza viruses in humans was thought to be a barrier to the emergence of new pandemic strains by direct avian-to-human transmission. However, this perception changed in 1997, when an H5N1 avian virus was transmitted directly to humans from birds.

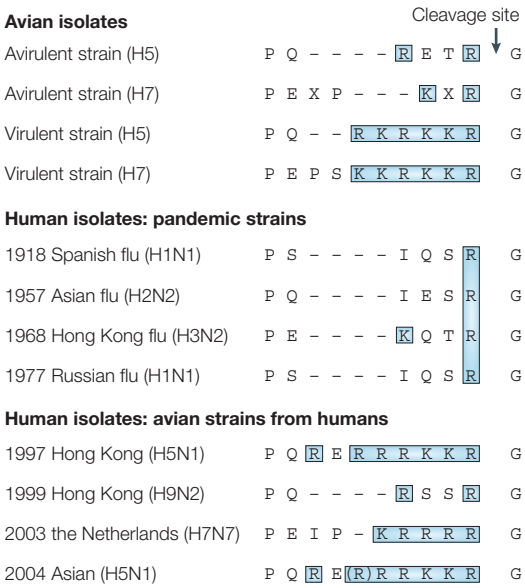

Figure 3 | **HA cleavage site sequence of influenza A viruses.** Basic amino acids are shown in blue boxes. Dashes are for the purpose of alignment only.

H5N1 virus. In May 1997, an H5N1 virus (A/Hong Kong/156/97) was isolated from a 3-year-old boy in Hong Kong[39–41], who later died of extensive influenza pneumonia complicated by REYE SYNDROME. By the end of 1997, a total of 18 people had been infected by this virus, six of whom died. The clinical features of infection included onset of fever and upper-respiratory-tract infection, typical of classical influenza. Some patients had severe complications, mainly pneumonia, gastrointestinal manifestations, elevated liver enzymes and renal failure[42]. In general, children fared better than adults. Epidemiological studies suggested direct transmission of the virus from birds and serological evidence of human-to-human transmission was limited to a few cases[43], indicating that the virus had not become fully adapted to its human host. The slaughter of all poultry in Hong Kong successfully eradicated the threat of a major outbreak of H5N1 virus infection.

The human H5N1 isolates were not reassortants like the 1957 and 1968 pandemic strains; instead, all of the viral genes originated from a Eurasian avian virus[40,41]. The gene that encodes HA was derived from a H5N1 virus first isolated from a goose that died in Guangdong Province, China (A/goose/Guangdong/1/96)[44]. From 1997 through 2001, H5N1 viruses with an HA of the same genetic lineage continued to circulate in birds in southeastern China[45–47]. In 2002, another H5N1 virus showing ANTIGENIC DRIFT emerged in Hong Kong and was highly pathogenic in ducks and other aquatic birds, a property rarely associated with HPAI viruses[48]. In early 2003, an H5N1 virus infected a family in Hong Kong[49] — the father and son (from whom the H5N1 virus was isolated) developed severe respiratory illness and the father died. The daughter also died of a respiratory infection of undiagnosed origin.

The most devastating outbreak of influenza associated with H5N1 HPAI viruses occurred in 2003–2004 in Asian countries, including Vietnam, Thailand, Indonesia, Cambodia, Laos, Korea, Japan and China (and later Malaysia)[50]. Although not officially reported, such viruses first appeared in July 2003 in poultry in Vietnam, Indonesia and Thailand. Extensive phylogenetic analysis of the viruses isolated from poultry in Hong Kong and mainland China revealed multiple genetic reassortants representing multiple genotypes (proposed definition: genotypes A, B, C, D, E, V, W, X_{0-3}, Y, Z and Z^+), although each of the reassortant viruses possessed an HA similar to that of the HA from the A/goose/Guangdong/1/96 strain[51]. Similarly, nine different genotypes were identified in domestic-duck isolates in mainland China (proposed definition: A–I), which were not related to the genotypes that were described for poultry isolates[52].

The H5N1 viruses with a dominant Z genotype were responsible for the outbreaks in Vietnam, Thailand and Indonesia[51,53], and had been detected in wild, migrating aquatic birds in Hong Kong in November 2002. Although the 2003 human isolate in Hong Kong showed genetic similarity to this aquatic-bird virus, it lacked a deletion in the NA 'stalk' region that is found in Z genotype viruses and was therefore given a Z^+ genotype[51]. By contrast, viruses isolated in 2004 in Japan, where one human case of H5 ANTIBODY SEROCONVERSION was confirmed[54], and in Korea belonged to genotype V. Their polymerase acid protein (PA) gene segments differed considerably from those of the genotype Z virus, although the remaining segments are related to this virus[55,56]. Therefore, at least two H5N1 genotypes, Z and V, seem to be responsible for the Asian outbreak in 2003–2004. Whether changes in the gene that encodes PA alone account for the different capacities of these viruses to infect humans and cause severe disease is unknown. Taken together, these observations indicate that domestic ducks and land-based poultry in southern China probably had a central role in the generation and maintenance of the H5N1 virus and that wild birds might have contributed to the broad spread of the virus.

Although outbreaks of HPAI viruses were confined to poultry (mainly chickens) in most countries, there was substantial transmission to humans, resulting in a total of 53 deaths in three countries: 37 of 76 infected persons in Vietnam, 12 of 17 infected persons in Thailand and all of four infected persons in Cambodia. The clinical presentations of fever, cough, diarrhoea, shortness of breath, rapid respiratory rate, lymphopaenia and abnormalities on chest radiography were similar to those noted during the 1997 H5N1 outbreak in Hong Kong, although diarrhoea was a more prominent feature in the Vietnamese patients[57]. The mortality rate in this outbreak was significantly higher than in the 1997 outbreak (54.6% versus 33.3%, calculated within the numbers of hospitalized cases reported officially), although both rates are undoubtedly overestimated owing to a lack of reliable denominators. Unlike the 1997 isolates, the Vietnamese human isolates were

REYE SYNDROME
A rare syndrome characterized by encephalopathy and fatty degeneration of the liver, occuring primarily in children upon a viral infection, including influenza. Although this syndrome has been epidemiologically associated with the use of aspirin and other salicylates, the exact pathogenesis of the disease remains unknown.

