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Avian influenza: our current understanding

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Abstract

Avian influenza (AI) virus is one of the most important diseases of the poultry industry around the world. The virus has a broad host range in birds and mammals, although the natural reservoir is wild birds where it typically causes an asymptomatic to mild infection. The virus in poultry can cause a range of clinical diseases and is defined either as low pathogenic AI (LPAI) or highly pathogenic AI (HPAI) depending on the type of disease it causes in chickens. Viruses that replicate primarily on mucosal surfaces and cause mild disease with low mortality are termed LPAI. Viruses that replicate on mucosal surfaces and systemically and cause severe disease with a mortality rate of 75% or greater in experimentally infected chickens are referred to as HPAI. A virus that is highly pathogenic in chickens may infect but result in a completely different disease and replication pattern in other host species. Outbreaks of HPAI have been relatively uncommon around the world in the last 50 years and have had limited spread within a country or region with one major exception, Asian lineage H5N1 that was first identified in 1996. This lineage of virus has spread to over 60 countries and has become endemic in poultry in at least four countries. AI virus also represents a public health threat, with some infected humans having severe disease and with a high case fatality rate. AI remains a difficult disease to control because of the highly infectious nature of the virus and the interface of domestic and wild animals. A better understanding of the disease and its transmission is important for control.

Keywords: avian influenza, wild birds, LPAI, HPAI, H5N1

Origins of avian influenza

Wild birds, primarily wild ducks, gulls, and shorebirds are the natural host and reservoir for all type A influenza viruses (Slemons *et al.*, 1974; Kawaoka *et al.*, 1988; Stallknecht, 1998). The virus, however, has an extremely wide host range and can on rare occasions spread from wild birds to either domestic poultry or mammalian species. The viruses typically are not well adapted to these new hosts and replicate and transmit poorly, resulting in dead end hosts. Therefore most introductions of virus into new hosts are often not recognized and rarely cause clinical disease (Suarez, 2000). However, in rare cases, avian influenza (AI) viruses can become adapted to the new host. Often our commercial animal rearing practices, which concentrate large numbers of susceptible animals in confined spaces, aid in the transmission of virus. Poor biosecurity can also facilitate transmission of viruses between different poultry farms. The longer a virus is allowed to persist in a poultry population, the more likely it is that the virus can become adapted to the new host. This adaptation process results in an increased ability of the virus to replicate within and transmit more efficiently in the new host species. The result may be a more severe clinical disease with the potential for the virus to mutate to the highly pathogenic (HP) phenotype with some subtypes of AI viruses (H5 or H7). The more adapted a virus is to the new host species, the more difficult it will be to eradicate the virus from that species.

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Host susceptibility

AI has been isolated from over 100 species of birds, which represent at least 12 different Orders. However, birds of the Orders Anseriformes and Charadriiformes, which include ducks, gulls, and shorebirds, are considered to be the primary natural reservoir of the virus because the virus

is reliably isolated from certain species from these Orders of birds (Stallknecht and Brown, 2008). However, the Anseriformes and Charadriiformes Orders of birds represent a diverse group of bird species, and it is clear that particular bird species are infected at a high prevalence every year and are important for the maintenance of the virus in the environment. Mallards, Northern Pintail, Green-winged teal, and Northern Shoveller ducks are four Anseriformes species from which AI virus can be commonly isolated, particularly in the autumn as the birds are marshalling for or traveling to wintering grounds on their southern migration (Stallknecht and Brown, 2008). Other Anseriformes species are susceptible to infection based on experimental studies; these include Canada geese or wood ducks, but based on the rates of isolation in the wild, these species do not appear to be important in the natural maintenance of the virus (Deibel *et al.*, 1985; Slemons *et al.*, 1991; Brown *et al.*, 2006, 2008). In the Charadriiformes Order of birds, the only species from which AI virus is isolated with high frequency is the Ruddy Turnstones in the spring in Delaware Bay. The high isolation rate from Ruddy Turnstones has been proposed to be the result of the unique concentration of migratory birds in Delaware Bay because of the abundance of high-quality feeding grounds at that time of year (Hanson *et al.*, 2008).

