Microbial adaptation and change: avian influenza

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Summary
The evolution of influenza is a continuing process involving viral and host factors. The increasing frequency of emergence of the highly pathogenic H5N1, H7N3 and H7N7 influenza viruses and the panzootic spread of H9N2 influenza virus, all of which can be potentially transmitted to humans, are of great concern to both veterinary and human public health officials. The question is how soon the next pandemic will emerge. A convergence of factors, including the population densities of poultry, pigs and humans, are likely factors affecting the evolution of the virus. Highly concentrated poultry and pig farming, in conjunction with traditional live animal or ‘wet’ markets, provide optimal conditions for increased mutation, reassortment and recombination of influenza viruses. Strategies to reduce the evolution of influenza and the emergence of pandemics include the separation of species, increased biosecurity, the development of new vaccine strategies and better basic knowledge of the virus. More effective co-operation between scientists and veterinary and public health officials is required to achieve these goals.

Keywords

Introduction
The emergence and re-emergence of influenza viruses with pandemic potential for both human and veterinary public health is of great concern to humans globally. The convergence of factors affecting contemporary human and animal health issues has led to changing roles for veterinarians and public health officials worldwide. Humans influence global ecology with their increasing population density, growing demands on land use and high intensity farming to feed ever-increasing human populations. In addition, the development of rapid transportation ensures the global spread of any virus. Each of these factors has implications for the emergence of novel disease agents. This is particularly the case for influenza, since poultry, pigs and humans are all factors in its viral evolution. These issues were addressed in a recent Institute of Medicine report of the National Academy of Sciences of the United States of America (USA) (35). In this paper, the authors will consider the adaptation of influenza viruses to the changing world and the urgency for co-operation among disciplines in planning for a pandemic that many believe is imminent.

There are 16 haemagglutinin (HA) subtypes of influenza viruses, which are perpetuated in the aquatic birds of the world. This gene pool of influenza viruses in aquatic birds is largely benign, but evolves rapidly after transfer to domestic avian and mammalian species. Of the 16 HA subtypes, two that can evolve into highly pathogenic strains (H5 and H7) are of great concern to agricultural authorities, including the World Organisation for Animal Health. Other subtypes, including H9, H6 and H3, have established, or are in the process of establishing, permanent lineages in chickens and the severity of disease depends on co-infecting agents. From a human perspective, H1, H2 and H3 have caused pandemic and epidemic influenza in humans, while H5, H7 and H9 have
been transmitted to humans with mortality caused by Asian H5N1 strains. The remaining subtypes of influenza A viruses may evolve into pathogens of concern in the future but, at present, they are not considered to have pandemic potential. Nevertheless, all influenza A subtypes have the potential to contribute to the emergence of a pandemic strain through genetic reassortment. This is a continuous process in nature, which allows the exchange of gene segments (22, 24, 35).

Influenza virus

This section will consider the mechanisms used by influenza viruses to adapt to their host range, as well as possible control strategies. Special attention will be given to the H5, H7 and H9 subtypes, which are considered to have high pandemic potential.

Avian influenza is classified as a member of the Orthomyxoviridae family. The virus is enveloped and the genome consists of eight segments of linear negative-sense, single-stranded ribonucleic acid (RNA). The negative-stranded RNA of influenza serves as a template for the synthesis of messenger RNAs. It also serves as a template for the positive-sense strand. The eight segments of viral RNA encode for ten proteins. Three of these are polymerase proteins: PA, PB1 and PB2. Two are surface proteins: neuraminidase (NA) and HA. One is a nucleocapsid (NP), two are matrix proteins (M1 and M2) and two are non-structural proteins (NS1 and NS2).

A constantly varying virus: mechanisms of variation

Frequent mutations: antigenic drift

Influenza viruses lack ‘proof-reading’ mechanisms and are therefore unable to repair errors that occur during replication (46). These mutations accumulate amino acid changes within the viral genome, resulting in the existing strain being replaced by a new antigenic variant (Fig. 1). This mechanism of acquiring new mutations is known as ‘antigenic drift’ and the accumulation of point mutations is seen in each of the gene products of the virus. However, it is most pronounced in the surface proteins HA and NA, where selection is antibody-mediated.