ANTIGENIC DRIFT
Change in the antigenicity of the haemagglutinin and neuraminidase owing to point mutations.

ANTIBODY SEROCONVERSION
The presence in serum of antibodies specific for antigens as a result of an infection.

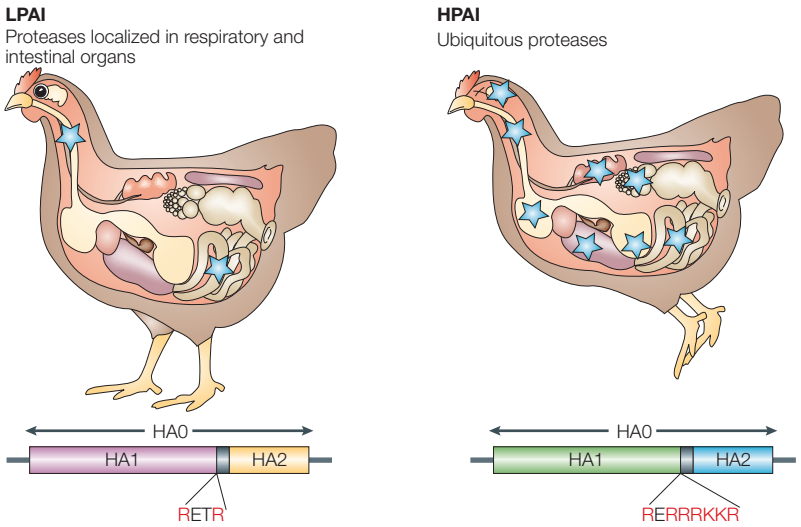

Figure 4 | **Haemagglutinin (HA) as a major determinant of the pathogenicity of avian influenza viruses in poultry.** Post-translational proteolytic cleavage of the HA precursor molecule (HA0) into HA1 and HA2 subunits by host proteases generates a fusogenic domain at the amino terminus of HA2 (shown in grey), which mediates fusion between the viral envelope and the endosomal membrane. Therefore, proteolytic activation of the HA molecule is essential for viral infectivity. The HAs of low-pathogenicity avian influenza (LPAI) viruses do not contain a series of basic amino acid (RETR) at the protease cleavage site and are cleaved by proteases that are localized in respiratory and intestinal organs, resulting in mild localized infections. By contrast, the HAs of high-pathogenicity avian influenza (HPAI) viruses possess multiple basic amino acids at the cleavage site (RERRRKKR), which are cleaved by ubiquitous proteases in a wide range of organs, resulting in lethal systemic infection.

resistant to the anti-influenza drugs amantadine and rimantadine (an amantadine derivative). There was no compelling evidence of human-to-human transmission in the more recent outbreak, with the exception of a few cases in Vietnamese[57] and Thai[58] families.

Information on the pathological features of human infection associated with avian H5N1 viruses is limited. The results of postmortem examination, reported for only two fatal cases of infection with the 1997 virus, showed reactive haemophagocytic syndrome as the most prominent feature, which involves diffuse alveolar damage with interstitial fibrosis, extensive necrosis of the hepatic central lobe, acute renal tubular necrosis and lymphoid depletion[59]. Elevated levels of cytokines such as interleukin-6 and interferon-γ (IFN-γ) were also detected, indicating that initial virus replication in the respiratory tract might trigger hypercytokinaemia, leading to reactive haemophagocytic syndrome. Similarly, patients infected with the 2003 H5N1 virus had unusually high serum concentrations of chemokines, such as IFN-inducing protein-10 and monokines induced by IFN-γ[49]. Therefore, cytokine dysregulation might contribute to the pathogenesis of H5N1 disease in humans. A similar cytokine/chemokine disorder has been detected in mice infected with viruses that express the HA from the 1918 influenza virus[11,13].

Interestingly, H5N1 viruses isolated from apparently healthy domestic ducks in southern China became progressively more pathogenic in mice after 2000 (REF. 52). Also, domestic cats and tigers died after eating poultry infected with H5N1 viruses[60], and the susceptibility of cats to H5N1 (REF. 61) and H7N7 (REF. 62) viruses has been demonstrated by experimental infection. Therefore, cats might serve as a biological vector, facilitating the transmission of H5N1 viruses to humans.

H9N2 virus. In 1999, one of two antigenically different H9N2 influenza viruses (A/quail/Hong Kong/G1/97-like and A/duck/Hong Kong/Y280/97-like) was transmitted from birds to two persons in Hong Kong, causing mild respiratory disease. In mainland China, five human cases of H9N2 virus infection were confirmed[63,64]. The G1-like virus, isolated from a human case in Hong Kong, is thought to have been involved in the generation of the HPAI H5N1 virus in 1997, as the two viruses share the same evolutionary origin of their six internal gene segments[65]. The H9N2 viruses were also detected in pigs in Hong Kong and are now PANZOOTIC in poultry in Eurasia[66]. In December 2003, an H9N2 (Y280-like) virus again infected humans in Hong Kong, causing mild respiratory disease in a child[67]. Therefore, in addition to H5N1 viruses, H9N2 viruses also pose a pandemic threat to humans, with their capacity to be transmitted to, and cause disease in, immunologically naive humans.

H7 viruses. Previously, several self-limiting human infections with H7 viruses have been documented[68–70]. In 1996, an H7N7 avian influenza virus was isolated from a woman with conjunctivitis in England[71]. The source of the virus was considered to be waterfowl, as the woman tended a collection of ducks that mixed freely with feral waterfowl on a small lake. The entire HA gene of this human isolate showed close homology with an H7N7 virus isolate from a turkey in Ireland in 1995 (REF. 72).