We currently have a poor understanding of why some species appear to be important in the ecology of AI, while closely related species are not. Factors that appear to be common between infected species is that they concentrate in high numbers for at least part of the year, particularly at marshaling grounds, which likely facilitates transmission of the virus. Similar observations have been made with poultry and for mammalian species in which influenza has become endemic, in that the affected species are often crowded together, thereby facilitating transmission (Suarez, 2008). Other factors are likely to be involved. For example, the incidence of infection appears highest at the higher latitudes. This may be related to the hypothesis that AI viruses can overwinter in water, reinfecting returning migratory waterfowl in the spring (Zhang *et al.*, 2006). Mounting evidence supports this idea because AI viruses appear to be viable for long periods of time at low temperatures in water (Brown *et al.*, 2009a). Another proposed factor may be the biology of the birds themselves, as the feeding style and differences in the density of lamellae (filter plates) of ducks appear to be positively correlated with AI infection (Hill *et al.*, 2010). Our understanding of the ecology of AI viruses in the natural host, however, remains rudimentary, and the complete host range important for propagation of the virus may never be clearly defined.

The movement of AI viruses from the natural wild bird host to aberrant hosts including gallinaceous poultry species, such as chickens, turkeys, quail, and pheasants, appears to happen relatively frequently, but is likely not often recognized (Suarez, 2000). With the increased concern for H5N1 highly pathogenic AI (HPAI), the surveillance of poultry for AI virus infections has greatly increased and documents that a number of commercial poultry flocks are infected with a variety of different AI viruses every year. Most of these cases are recognized based on seroconversion of the flock without clear evidence of clinical disease, and most of these infections are transient with the virus not spreading to other flocks (Alexander, 2007; Senne, 2007). This lack of persistence is believed to be because most of these viruses are not well adapted to the new host and cannot efficiently maintain the transmission chain. However, some viruses do become established in the new host population, in part because of the natural variation of AI viruses that exist in the wild bird population that allows some viruses to more easily replicate and transmit (Swayne and Slemons, 2008). On rare occasions, an introduced virus can become established, and by an adaptation process, increase its ability to replicate and transmit in poultry populations. Although the exposure of low pathogenic AI (LPAI) viruses from wild birds to poultry species is likely to be similar, the number of outbreaks of HPAI in the last 20 years is higher than the number of HPAI outbreaks reported in the previous 40 years. One hypothesis for the increase in the number of unique HPAI outbreaks in recent years is that there has been an increase in the number of high-density poultry rearing operations, and this increased density of birds within a flock increases opportunities for virus transmission, providing greater opportunity for the virus to adapt and mutate to the HP form of the virus (Swayne, 2008a).

One method of evaluating virus adaptation for a particular species is to experimentally determine the minimal infectious dose 50 (MID_{50}) or the dose for a particular strain of virus that will infect half the birds of a particular species (Tumpey *et al.*, 2004; Spackman *et al.*, 2007b). A highly host-adapted virus is expected to have a low MID_{50} and a poorly adapted virus will have a high MID_{50}. However, the MID_{50} can be influenced by a number of factors including the age of the bird, the route of inoculation, the breed of animal, and concurrent infection (Swayne and Slemons, 2008; Costa *et al.*, 2010; Pantin-Jackwood *et al.*, 2010; Ramirez-Nieto *et al.*, 2010). Calculating the MID_{50} can be difficult because it requires the inoculation of a large number of animals, which must be separated based on challenge dose. For this reason relatively few viruses have been characterized with respect to infectious dose, and almost all of these have been in chickens. In the studies performed in chickens with virus given by the mucosal route, viruses in three general groups have been tested: (i) LPAI from wild birds, (ii) LPAI of poultry origin, and (iii) HPAI (Table 1) (Chen *et al.*, 2003; Subbarao *et al.*, 2003; Tumpey *et al.*, 2004; Swayne and Beck, 2005; Brown *et al.*, 2007; Okamatsu *et al.*, 2007; Spackman *et al.*, 2007b; Tsukamoto *et al.*, 2007). For the wild-bird origin LPAI viruses, the chicken

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Table 1. Chicken infectious doses for viruses in various pathogenicity categories