Antigenic drift is a continuous process in mammalian influenza viruses and is the basis for the evolution of human epidemic influenza. Antigenic drift is most apparent in human influenza viruses, less so in swine and equine influenza viruses and usually least apparent in

Fig. 1
The three mechanisms used by influenza viruses to adapt to their host ranges
A. Antigenic drift occurs through errors during the replication of influenza viruses, which are unable to be repaired. These mutations accumulate amino acid changes within the viral genome, resulting in the existing strains being replaced by a new antigenic variant
B. Reassortment or antigenic shift is the exchange of ribonucleic acid (RNA) segments between two genotypically different influenza viruses infecting a single cell, which can result in the generation of a novel strain and/or subtype
C. Recombination occurs when two different sources of RNA contribute to a single influenza RNA segment
avian influenza viruses. It can be mimicked in the laboratory by the selection of escape mutants with monoclonal antibodies (6).

Antigenic drift in avian influenza viruses in their original aquatic bird reservoirs is limited but, after the virus has spread into domestic poultry, it can become more pronounced. Recent examples of antigenic drift, which are relevant to recently emerging influenza viruses, are found in the H5 and H9 subtypes in Asia (Tables I and II). Since antigenic drift is mediated by antibody pressure, researchers must accept that antigenic variation in influenza viruses is caused by immune pressure in poultry. Thus, vaccination can potentially contribute to increasing the rate of antigenic drift (20).

Reassortment: antigenic drift

Influenza viruses exhibit a high frequency of reassortment. Owing to the segmented nature of the influenza viral genome, ‘reassortment’, or the exchange of RNA segments between two genotypically different influenza viruses infecting a single cell, can result in the generation of a novel strain and/or subtype (Fig. 1). Theoretically, one pair of influenza viruses with eight segments each can produce 256 different combinations. Reassortment has been detected between influenza viruses in wild aquatic birds (12, 49), in live poultry markets (24) and in the evolution of the Z genotype of H5N1, which spread across Asia in 2004 (22) (Fig. 2).

The available evidence is that the human pandemics of 1957 (the Asian pandemic) and 1968 (the Hong Kong pandemic) each arose from reassortment between human and avian influenza viruses (18). The acquisition of novel HA and NA glycoproteins, together with a novel avian PB1 gene, allowed the reassorted viruses to circumvent the humoral immunity of the host while they gained the ability to spread from human to human from their human influenza precursor.

Other recent examples of reassortment among the influenza viruses of humans, pigs and birds are the H3N2 viruses that are circulating in pigs in the USA (16, 42, 50).

### Table I
Cross-reactivity (titres determined by haemagglutinin inhibition) of H9N2 viruses (isolated between 1997 and 2003) with post-infection serum to the H9N2 viruses

<table>
<thead>
<tr>
<th>Post-infection sera from H9N2 virus strains</th>
<th>A/quail/HK/G1/97</th>
<th>A/ck/HK/G9/97</th>
<th>A/ck/HK/FY313/00</th>
<th>A/ck/Nan/1-0016/00</th>
<th>A/duck/Shantou/4808/03</th>
<th>A/HK/2108/03</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/quail/HK/G1/97</td>
<td>320</td>
<td>&lt; 10</td>
<td>20</td>
<td>&lt; 10</td>
<td>40</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>A/ck/HK/G9/97</td>
<td>20</td>
<td>≥ 1,280</td>
<td>≥ 1,280</td>
<td>640</td>
<td>160</td>
<td>80</td>
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<tr>
<td>A/ck/HK/FY313/00</td>
<td>20</td>
<td>≥ 1,280</td>
<td>≥ 1,280</td>
<td>640</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>A/ck/Nan/1-0016/00</td>
<td>10</td>
<td>≥ 1,280</td>
<td>≥ 1,280</td>
<td>640</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>A/duck/Shantou/4808/03</td>
<td>&lt; 10</td>
<td>40</td>
<td>160</td>
<td>40</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>A/HK/2108/03</td>
<td>&lt; 10</td>
<td>80</td>
<td>320</td>
<td>160</td>
<td>320</td>
<td>≥ 1,280</td>
</tr>
</tbody>
</table>