Between February and May 2003, an HPAI H7N7 virus infected poultry in the Netherlands and was then transmitted to at least 89 people, 83 of whom presented with conjunctivitis. Cases of influenza-like illness were limited and mild[73,74], although there was one fatal case of pneumonia in combination with acute respiratory distress syndrome[75]. Human-to-human transmission of the H7N7 virus was documented in three cases, in which the virus was acquired from a family member with conjunctivitis. Antibodies were also detected in 59% of the household contacts of infected poultry workers. Approximately 50% of the 500 persons examined who had contact with infected poultry during the epidemic had antibodies against the virus. It is estimated that at least 1,000 individuals were infected with the H7N7 virus during this epidemic in the Netherlands[76].

In 2004, an HPAI H7N3 virus emerged in poultry in British Columbia, Canada, and was apparently transmitted to two persons, whose clinical signs included conjunctivitis and mild respiratory symptoms[77]. The molecular basis for the ability of H7, but not H5, viruses to cause conjunctivitis in humans is unknown.

PANZOOTIC
Global prevalence of an infection in non-human animal hosts.

Molecular determinants of human infection

It should be stressed that multiple genetic factors contribute to the efficient growth and pathogenicity of influenza viruses in any host. Nonetheless, key determinants have been elucidated and others are beginning to emerge.

HA. As already mentioned, the HA glycoprotein is involved in host-cell recognition and is therefore an important determinant of host range. Human viruses preferentially recognize host-cell receptors containing sialyloligosaccharides terminated by *N*-acetyl sialic acid linked to galactose with an α2,6 linkage (NeuAcα2,6Gal), whereas avian viruses preferentially recognize host-cell receptors containing *N*-acetyl sialic acid linked to galactose by an α2,3 linkage (NeuAcα2,3Gal)[78–81]. The epithelial cells in the human trachea contain mainly NeuAcα2,6Gal (REF. 82), whereas those in duck trachea and intestine contain mainly NeuAcα2,3Gal linkages[80] (FIG. 5). In the pig trachea, epithelial cells contain both NeuAcα2,6Gal and NeuAcα2,3Gal linkages[83], explaining why pigs are highly susceptible to both human and avian viruses and are thought by some to be a 'mixing vessel' for avian and human viruses, reassortment of which might give rise to pandemic strains. However, there is no evidence that the 1957 Asian and 1968 Hong Kong pandemic viruses were generated in pigs.

The crucial residues determining α2,3 or α2,6 specificity differ among HA subtypes. For example, for the H3 HA, Leu226 found in human virus HAs, instead of Gln226 found in avian viruses, confers α2,6 specificity[79], whereas for the H1 HA, Asp190 found in swine and human viruses, but not Glu190 found in avian viruses, is responsible for this specificity, as predicted by sequence comparison[84] and crystallographic analysis of the 1918 virus HA[9,10], and confirmed by a direct receptor specificity assay[11].

The index 1997 human-isolated H5N1 virus preferentially recognized the avian receptor, NeuAcα2,3Gal (REF. 85). Recently, α2,3-linked sialic acids were identified on ciliated cells in differentiated cultures of human tracheobronchial epithelium, and avian, but not human, viruses infected these cells[86]. So, there are cells in the human airway that are susceptible to avian viruses. The prevalence of cells possessing α2,3 and/or α2,6-linked sialic acids in the lower respiratory tract, including epithelial cells in bronchiole and alveolar cells, is still unknown. Also, it is highly probable that the receptor specificity of the H5N1 virus could be converted to a human type during repeated replication in humans or pigs[87]. The earliest isolates from the 1918, 1957 and 1968 pandemics preferentially recognized NeuAcα2,6Gal (REF. 84), even though their HAs were derived from an avian virus. This indicates that conversion of receptor specificity to NeuAcα2,6Gal is an alteration that might be necessary for the generation of a virus with pandemic potential.

An intriguing concept that might account for the emergence of some pandemic viruses was recently proposed. It states that the receptor specificity of viruses circulating in land-based poultry differs from that of viruses circulating in aquatic birds[85,88]. In support of this hypothesis, H5N1 viruses isolated from chickens had a substantially lower affinity for NeuAcα2,3Gal than did aquatic bird viruses[85], and this reduced affinity was similar to that of human influenza viruses, although the H5N1 chicken isolates did not show NeuAcα2,6Gal specificity. Moreover, the H5N1 chicken isolates with reduced receptor-binding affinity had acquired an additional glycosylation site in the globular head region of the HA, as well as a deletion in the NA stalk region — molecular features found in the glycoproteins of human viruses[85]. Strikingly, H9N2 viruses isolated

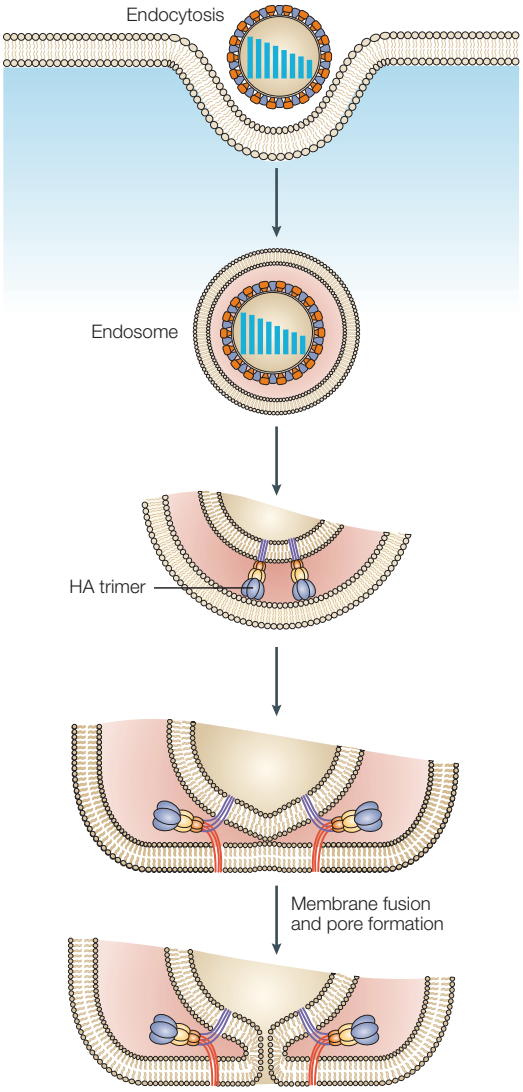

Figure 5 | **Schematic representation of mechanism of action of influenza A haemagglutinin (HA).** HA mediates virus binding to sialic-acid-containing host-cell receptors. Following binding, the virus is internalized by endocytosis. Acidification of the endosome environment induces conformational changes in the HA trimer, mediating fusion between the viral envelope and the endosomal membrane, which allows delivery of the viral ribonucleoprotein into host cells. Image modified with permission from REF. 81 © (2000) Annual Reviews.