Isolate	Subtype	Chicken infectious dose (log 10)	Reference
Wild bird low pathogenic isolate			
Mallard/Ohio/338/1986	H4N8	4.4	Swayne and Slemons (2008)
Mallard/Ohio/184/1986	H5N1	6.7	Swayne and Slemons (2008)
Cinnamon Teal/Bolivia/4537/2001	H7N3	6.2	Spackman *et al.* (2006)
Mallard/Maryland/791/2002	H5N2	>5.3	Spackman *et al.* (2007a)
Ruddy Turnstone/New Jersey/1148676/2004	H5N7	>7.5	Spackman *et al.* (2007b)
DK/Pennsylvania/454069-9/2006	H5N1	7.5	Spackman *et al.* (2007b)
Mute Swan/Michigan/451072-2/2006	H5N1	6.3	Spackman *et al.* (2007b)
Poultry low pathogenic isolate			
CK/Alabama/1975	H4N8	3.0	Swayne and Slemons (2008)
Emu/Texas/39924/1993	H5N2	4.2	Swayne *et al.* (1996)
Rhea/North Carolina/39482/1993	H7N1	6.9	Swayne *et al.* (1996)
CK/Hong Kong/G9/1997	H9N2	<2.9	Chen *et al.* (2003)
TK/Virginia/15821/2002	H7N2	2.8	Tumpey *et al.* (2004)
Parrot/California/D0406032/2004	H5N2	4.1	Swayne and Slemons (2008)
HP isolate			
CK/Rostock(Germany)/1934	H7N1	1.2	Swayne and Slemons (2008)
CK/Scotland/1959	H5N1	3.0	Swayne and Slemons (2008)
Tern/South Africa/1961	H5N3	3.4	Swayne and Slemons (2008)
TK/Ontario/7732/1966	H5N9	3.4	Swayne and Slemons (2008)
CK/Victoria/1975	H7N7	2.9	Swayne and Slemons (2008)
TK/Ireland/1983	H5N8	4.7	Swayne and Slemons (2008)
CK/Pennsylvania/1370/1983	H5N2	3.0	Swayne and Beck (2005)
Turkey/England/50-92/1991	H5N1	3.9	Swayne and Slemons (2008)
CK/Queretaro/14588-19/1995	H5N2	3.0	Swayne and Slemons (2008)
Hong Kong/486/1997	H5N1	2.4	Swayne and Slemons (2008)
Hong Kong/491/1997	H5N1	2.3	Subbarao *et al.* (2003)
TK/Italy/4580/1999	H7N1	2.0	Swayne and Slemons (2008)
CK/Korea/ES/2003	H5N1	2.5, 3.1	Swayne and Beck (2005)
CK/Yamaguchi/7/2004	H5N1	2.0	Nakamura *et al.* (2008)
Whooper Swan/Mongolia/7/2005	H5N1	2.8	Brown *et al.* (2007)

MID_{50} was greater than 10^6 EID_{50} with one exception (A/Mallard/Ohio/338/1986 at a MID_{50} of $10^{4.4}$). For LPAI viruses of poultry origin, most of the MID_{50}s were in the range of $10^{2.8}$–$10^{4.2}$. This difference in the infectious dose clearly highlights that wild-bird origin viruses are generally not well adapted to chickens. Most of the wild-bird origin viruses required 100–1000 times more virus to infect a chicken than did the poultry-adapted viruses. Some of the poultry origin viruses have a well-described history of infection in chickens or other gallinaceous birds, such as A/Turkey/Virginia/15821/2002 (H7N2), with a MID_{50} of $10^{2.8}$. The roots of this H7N2 virus can be documented in poultry back to 1994 in the live bird markets (LBMs) in the Northeast United States, and it is considered a poultry-adapted virus. This virus also highlights the differences within poultry species, because this strain infected turkeys at an infectious dose 50 of $10^{0.8}$, which is almost 100 times lower than that required to infect chickens. HPAI viruses have a MID_{50} that ranges from $10^{1.2}$ to $10^{4.7}$, with an average of around $10^{3.0}$, which demonstrates that these viruses are also well adapted to poultry (Subbarao *et al.*, 2003; Swayne and Beck, 2005; Brown *et al.*, 2007; Swayne and Slemons, 2008).

The introduction, transmission, and adaptation of a wild bird AI virus to a poultry-adapted AI virus can be a complex chain of events, and the steps required are rarely clear. The term 'poultry' typically is defined as domesticated birds that are kept for their food, feathers, or sport and include several orders of birds. The actual list of poultry species is large, but ducks, chickens, turkeys, quail, pheasant, and pigeons are commonly kept species. Many backyard or village farmers have multiple species in close association, including ducks and gallinaceous birds, and LBMs often sell multiple species under crowded conditions. It is presumed, although not well tested experimentally, that domestic ducks are more susceptible to wild duck AI viruses than are gallinaceous birds (Spackman *et al.*, 2007b). Domestic ducks also often have closer association with wild birds because duck rearing practices often allow access to open lakes where wild and domestic birds can mingle. This provides the potential for wild ducks to infect domestic ducks, which then have opportunities to spread the virus to gallinaceous poultry (Swayne, 2008b). The role of LBMs in harboring and potentiating the adaptation of AI viruses to gallinaceous poultry is also well known (Senne *et al.*, 1992; Suarez *et al.*, 1999; Bulaga *et al.*, 2003; Liu *et al.*, 2003; Spackman

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et al., 2003; Choi *et al*., 2005; Nguyen *et al*., 2005; Yee *et al*., 2009).