HK: Hong Kong
ck: chicken
Nan: Nanchang

### Table II
Cross-reactivity (titres determined by haemagglutinin inhibition) of H5N1 viruses (isolated between 1997 and 2004) with post-infection serum to the H5N1 viruses

<table>
<thead>
<tr>
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<tbody>
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<td>A/HK/156/97</td>
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<td>160</td>
<td>80</td>
<td>≥ 1,280</td>
<td>≥ 1,280</td>
</tr>
<tr>
<td>A/gs/HK/437-4/99</td>
<td>80</td>
<td>640</td>
<td>160</td>
<td>80</td>
<td>640</td>
<td>≥ 1,280</td>
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<td>A/HK/213/03</td>
<td>40</td>
<td>320</td>
<td>640</td>
<td>320</td>
<td>640</td>
<td>80</td>
</tr>
<tr>
<td>A/VN/1203/03</td>
<td>10</td>
<td>&lt; 10</td>
<td>160</td>
<td>160</td>
<td>≥ 1,280</td>
<td>640</td>
</tr>
<tr>
<td>A/ck/VN/c58/04</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>40</td>
<td>40</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>A/VN/3046/04</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>40</td>
<td>40</td>
<td>640</td>
<td>320</td>
</tr>
</tbody>
</table>

HK: Hong Kong
gs: goose
VN: Vietnam
ck: chicken
Thus, reassortment occurs much more frequently than was previously realised and live bird markets in both Asia and the USA provide optimal conditions for the reassortment of influenza viruses (32, 44).

It has been published that pigs can serve as the intermediate host or reassortment vessel (31). Both α2-3 and α2-6 terminal sialic acid linkages are present in the cells found in pig tracheas. Having both receptor specificities could allow for human and avian viruses to replicate in the same cell, allowing reassortment to occur. Pigs not only provide ideal conditions for reassortment, they have also been implicated in interspecies transmission events between swine, avian and human hosts (1). Quail have also been identified as intermediate hosts, since they permit the replication of all the subtypes of influenza found in wild aquatic birds and may be involved in transmitting these viruses to chickens (28).

**Recombination**

Recombination results in a single influenza RNA segment containing genetic material from two different sources (Fig. 1). Recombination is a rare occurrence in influenza viruses. However, it has recently been documented more frequently (40). Recombination can occur through several mechanisms, including the ‘crossing-over’ or ‘copy-choice’ mechanism.

**Current status of H5, H7 and H9 avian influenza strains and their microbial adaptation**

Influenza A viruses of all 16 subtypes are perpetuated in aquatic birds throughout the world. After transfer to an alternative avian or mammalian host, influenza viruses undergo rapid evolution (26, 51). For avian influenza viruses, including H5, this transfer can occur in backyard poultry flocks or live poultry markets where ducks, geese, quail, pheasants, chickens, etc. are raised or housed together (44). Since avian influenza viruses are primarily transmitted through faeces, inadequate disease control measures between the live markets and industrial poultry farms, such as insufficient hygiene measures for people and trucks, can result in the spread of influenza. Once an influenza virus invades a commercial poultry farm, it has an optimum number of susceptible poultry for rapid viral evolution.

**H9 subtype: a low-pathogenic avian influenza problem**

The H9 subtypes in Asia became established in domestic poultry some time before 1990 and have evolved into three phylogenetic sub-lineages, as follows:

- G1
- Y280 (a G9-like subtype)
- Y439 (7, 21).
The G1 lineage is thought to have undergone reassortment with H5N1/97 viruses to generate new H9N2 viruses, which infected humans (23), as well as generating new highly pathogenic H5N1 viruses (7). The Y280 lineage was also involved in human infections, which arose from the virus undergoing reassortment with A/chicken/HongKong/G9/97 virus (10, 11). The H9N2 viruses have acquired the ability to bind to both the α2-3 and α2-6 sialic acid linkages, indicating specificity for both human and avian cells (27). It has been reported that H9N2 viruses in the People’s Republic of China were transmitted from chickens or quail back to domestic ducks, which provided a perfect environment for further reassortment. These multiple genotypes of H9N2 were generated between 2000 and 2001 in the People’s Republic of China (21). A recent study of H9 viruses circulating in Hong Kong bird markets in 2003 showed that the G1-like viruses were no longer present (3). This was most probably due to the removal of quail from the live bird markets. However, these viruses were replaced by a new, highly diverse set of H9N2 viruses. The haemagglutinin inhibition test shows the cross-reactivity of some of these H9N2 viruses and indicates that antigenic drift is occurring (Table I). Reassortment, linked with the re-introduction of H9N2 viruses from domestic poultry into ducks, contributes to the constant evolution of the viruses.