Box 1 | **Options for pandemic control**

Antivirals

Amantadine inhibits virus uncoating by interfering with the activity of the M2 ion-channel protein[107]. However, a single mutation in human and avian influenza viruses can confer resistance to amantadine[108]. Moreover, the 2004 H5N1 Vietnam and Thailand isolates were resistant to amantadine because of mutations in the M2 gene segment[51]. Therefore, the potential of amantadine and amantadine derivatives for controlling pandemics might be limited. By contrast, anti-influenza neuraminidase (NA) inhibitors, such as oseltamivir, which interfere with virus release from cells[109,110], efficiently blocked the NA activity of 2004 H5N1 viruses in *in vitro* assays[55], indicating that they would be effective for chemotherapy and prophylaxis against H5N1 virus infection. Although resistance to these compounds emerges less frequently than resistance to amantadine, resistant viruses have been identified *in vitro*[111] and *in vivo*[112], emphasizing the need for careful monitoring whenever NA inhibitors are used to control pandemic influenza.

Vaccines

Although vaccination is one of the most effective means to control influenza, the pathogenicity of the H5N1 viruses creates many problems and safety concerns for vaccine manufacture. New vaccines are being developed, based on reverse genetics technology[113–115] and the knowledge that high-pathogenicity avian influenza (HPAI) viruses carry multiple basic amino acids at the haemagglutinin (HA) cleavage site, whereas low-pathogenicity avian influenza (LPAI) viruses lack such sequences[31] (TABLE 1). Conversion of the HA cleavage site sequence of HPAI viruses to that of LPAI viruses attenuates viral virulence but does not affect antigenicity[32]. Using this rationale, several research groups have produced candidate H5N1 vaccine strains whose HA and NA are derived from a human H5N1 virus, with the remainder of the genes coming from a virus that grows well in eggs[116–118]. Clinical trials with one of these strains[119,120] will be carried out in several different countries in the near future. One of the main concerns with this vaccine is that it might not induce sufficient protective immunity, as exemplified by recent experience with alternative H5 (REFS 121,122) or H9 (REFS 123,124) vaccines. Therefore, an adjuvant or an alternative immunization route (for example, nasal inoculation) might be required. A live H5N1 vaccine based on a recently licensed product, FLUMIST (MedImmune Vaccines, Inc.), is also an option[125]; however, it could not be used until the H5N1 virus has become widespread among humans, unless the vaccine strain could be genetically modified to ensure that it does not reassort with human field strains.

from land-based poultry, but not from aquatic birds, showed a receptor specificity that was similar to that of human isolates[88,89]. These findings indicate that avian viruses in land-based poultry might pose a greater infectious threat to humans than the same viruses in aquatic birds.

Enhanced HA cleavability owing to the presence of multiple basic amino acids at the cleavage site is essential for the high pathogenicity of avian viruses in poultry. All of the avian viruses that have killed humans possessed a highly cleavable HA[40,41,73], indicating that this requirement also extends to pathogenicity in humans. Indeed, the virulence of an H5N1 mutant virus in which the HA cleavage-site sequence was changed to an avirulent type was attenuated in a mouse model[90]. The H9N2 viruses isolated from humans in 1999 displayed a human-receptor specificity[88,89], but did not cause fatal infections, probably because they lacked a highly cleavable HA. The evidence reported to date indicates that HPAI viruses, of either the H5 or H7 subtype, have the potential to cause devastating pandemics in the future.

PB2. The 1997 H5N1 isolates from humans in Hong Kong formed two groups based on their pathogenicity in mice, which generally corresponded to the severity of disease in humans[91,92]. A reverse genetics study revealed that the amino acid at position 627 of PB2, a subunit protein of the viral RNA polymerase, determines the efficiency of virus replication in mice: Lys627, instead of the Glu627 that is found in avian viruses, is crucial for high virulence[90]. However, this amino acid does not determine the cell tropism of the virus[93], but instead enhances viral growth in mice and probably in humans. This finding is supported by earlier studies of a single-gene reassortant virus (containing the PB2 gene from an avian virus and all other gene segments from a human virus) that displayed a restricted host-range phenotype characterized by efficient replication in avian, but not mammalian, cells[94]. In this virus, residue 627 in the PB2 protein was shown to be responsible for host-cell restriction (Glu627 in avian and Lys627 in human viruses)[95]. Interestingly, the 2003 H7N7 virus isolated from the fatal human case of pneumonia in the Netherlands also possessed Lys627 in PB2, in contrast to avian viruses isolated during the outbreak and other human isolates from non-fatal cases of conjunctivitis[73]. Similarly, many, but not all, of the 2004 H5N1 viruses isolated from humans in Vietnam harboured Lys627 in PB2. These findings implicate Lys627 in PB2 as an important determinant of virus replicative ability and host-cell tropism in humans.