**H7 subtype: shifts in virulence cause health concerns for poultry and people**

The H7 subtype of avian influenza has always been of concern, due to the evolution of highly pathogenic variants, which have caused large economic losses in poultry flocks. Recently, it has also become a human health concern. In March 2003, the Netherlands experienced a large outbreak of highly pathogenic H7N7. Eighty-five people became infected, with the virus causing conjunctivitis and one death (4, 19). This highly pathogenic H7N7 variant originated from another virus which was isolated from a duck in the Netherlands in 2000. All of the internal genes of the H7N7 isolate were of avian origin (19).

The variant H7N2 has been a recurring problem for the live bird markets of the northeastern USA since 1994, and can be linked to three outbreaks in domestic poultry (39). These viruses once again caused problems in 2004. Data indicate that reassortment between HA, NA, M and NS genes played a large role in the genetic diversity found in H7N2 viruses before 1997 (38). However, after 1997, there does not seem to be further evidence of reassortment (36).

In 2002, a highly pathogenic H7N3 outbreak occurred in Chile (29). The highly pathogenic avian influenza (HPAI) virus emerged from a low pathogenic virus that had been isolated a few months earlier. Interestingly, the increase in virulence of the HPAI virus was linked with the insertion of 10 amino acids in the HA by a recombination event (40). The sequence inserted into the HA was similar to a portion of the NP gene of A/gull/Maryland/704/77 (H1N3) from positions 1,268 to 1,297 (40). The exact mechanism of the recombination event is unclear.

In February 2004, an HPAI H7N3 outbreak occurred in British Columbia, Canada. This outbreak, like the H7N3 outbreak in Chile, was also linked to a virulence shift from low pathogenic avian influenza to HPAI. This shift in virulence was also caused by a recombination event. However, in this British Columbian outbreak, the recombination occurred between the HA and M genes (14). Two human cases of H7N3 infections have been reported. Such recent occurrences indicate that recombination events can play a significant role in creating genetic diversity, resulting in virulence shifts for H7 viruses.

**H5N1: the continuing evolution of avian strains**

In 1997, H5N1 infected humans, killing six of the eighteen people who were clinically infected. The H5N1 virus that infected these people acquired all eight gene segments from Eurasian avian sources. It is speculated that this 1997 H5N1 virus was generated from an H5N1 goose-Guangdong-like virus from its natural reservoir in geese, which then crossed into chickens, hence acquiring internal genes from H9N2 and/or H6N1 by reassortment (8). The 1997 H5N1 strain was eradicated in Hong Kong. However, the HA gene of the 1997 strain continued to circulate in geese in the southeastern People’s Republic of China (48). In 2000, H5N1 viruses were isolated from aquatic poultry and from 2001 onwards, these viruses were isolated from aquatic birds as well as domestic chickens. In 2002, the HA gene of the H5N1 strains showed significant antigenic drift (9). The antigenic drift was pronounced in the genotype Z and Z+ viruses isolated in late 2002, as well as the human viruses isolated in early 2003 (9). In addition, these Z genotypes of H5N1 were lethal for aquatic poultry (37).

Between 2003 and 2004, the H5N1 epidemic spread across much of East Asia. Outbreaks in Indonesia, Thailand and Vietnam in late 2003 and early 2004 were all of genotype Z viruses (22) (Fig. 2). Moreover, H5N1 viruses were also isolated from the People’s Republic of China, Japan, South Korea, Cambodia, Laos and Malaysia. To date, 11 human deaths from 16 infections of H5N1 have been reported from Thailand and 20 human deaths out of 27 infections have been reported from Vietnam. The antigenic drift of these viruses can be illustrated by showing their cross-reactivity (Table II). Post-infection sera collected from earlier strains (between 1997 and 2003) no longer react with the new 2004 viruses. Interestingly, the new 2004 virus post-infection sera react more strongly with the older viruses than with the homologous 2004 virus.
The resurgence of HPAI H5N1 viruses on poultry farms in Thailand, Vietnam, the People’s Republic of China and Malaysia in the summer months (between July and September 2004) and the subsequent infection and deaths in humans in August 2004 raise the possibility that HPAI H5N1 viruses are now endemic in poultry in Asia. The available evidence is that H5N1 is widespread in domestic ducks in the southern People's Republic of China (2), and the above information supports the likelihood that H5N1 is endemic in domestic ducks throughout southern Asia. Although the Z genotype of H5N1 influenza virus has been dominant in Asia, there is heterogeneity in pathogenicity in ducks. Examination of multiple H5N1 isolates of the Z genotype for pathogenicity in ducks reveals that these isolates can be divided into three broad groups, as follows:

- those that kill all inoculated ducks with neurological signs of disease
- those that kill a percentage of ducks without neurological signs of disease
- those that replicate but cause no signs of disease (K.M. Sturm-Ramirez et al., unpublished findings).

All these H5N1 viruses found in ducks are highly pathogenic in gallinaceous poultry.

Controlling the spread of avian influenza

Whether the highly pathogenic H5N1 viruses that are probably endemic in domestic ducks have also been transmitted back into wild migrating birds is of great concern. This question is currently unresolved. If the hypothesis is correct, how will it affect control strategies?

Since wild birds are the source of all influenza viruses (47), they must be excluded from poultry farms. Thus, the biosecurity measures of screening commercial poultry houses for the presence of virus, treating water and food supplies and controlling access to farms are crucial, whether it is migrating or local birds which are spreading highly pathogenic or non-pathogenic influenza viruses. While poultry raised indoors can be separated from wild birds, poultry raised in open sheds cannot be separated. Thus, the practice of raising ducks on rice farms in Asia for insect control and retrieving residual grain at harvest is not in accordance with influenza control. Another practice that is not commensurate with influenza control is fish farming in Asia, where the faecal wastes from poultry sheds are dropped directly into fish ponds as fertiliser (30). The recently evolving intensive farming practice in the USA, of raising pigs and poultry in adjacent sheds with the same staff, is yet another unsound agricultural practice.

The first strategy for controlling influenza is preventing the virus from gaining access to domestic poultry and pigs. However, if biosecurity measures fail to prevent the transmission of an influenza virus to domestic animals and the virus becomes highly pathogenic, what are the control options?

Culling is the time-honoured strategy recommended by agricultural authorities. This involves:

- the establishment of quarantine zones
- banning the movement of poultry, poultry products and people to and from the affected areas
- repopulating the affected areas with livestock only after the ‘clean-up’ (successful application of disease-control measures) is complete and the area is free of the virus.

There is concern that the second wave of H5N1 in Asia between July and September 2004 was due, in part, to an inadequate clean-up, insufficient testing and premature restocking.

If the virus is not widespread, and it is not endemic in local poultry and wild birds, then culling is likely to be a successful strategy. Culling has been successfully used in many countries, including the USA for H5N2 in Pennsylvania in the 1980s, and in Japan for H5N1 in early 2004. If the virus is widespread in domestic animals or has become endemic in domestic animals and wild birds, then it is unlikely that culling alone will be successful.

The alternative strategy is culling plus vaccination. This method was adopted by the People’s Republic of China and Indonesia in 2004 for the control of highly pathogenic H5N1/04 influenza virus. There are diverse opinions on this strategy, which was used by Mexico to control highly pathogenic H5N2 in the 1990s. Although highly pathogenic H5N2 has not been reported in poultry in Mexico since this measure, the precursor virus was not eradicated, continues to circulate and has become highly pathogenic in poultry in Central America and the USA in 2004 (20). The arguments against the use of vaccination are as follows:

a) the use of vaccine does not encourage farmers to improve isolation, disease control or biosecurity measures
b) vaccination can successfully prevent signs of the disease but does not completely prevent virus shedding
c) vaccine use has been reported to promote the selection of antigenic drift variants (20)
d) vaccine use may cause the virus to become endemic, as appears to have happened in Mexico and Central America and is probably occurring in Asia
e) the use of vaccine can affect trade and mask residual infection.
Poultry-exporting countries do not use vaccine. Thus, at the time of writing, Thailand has made the use of vaccination illegal.