NS1. Unlike most human, avian and swine viruses, the 1997 H5N1 viruses are resistant to the antiviral effects of IFNs and tumour-necrosis factor-α (TNF-α). Therefore, a recombinant human H1N1 virus that contains the non-structural (NS) gene segment from the 1997 H5N1 virus showed increased pathogenicity in pigs[96,97]. Indeed, previous studies had indicated that the NS1 gene functions as an antagonist of the host IFN defence system in multiple ways[98–100]. The idea of a crucial role for the NS1 gene segment of the 1997 H5N1 virus in determining pathogenicity in humans is supported by

FLUMIST
A live attenuated influenza vaccine based on cold-adapted viruses.

another report, which shows that a recombinant virus containing the NS gene from the 1918 Spanish influenza virus blocked the expression of IFN-regulated genes in human lung cells more efficiently than a wild-type virus[101]. So, counteracting the host's innate immunity at an early phase of infection seems to be an important step for the efficient replication of avian viruses in human cells. Interestingly, infection with viruses containing the NS gene from the 1997 Hong Kong H5N1 virus led to increased transcription of proinflammatory cytokine genes (such as TNF-α and IFN-β) in human primary monocyte-derived macrophages *in vitro* compared with infection with other types of viruses[102]. Such an upregulation of cytokine function at later phases of infection might explain the unusual clinical presentation and severity of disease associated with human H5N1 infection. Overall, these results indicate that an NS1-induced cytokine imbalance, similar to the hypercytokinaemia associated with the 1997 and 2003 H5N1 viruses, probably contributes to the extreme pathogenicity of avian viruses in humans.

Other gene products. The 1997 H5N1 chicken and human viruses contained a 19-amino-acid deletion in the NA stalk region that probably decreased NA enzymatic activity[85]. Whether this deletion is involved in the pathogenicity of the H5N1 viruses in humans is unclear, although a similar change is probably required for the adaptation of influenza viruses from wild aquatic birds to chickens and has been recognized in HPAI viruses[103].

The genes encoding the internal proteins of viruses other than PB2 also probably influence host specificity[104,105]. For example, replacement of the PB1 gene of a human virus with that of an avian virus resulted in attenuation of viral replicative ability in cells of mammalian origin (MDCK cells) and in squirrel monkeys, but not in chicken kidney cells[106]. However, these findings can be interpreted either as incompatibility among viral gene products or solely as a contribution of these gene products to host range restriction. Together with the lack of appropriate animal models, this type of ambiguity in interpreting experimental results poses considerable difficulties in determining the factors responsible for the restriction of avian viruses in humans.

Emergence of pandemic strains

Historically, influenza pandemics have been caused by viruses possessing avian-virus-derived HAs to which human populations lack immunity; this mechanism of emergence will probably continue. Whether these viruses are introduced directly or indirectly into human populations is uncertain. Pandemics in the previous century were caused by the introduction of a wholly avian virus or an avian–human reassortant[14]. Phylogenetic and virological studies of isolates from these global outbreaks suggest the following two possibilities. Firstly, transmission of viruses from land-based poultry. In this scenario, viruses from quails, chickens or other types of land-based poultry, for which the receptor specificity has been altered during adaptation in these birds towards that of a human virus[85,88], directly infect humans and undergo further adaptation, with mutations in genes that encode both surface and internal proteins, or reassort with a human virus, acquiring genes that encode internal proteins required for efficient viral replication. Second, involvement of pigs. In this scenario, avian viruses are transmitted to pigs, where they adapt for efficient growth in humans by acquiring mutations in the HA that are necessary to recognize α2,6-linked sialic acids[84] and possibly also in the genes encoding internal proteins. Alternatively, both avian and human viruses are transmitted to pigs, where they reassort, and the resultant reassortant viruses can be transmitted to humans: the classic 'pigs-as-mixing vessels' concept.

Regardless of the scenario, further adaptive mutations in the gene segments that encode both surface and internal proteins are probably introduced after the viruses enter human populations. Therefore, viruses can infect humans and trigger pandemics even when they are not optimally adapted for growth in the human host, while continued replication in humans allows the viruses to 'fine tune' their replicative machinery.

In addition, accidental release of a virus that was previously circulating in, but had since disappeared from, human populations might also lead to a pandemic. In fact, this was probably the case for the outbreak of Russian influenza in 1977.

Concluding remarks

The recent influenza outbreaks in Asia provide a warning that any type A influenza virus has the potential to trigger a pandemic and that avian viruses do not need to reassort with a human virus first, nor do they require an intermediate mammalian host. Although Spanish influenza exacted a frightening toll of victims in 1918–1919, its mortality rate seems to have been lower than the rates reported for the recent H5N1 human influenza in Hong Kong, Vietnam and Thailand. Whether H5N1 viruses will acquire the ability to spread rapidly through human populations is uncertain. Past pandemics indicate that a change in host-cell-receptor specificity, and possibly in other properties of the viral gene products, is required for efficient human-to-human transmission of the virus. If this occurs, and if the virus continues to have such a high mortality rate, the resultant pandemic will have a devastating global impact. Additionally, it is uncertain whether H5N1 viruses will emerge that are resistant to anti-influenza drugs, especially the NA INHIBITORS. Nonetheless, a lack of other options has led to efforts to stockpile these drugs, while work to produce an effective vaccine is underway (BOX 1). Alternatively, one could efficiently prevent a pandemic outbreak by stopping the transmission of H5N1 viruses from poultry to humans. Although national and regional governments have been collaborating on strategies to achieve this goal, the results remain inadequate. The investment of time and money needed to control H5N1 viruses in poultry might seem excessive, but could easily be justified in terms of the number of human lives spared.

NA INHIBITORS
Compounds, produced based on the three-dimensional structure of the neuraminidase (NA), that inhibit NA sialidase activity, preventing efficient viral release from cells.