The arguments for the use of vaccine are as follows:

a) vaccines can prevent disease and save the poultry industry of a country from collapse, especially if the outbreak is widespread and culling is beyond the economic capacity of the country concerned

b) vaccines reduce the virus load and the likelihood of transmission, including transmission to humans and the possibility of emergence of a pandemic strain

c) the use of sentinel unvaccinated birds can detect transmission in a flock and warn of vaccine failure

d) vaccinated birds are eventually eliminated by slaughter and processing for food, and virus eradication is declared only when all the poultry in the area are serologically negative

e) there is no disagreement on control strategies for viral diseases in humans: vaccination is the first and most efficacious approach.

H5 vaccines for poultry

Two strategies are currently being used to prepare H5 poultry vaccines. One strategy is the use of fowl pox recombinants containing the HA gene; such a vaccine has been approved for use in Mexico. The other strategy is the use of conventional, egg-grown inactivated vaccine. A/chicken/Mexico/232/94 (H5N2) vaccine, which has been used in Hong Kong (25, 34). A/turkey/England/N-28/73 (H5N2) has been used on the mainland of the People's Republic of China. Studies on the efficacy of the A/chicken/Mexico/232/94 (H5N2) vaccine against the H5N1 virus from Hong Kong in 2001, which has 94% homology in the HA gene between the vaccine and challenge virus, showed that the vaccine gave complete protection from signs of disease but some challenged birds shed low levels of the virus (25). The use of A/chicken/Mexico/232/94 (H5N2) vaccine on Hong Kong poultry farms in 2004 probably contributed to the absence of H5N1 in domestic poultry in Hong Kong during that year, while surrounding countries, including the rest of the People's Republic of China, were affected.

Reverse genetic vaccines for poultry and humans

A new technique for the rescue of infectious influenza A virus from cloned complementary deoxyribonucleic acid (DNA), using an eight-plasmid DNA transfection system, was developed recently (15). This technique provides a powerful new tool for the generation of vaccines for both poultry and humans. A recent study showed the efficacy of a reverse-genetic-derived H5N3 vaccine for agricultural use (25). The H5N3 vaccine was developed as follows: the HA gene came from A/goose/HK/437-4/09, which was altered by the deletion of basic amino acids from the connecting peptide region. The internal genes were from A/PuertoRico/8/34, and the NA was derived from A/duck/Germany/1215/73 (H2N3), which allowed differentiation between vaccinated and non-vaccinated birds (Fig. 3) (25). The vaccine induced antibodies in the vaccinated birds, prevented death and clinical signs of disease and reduced virus shedding after challenge (25).

There are several advantages in using reverse genetics to generate vaccines for poultry, as follows:

- rapid and easy development of vaccines
- uniform vaccination
- low cost
- safety and effectiveness.

Rapid and easy development of vaccines

With the constant evolution of pathogenic viruses in the field, it is essential to find a method for developing vaccines that are immediately protective, so that action can be taken as soon as a problem is detected.

Uniform vaccination

At present, the commercial H5N2 vaccine is not standardised for antigen content. As a result, H5N2 vaccines have been shown to vary substantially among the batches of prepared virus (5). Conventional methods, which are used to standardise the human influenza vaccine, can be applied to standardise the antigen content of the reassortant vaccine in the unconcentrated allantoic fluid. This allows the vaccine to have standardised antigen content. Thus, during vaccination, vaccinated poultry will consistently receive the same amount of antigen, resulting in uniform protection.

Inexpensive vaccine

Using reverse genetics, it is possible to generate high-yield virus that does not require concentration or purification (25). Since the vaccine virus is non-pathogenic, it does not kill embryonated chicken eggs, allowing the virus to reach high titres.

Safety and effectiveness

Vaccines generated by reverse genetics are safe, due to the removal of the basic amino acid peptides which are necessary for H5 viruses to retain their high pathogenicity.
Stability testing of the reverse genetic H5N3 vaccine showed that, after 14 passages in chicken embryos, the virus had not reverted to an increased virulence and the HA sequence remained the same (25). The vaccine used is inactivated, which also increases safety. The reverse genetic vaccine was also shown to be effective. While it did not provide sterilising immunity in a single dose, it did protect the chickens from clinical signs of disease and death, and reduced shedding after challenge. At present, a chicken vaccine virus to the 2004 Vietnam H5N1 strains, which has been produced by the same methods, is about to enter clinical trials.

Reverse genetics techniques may also be applied to the production of human vaccines. In 2003, a pandemic alert was issued by the World Health Organization due to human infections caused by H5N1 influenza viruses. In response, the rapid development of a human vaccine generated by reverse genetics was initiated (43). The vaccine virus that was generated contained the HA from A/Hong Kong/213/03, which again had the basic amino acids at the HA cleavage site removed, and the internal genes came from A/Puerto Rico/8/34 (43). The new vaccine was found to be:

- non-pathogenic for chickens and ferrets
- stable after several passages in chicken eggs
- rapid, in that it took less than four weeks to generate
- effective in conferring protection.

Avian H5 vaccines, unlike human vaccines, do not need to be so closely matched antigenically to provide protection from signs of disease. The basic mechanism of cross-protection between widely divergent antigenic variants in poultry is unresolved. It may, in part, be due to differences between the systemic spread of a highly pathogenic virus in gallinaceous poultry when compared to the respiratory tract infection of mammals, including humans. An important consideration with poultry vaccines is that they do not necessarily prevent virus shedding and unvaccinated sentinel birds are required in each poultry house to monitor viral spread to contacts. Unfortunately, agricultural vaccines are not required to be standardised for antigen content, as human influenza vaccines are, and unvaccinated sentinel birds are not obligatory in the field. Thus, it is probable that antigenic drift is being driven by the use of poultry H5 vaccines. There is clearly room for improvement of agricultural vaccines; the methodology is available but to date the political will is missing.

**Conclusions**

The transmission of H5N1, H7N3, H7N7 and H9N2 avian influenza viruses to humans since 1997, and the accompanying occurrence of disease outbreaks in domestic poultry, are a continuing threat to veterinary and human public health. What has changed since 1997? Before 1997, sporadic transmissions of H7N7 to humans caused conjunctivitis (41, 45), but since then multiple human deaths have occurred. Human infections have been more frequent in Eurasia (H5N1, H7N7, H9N2) and have been associated with changes in virus genes, including the acquisition of multiple basic amino acids at the cleavage site of the HA (17), mutations in the PB2 gene resulting in...
a lysine residue at position 627 (13), and mutations in the NS gene resulting in a glutamic acid at position 92 that modulates the host cytokine responses (33). These changes are the result of mutational, reassortant and recombination events and result from huge numbers of replicative cycles in susceptible hosts. Highly concentrated poultry raising provides optimal conditions for the evolution of influenza viruses. Acquisition of the multiple basic amino acids occurs mainly in gallinaceous poultry, while the PB2 and NS mutations are detected after transmission to humans. It is unknown if they are first selected in poultry or in humans.

The much-dreaded event of continued human-to-human transmission of novel H5, H7 and H9 influenza viruses would most easily be realised through reassortment with a circulating human strain, as occurred in the human pandemics of 1957 (Asian) and 1968 (Hong Kong) (18). Alternatively, the viruses could continue to acquire multiple mutations that would support human-to-human transmission, as may have happened with Spanish influenza in 1918. Optimal conditions for reassortment occur when influenza viruses from different species are brought together in ‘wet’ or live bird markets (44). What has changed is the direct and rapid transmission of infection between wet markets and highly concentrated poultry farming. Traditional wet markets were village operations. Now, poultry can move from a mega-farm to a wet market and the cages, trucks and humans can return to the mega-farm (with viruses) in sufficient time to ensure occasional transmission. The continuing problems with H7N2 influenza in the wet-market system of the northeastern USA is indicative of this process, with an apparent inability to prevent non-pathogenic viruses becoming pathogenic by the steady acquisition of basic amino acids at the cleavage site of the HA.