1. Lamb, R. A. & Krug, R. M. In *Fields Virology* 4th edn (eds Fields, B. N., Knipe, D. M. & Howley, P. M.) Ch. 46 (Lippincott-Raven, Philadelphia, 2001).
2. Wright, P. F. & Webster, R. G. In *Fields Virology* 4th edn (eds Fields, B. N., Knipe, D. M. & Howley, P. M.) Ch. 47 (Lippincott-Raven, Philadelphia, 2001).
3. 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).
4. 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).
5. Johnson, N. P. & Mueller, J. Updating the accounts: global mortality of the 1918–1920 "Spanish" influenza pandemic. *Bull. Hist. Med.* **76**, 105–115 (2002).
6. Reid, A. H. & Taubenberger, J. K. The origin of the 1918 pandemic influenza virus: a continuing enigma. *J. Gen. Virol.* **84**, 2285–2292 (2003).
7. Simonsen, L. *et al.* Pandemic versus epidemic influenza mortality: a pattern of changing age distribution. *J. Infect. Dis.* **178**, 53–60 (1998).
8. Reid, A. H, Taubenberger, J. K. & Fanning, T. G. Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. *Nature Rev. Microbiol.* **2**, 909–914 (2004).
9. Gamblin, S. J. *et al.* The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science* **303**, 1838–1842 (2004).
10. Stevens, J. *et al.* Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* **303**, 1866–1870 (2004).
References 9 and 10 suggest a preferential binding of the HA of the Spanish influenza virus to human cell receptors based on its three-dimensional structure.
11. Kobasa, D. *et al.* Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature* **431**, 703–707 (2004).
Describes, in a mouse model, the crucial role of the HA of the Spanish influenza virus that plays a part in the extreme pathogenicity of this virus through a mechanism involving cytokine dysfunction.
12. 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).
13. Kash, J. C. *et al.* Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. *J. Virol.* **78**, 9499–9511 (2004).
14. Cox, N. J. & Subbarao, K. Global epidemiology of influenza: past and present. *Annu. Rev. Med.* **51**, 407–421 (2000).
15. Scholtissek, C., Rohde, W., Von Hoyningen, V. & Rott, R. On the origin of the human influenza virus subtype H2N2 and H3N2. *Virology* **87**, 13–20 (1978).
16. Kawaoka, T., Krauss, S. & Webster, R. G. Avian-to-human transmission of the PB1 gene of influenza A virus. *J. Virol.* **63**, 4603–4608 (1989).
17. Zhou, N. N. *et al.* Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *J. Virol.* **73**, 8851–8856 (1999).
18. Nakajima, K., Desselberger, U. & Palese, P. Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. *Nature* **274**, 334–339 (1978).
19. Alexander, D. J., Parsons, G. & Manvell, R. J. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol.* **15**, 647–662 (1986).
20. Mo, I. P., Brugh, M., Fletcher, O. J., Rowland, G. N. & Swayne, D. E. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. *Avian Dis.* **41**, 125–136 (1997).
21. Kobayashi, Y., Horimoto, T., Kawaoka, Y., Alexander, D. J. & Itakura, C. Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. *Avian Pathol.* **25**, 285–304 (1996).
22. White, J., Kartenbeck, J. & Helenius, A. Membrane fusion activity of influenza virus. *EMBO J.* **1**, 217–222 (1982).
23. Klenk, H.-D., Rott, R., Orlich, M. & Blodorn, J. Activation of influenza A viruses by trypsin treatment. *Virology* **68**, 426–439 (1975).
24. Webster, R. G. & Rott, R. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. *Cell* **50**, 665–666 (1987).
25. Gotoh, B. *et al*. An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. *EMBO J.* **9**, 4189–4195 (1990).
26. Kido, H. *et al.* Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. *J. Biol. Chem.* **267**, 13573–13579 (1992).
27. Murakami, M. *et al.* Mini-plasmin found in the epithelial cells of bronchioles triggers infection by broad-spectrum influenza A viruses and Sendai virus. *Eur. J. Biochem.* **268**, 2847–2855 (2001).
28. Stieneke-Grober, A. *et al.* Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J.* **11**, 2407–2414 (1992).
29. Horimoto, T, Nakayama, K., Smeekens, S. P. & Kawaoka, Y. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avain influenza viruses. *J. Virol*. **68**, 6074–6078 (1994).
30. Kawaoka, Y., Naeve, C. W. & Webster, R. G. Is virulence of H5N2 influenza viruses in chicken associated with loss of carbohydrate from the hemagglutinin? *Virology* **139**, 303–316 (1984).
31. Kawaoka, Y. & Webster, R. G. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. *Proc. Natl Acad. Sci. USA* **85**, 324–328 (1988).
32. Horimoto, T. & Kawaoka, Y. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. *J. Virol.* **68**, 3120–3128 (1994).
33. Horimoto, T. *et al.* Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. *Virology* **213**, 223–230 (1995).
34. Garcia, M., Crawford, J. M., Latimer, J. W., Rivera-Cruz, E. & Perdue, M. L. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. *J. Gen. Virol.* **77**, 1493–1504 (1996).
35. Banks, J. *et al.* Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. *Arch. Virol.* **146**, 963–973 (2001).
36. Suarez, D. L. *et al.* Recombination resulting in virulence shift in avian influenza outbreak, Chile. *Emerg. Infect. Dis.* **10**, 693–699 (2004).
37. Hirst, M. *et al.* Novel avian influenza H7N3 strain outbreak, British Columbia. *Emerg. Infect. Dis.* **10**, 2192–2195 (2004).
38. Beare, A. S. & Webster, R. G. Replication of avian influenza viruses in humans. *Arch. Virol.* **119**, 37–42 (1991).
39. de Jong, J. C., Claas, E. C. J., Osterhaus, A. D. M. E., Webster, R. G. & Lim, W. L. A pandemic warning. *Nature* **389**, 554 (1997).