What can be done? Hong Kong has demonstrated that changes in poultry marketing practices probably prevented an H5N1 outbreak in 2004, when the rest of the People’s Republic of China and surrounding countries were experiencing a widespread epidemic. The steps that were taken included: banning all aquatic birds (the original source of influenza viruses) and quail from the markets and the introduction of two ‘clean days’ per month, during which all markets were simultaneously closed, emptied and cleaned. Subsequently, all birds sold in the markets were vaccinated and screening was conducted to ensure the desired level of antibody (34).

There is continuing debate in Hong Kong on whether to close all wet markets and move to poultry that is killed and chilled in the abattoir. While this debate may serve as an illustration of what could be done to reduce the evolution of influenza viruses, it is unlikely to reduce the risk of an emergence of pandemic influenza in Hong Kong, unless similar changes are introduced in the rest of the People’s Republic of China. Severe acute respiratory syndrome is the most recent example of transmission of a newly emerging virus to neighbouring areas.

Thus, it is apparent that influenza will continue to be a re-emerging, zoonotic infectious disease that requires attention from researchers in veterinary and human infectious diseases. Much remains to be clarified about the molecular mechanisms of interspecies transmission of influenza viruses and how to prevent it. The tools to produce better vaccines are available and more rigorous standards of quality control are greatly needed.

Acknowledgements

The authors thank Carol Walsh for preparing the manuscript and Julia Cay Jones for editorial assistance. This work was supported by Public Health Service grants AI-95357 and CA-21765 and by the American Lebanese Syrian Associated Charities.
L’influenza aviaire : un exemple de modification et d’adaptation microbienne

R.G. Webster & D.J. Hulse

Résumé
L’évolution de l’influenza s’inscrit dans un processus continu mettant en jeu des facteurs liés au virus et à ses hôtes. L’émergence de plus en plus fréquente des souches H5N1, H7N3 et H7N7 hautement pathogènes du virus de l’influenza et la propagation panzootique de sa souche H9N2 suscitent une vive inquiétude auprès des responsables de la santé publique et de la santé animale dans la mesure où toutes ces souches sont susceptibles d’être transmises à l’homme. La question est de savoir combien de temps nous sépare de la prochaine pandémie. Un faisceau de facteurs convergents (la densité des populations avicoles, porcines et humaines, par exemple) peuvent influer sur l’évolution du virus. Les élevages à forte concentration de volailles et de porcins, ainsi que les marchés traditionnels d’animaux vivants à ciel ouvert, créent des conditions optimales pour les mutations, les réassortiments et la recombinaison des gènes des virus de l’influenza. La séparation des espèces, le renforcement de la biosécurité, la mise au point de nouvelles stratégies de vaccination et l’amélioration de notre connaissance du virus sont autant de stratégies capables de ralentir l’évolution de l’influenza et l’apparition de pandémies. La réalisation de ces objectifs passe par une coopération plus efficace entre les chercheurs en recherche fondamentale et les responsables de la santé publique et de la santé animale.

Mots-clés

La influenza aviar como ejemplo de adaptación y cambio microbiano

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Resumen
La evolución de la influenza es un proceso continuo en el que intervienen factores ligados tanto al virus como a sus anfitriones. La creciente frecuencia con la que aparecen los virus de la influenza altamente patógenos H5N1, H7N3 y H7N7, así como la diseminación panzootica del virus H9N2, todos los cuales pueden transmitirse al ser humano, preocupan sobremanera a los responsables de la salud pública y veterinaria. La cuestión es cuánto va a tardar en surgir la próxima pandemia. Hay una serie de factores convergentes, entre ellos la densidad de población de las aves de corral, los porcinos y el ser humano, que probablemente influirán en la evolución del virus. Las explotaciones avícolas y porcinas con poblaciones animales muy densas, combinadas con los tradicionales mercados de animales vivos, ofrecen un contexto idóneo para que los virus experimenten más mutaciones, redistribuciones y recombinaciones.
génicas. Para frenar la evolución de la influenza y reducir la aparición de pandemias se requieren, entre otras cosas, la separación de las especies, el aumento del nivel de seguridad biológica, la concepción de nuevas estrategias en materia de vacunas y un mejor conocimiento básico del virus, objetivos todos ellos que exigen una colaboración más eficaz entre los científicos dedicados a la investigación fundamental, por un lado, y las instancias de salud pública y veterinaria por el otro.

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