40. 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).
41. Claas, E. J. *et al.* Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* **351**, 472–477 (1998).
References 40 and 41 analysed the index case of H5N1 human infection in 1997 in Hong Kong, and revealed that a purely avian virus was transmitted to a human for the first time.
42. Yuen, K. Y. *et al*. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet* **351**, 467–471 (1998).
43. Buxton Bridges, C. *et al.* Risk of influenza A (H5N1) infection among health care workers exposed to patients with influenza A (H5N1), Hong Kong. *J. Infect. Dis.* **181**, 344–348 (2000).
44. 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 outbreaks in Hong Kong. *Virology* **261**, 15–19 (1999).
45. Cauthen, A. N., Swayne, D. E., Schultz-Cherry, S., Perdue, M. L. & Suarez, D. L. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. *J. Virol.* **74**, 6592–6599 (2000).
46. Guan, Y. *et al.* Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. *Proc. Natl Acad. Sci. USA* **99**, 8950–8955 (2002).
47. Guan, Y. *et al.* H5N1 influenza: A protean pandemic threat. *Proc. Natl Acad. Sci. USA* **101**, 8156–8161 (2004).
48. Sturm-Ramirez K. M. *et al.* Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J. Virol*. **78**, 4892–4901 (2004).
49. Peiris, J. S. *et al*. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* **363**, 617–619 (2004).
50. World Health Organization. Avian influenza A (H5N1). *Weekly Epidemiol. Rev.* **79**, 65–70 (2004).
51. Li, K. S. *et al.* Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**, 209–213 (2004).
Describes the phylogenetic relationships of the genes of Asian H5N1 viruses, showing a series of genetic reassortment events that are traceable to the precursor virus in 1997 and subsequent avian outbreaks in 2001 and 2002, which gave rise to a dominant genotype responsible for the outbreaks in 2003–2004.
52. Chen, H. *et al.* The evolution of H5N1 influenza viruses in ducks in southern China. *Proc. Natl Acad. Sci. USA* **101**, 10452–10457 (2004).
Describes that H5N1 viruses, isolated from healthy domestic ducks in China after 1999, became progressively more pathogenic in a mammalian model.
53. Viseshakul, N. *et al.* The genome sequence analysis of H5N1 avian influenza A virus isolated from the outbreak among poultry populations in Thailand. *Virology* **328**, 169–176 (2004).
54. ProMED-mail. Avian influenza, humans — Japan (Kyoto) (03) [online], <http://www.promedmail.org> (Archive Number 20041222. 3372, 22 Dec 2004).
55. Mase, M. *et al.* Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. *Virology* **332**, 167–176 (2005).
56. Lee, C. W. *et al.* Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J. Virol.* **79**, 3692–3702 (2005).
References 55 and 56 describe, respectively, that the H5N1 viruses isolated in Japan and South Korea were genotypically and pathologically different from those prevalent in southeast Asia.
57. Tran, T. H. *et al.* Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med.* **350**, 1179–1188 (2004).
58. Ungchusak, K. *et al.* Probable person-to-person transmission of avian influenza A (H5N1). *N. Engl. J. Med.* **352**, 333–340 (2005).
A report on probable human-to-human transmission of the H5N1 virus.
59. To, K. F. *et al.* Pathology of fatal human infection associated with avian influenza A H5N1 virus. *J. Med. Virol.* **63**, 242–246 (2001).
60. Enserink, M. & Kaiser, J. Avian flu finds new mammal hosts. *Science* **305**, 1385 (2004).
61. Kuiken, T. *et al.* Avian H5N1 influenza in cats. *Science* **306**, 241 (2004).
62. Nakamura, J. & Iwasa, T. On the fowl-pest infection in cat. *Jpn. J. Vet. Sci*. **4**, 511–523 (1942).
63. Peiris, M. *et al*. Human infection with influenza H9N2. *Lancet* **354**, 916–917 (1999).
64. Guo, Y. *et al.* Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology* **267**, 279–288 (2000).
65. 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).
66. Choi, Y. K. *et al.* Continuing evolution of H9N2 influenza viruses in southeastern China. *J. Virol.* **78**, 8609–8614 (2004).
67. World Health Organization disease alert: 10 December 2003. Influenza A (H9N2) in Hong Kong Special Administrative Region of China. [online], <http://www.who.int/csr/disease/avian_influenza/updates/en> (2003).
68. Campbell, C. H., Webster, R. G. & Breese, S. S. Jr. Fowl plague virus from man. *J. Inf. Dis.* **122**, 513–516 (1970).
69. Taylor, H. R. & Turner, A. J. A case report of fowl plague keratoconjunctivitis. *Br. J. Ophthalmol.* **61**, 86–88 (1977).
70. Webster, R. G., Geraci, J., Petursson, G. & Skirnission, K. Conjunctivitis in human beings caused by influenza A virus of seals. *N. Engl. J. Med.* **304**, 911 (1981).
71. Kurtz, J., Menvell, R. J. & Banks, J. Avian influenza virus isolated from a woman with conjunctivitis. *Lancet* **348**, 901–902 (1996).
72. Banks, J., Speidel, E. & Alexander, D. J. Characterization of an avian influenza A virus isolated from a human — is an intermediate host necessary for the emergence of pandemic influenza viruses? *Arch. Virol.* **143**, 781–787 (1998).
73. Fouchier, R. A. M. *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).
74. 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).
References 73 and 74 summarize human infections with a highly pathogenic avian H7N7 virus in the Netherlands in 2003.
75. van Kolfschooten, F. Dutch veterinarian becomes first victim of avian influenza. *Lancet* **361**, 1444 (2003).
76. Bosman, A. *et al.* Avian flu epidemic 2003: public health consequences. *RIVM Report* **630940004** (2004).

77. Tweed, S. A. *et al.* Human illness from avian influenza H7N3, British Columbia. *Emerg. Infect. Dis.* **10**, 2196–2199 (2004).
78. Rogers, G. N. & D'Souza, B. L. Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* **173**, 317–322 (1989).
79. 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).
80. Ito, T. & Kawaoka, Y. Host-range barrier of influenza A viruses. *Vet. Microbiol.* **74**, 71–75 (2000).
81. Skehel, J. J. & Wiley, D. C. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem*. **69**, 531–569 (2000).
82. Couceiro, J. N. S. S., Paulson, J. C. & Baum, L. G. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium: the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res.* **29**, 155–165 (1993).
83. Ito, T. *et al.* Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.* **72**, 7367–7373 (1998).
84. Matrosovich, M. *et al.* Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J. Virol*. **74**, 8502–8512 (2000).
85. Matrosovich, M., Zhou, N., Kawaoka, Y. & Webster, R. G. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. *J. Virol.* **73**, 1146–1155 (1999).
86. Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A. & Klenk, H-D. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc. Natl Acad. Sci. USA* **101**, 4620–4624 (2004).
The first demonstration that human airway epithelium contains cells possessing avian-type receptors.
87. Harvey, R., Martin, A. C., Zambon, M. & Barclay, W. S. Restrictions to the adaptation of influenza A virus H5 hemagglutinin to the human host. *J. Virol.* **78**, 502–507 (2004).
88. 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).
89. Saito, T. *et al*. Characterization of a human H9N2 influenza virus isolated in Hong Kong. *Vaccine* **20**, 125–133 (2001).
90. 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).
This paper describes that one amino acid in PB2, an RNA polymerase subunit, affects virulence of avian H5N1 virus in a mammalian model.
91. Gao, P. *et al.* Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J. Virol.* **73**, 3184–3189 (1999).
92. 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).
93. Shinya, K. *et al*. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. *Virology* **320**, 258–266 (2004).
94. Clements, M. L. *et al.* Use of single-gene reassortant viruses to study the role of avian influenza A virus genes in attenuation of wild-type human influenza A virus for squirrel monkeys and adult human volunteers. *J. Clin. Microbiol.* **30**, 655–662 (1992).
95. 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).
96. Seo, S. H., Hoffmann, E. & Webster, R. G. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nature Med.* **8**, 950–954 (2002).
97. Seo, S. H., Hoffmann, E. & Webster, R. G. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. *Virus Res.* **103**, 107–113 (2004).
References 96 and 97 indicate that one amino acid in NS1, an IFN antagonist, affects the virulence of an avian H5N1 virus in a mammalian model.
98. Garcia-Sastre, A. *et al.* Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* **252**, 324–330 (1998).
99. Garcia-Sastre, A. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology* **279**, 375–384 (2001).
100. Krug, R. M., Yuan, W., Noah, D. L. & Latham, A. G. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* **309**, 181–189 (2003).
101. Geiss, G. K. *et al.* Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc. Natl Acad. Sci. USA* **99**, 10736–10741 (2002).
102. Cheung, C. Y. *et al.* Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? *Lancet* **360**, 1831–1837 (2003).
103. Deshpande, K. L., Naeve, C. W. & Webster, R. G. The neuraminidases of the virulent and avirulent A/Chicken/Pennsylvania/83 (H5N2) influenza A viruses: sequence and antigenic analyses. *Virology* **147**, 49–60 (1985).
104. Zhou, N. N., Shortridge, K. F., Claas, E. C. J., Krauss, S. L. & Webster, R. G. Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. *J. Virol.* **73**, 3366–3374 (1999).
105. Katz, J. M. *et al.* Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *J. Virol.* **74**, 10807–10810 (2000).
106. Snyder, M. H., Buckler-White, A. J., London, W. T., Tierney, E. L. & Murphy, B. R. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses from monkeys. *J. Virol*. **61**, 2857–2863 (1987).
107. Wang, C., Takeuchi, K., Pinto, L. H. & Lamb, R. A. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. *J. Virol.* **67**, 5585–5594 (1993).
108. Crumpacker, C. In *Fields Virology* 4th edn (eds Fields, B. N., Knipe, D. M. & Howley, P. M.) Ch. 15 (Lippincott-Raven, Philadelphia, 2001).
109. von Itzstein, M. *et al.* Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363**, 418–423 (1993).
110. Gubareva, L. V., Kaiser, L. & Hayden, F. G. Influenza virus neuraminidase inhibitors. *Lancet* **355**, 827–835 (2000).
111. Gubareva, L. V. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. *Virus Res.* **103**, 199–203 (2004).
112. Kiso, M. *et al.* Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet* **364**, 759–765 (2004).
113. Neumann, G. *et al.* Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl Acad. Sci. USA* **96**, 9345–9350 (1999).
114. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R. G. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl Acad. Sci. USA* **97**, 6108–6113 (2000).
115. Fodor, E. *et al.* Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73**, 9679–9682 (1999).
116. 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).
117. Wood, J. M. & Robertson, J. S. From lethal virus to life-saving vaccine: the development of inactivated influenza vaccines for pandemic influenza. *Nature Rev. Microbiol.* **2**, 842–847 (2004).
An overview of development of H5N1 influenza vaccine using reverse genetics.
118. 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).
119. WHO Global Influenza Programme. Production of pilot lots of inactivated influenza vaccines from reassortant derived from avian influenza viruses: interim biosafety risk assessment. [online], < http://www.who.int/csr/resources/publications/influenza/en/influenzaRMD2003_5.pdf> (2003).
120. 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).
121. 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).
122. Treanor, J. J. *et al.* Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. *Vaccine* **19**, 1732–1737 (2001).
123. Hehme, N., Engelmann, H., Kunzel, W., Neumeier, E. & Sanger, R. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. *Med. Microbiol. Immunol.* **191**, 203–208 (2002).
124. 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).
125. Li, S. Q. *et al.* Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. *J. Infect. Dis.* **179**, 1132–1138 (1999).

Acknowledgements

We thank J. Gilbert for editing the manuscript. We also thank those in our laboratories who contributed to the data cited in this review. Our original research was supported by National Institute of Allergy and Infectious Diseases Public Health Service research grants, by CREST (Japan Science and Technology Agency) and by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Competing interests statement

The authors declare no competing interests.

Online links

DATABASES

The following terms in this article are linked online to:
Infectious Disease Information: http://www.cdc.gov/ncidod/diseases/index.htm
Influenza
Protein Data Bank: http://www.rcsb.org/pdb
HA | NA

FURTHER INFORMATION

Yoshihiro Kawaoka's homepages:
http://www.growingenius.com/firstpage.htm
http://www.vetmed.wisc.edu/pbs/kawaoka
WHO Influenza Information: http://www.who.int/csr/disease/influenza/en
WHO Avian Influenza Information: http://www.who.int/csr/disease/avian_influenza/en
Access to this interactive links box is free online.