In addition to the interaction between ducks and gallinaceous poultry, certain gallinaceous species have been identified as being more susceptible to infection with AI viruses and therefore may play a special role in the adaptation or spread of the virus to chickens. Japanese Quail, a commonly reared poultry species, has been proposed as an important bridge species, based on an increased susceptibility to some AI viruses compared to chickens and to the concentration of both α 2,3 and α 2,6 sialic acid receptors in the respiratory tract (Perez *et al*., 2003a, b; Wan and Perez, 2006; Sorrell and Perez, 2007). The concern about quail as a bridge species even prompted regulatory officials in Hong Kong in 2002 to ban the sale of live quail in LBMs just as they had banned the sale of ducks and geese in 1998 (Lau *et al*., 2007). Another species proposed as an important bridge species is the turkey (Tumpey *et al*., 2004; Pillai *et al*., 2010). In the United States, based on over 30 years of surveillance activity, turkeys are more commonly infected with avian and swine origin influenza viruses than chickens, supporting observations of an increased susceptibility of turkeys in the field (Halvorson *et al*., 1985; Senne, 2007). Changes in turkey rearing practices in the United States to a more confinement rearing approach, which reduces exposure to wild birds, has resulted in a greatly reduced number of outbreaks of AIV infection (Halvorson, 2008). The interactions among various species are critical in the spread and maintenance of AI in poultry, but because the relations between species vary considerable in different production systems and regions of the world, it remains difficult to model. Approaches to reduce contact, like banning certain species in LBMs, appear to have been successful in reducing the number of influenza outbreaks, but this approach has not been widely adopted (Lau *et al*., 2007). Increased biosecurity to reduce contact with wild birds, also shown to be effective, is also not a widely adopted practice in part because of the cost involved. Prevention, although in the long run perhaps the most cost effective approach, has been difficult to apply consistently around the world.

An alternative way to compare viruses is to measure virus shedding, typically oropharyngeal and cloacal shedding, after experimental inoculation. The presumption is that a well-adapted virus will replicate better and shed more virus compared to a poorly adapted virus. For example, a recent mute swan H5N1 LPAI virus from Michigan when challenged by the choanal mucosal route replicated to higher levels in domestic ducks as compared to chickens or turkeys (Spackman *et al*., 2007b). Viral shedding is also dependent on a number of host factors including the age of the bird, the challenge dose, the route of challenge, and the breed of bird.

Another common method of evaluating host adaptation is to determine transmission of the virus within a species. These types of experiments are usually performed as either direct exposure experiments, where uninfected birds are placed directly in contact with infected birds or indirect transmission experiments where the birds remain separated but share common air space or are otherwise in close proximity (Shortridge *et al*., 1998). The direct transmission model has also been used to model virus transmission to show the efficacy of vaccination in controlling disease outbreaks using the SEIR epidemic model (van der Goot *et al*., 2005). This model evaluates susceptible, latently infected, infectious, recovered (SEIR) animals to provide a statistical basis to determine the reproductive ratio (R) to estimate if the transmission chain is robust enough to result in an epizootic outbreak. If the transmission model shows an $R>1$, meaning one infected bird infects more than one susceptible bird, then an epidemic spread can occur in that species. If $R<1$, then an infected bird infects less than one animal, and the transmission chain will fizzle out (van der Goot *et al*., 2003). These types of experiments can provide a direct assessment of the transmissibility of a virus, but they can be difficult to repeat or replicate because of the many variables that contribute to the transmission of a virus.

Genetic markers of species adaptation

The adaptation process of influenza to a new host currently is unpredictable, with few genetic markers to predict host adaptation. As previously described, wild bird viruses generally replicate poorly in chickens, but several studies have shown that by repeated passaging of viruses in chickens, the viruses can replicate to higher levels and be more readily transmitted from chicken to chicken (Sorrell and Perez, 2007; Ramirez-Nieto *et al*., 2010). However, in these studies, genetic changes include amino acid differences that directly contribute to adaptation and those that are neutral, and it has been difficult to separate what changes are important. In addition, considerable variation in the external environment or the host can occur to allow a virus to be better adapted to the new host. In other words, a virus can adapt to the new host species in many ways that are not predictable. When comparing AI viruses in wild bird species, at the nucleotide level, considerable variation occurs so that viruses from the Americas can usually be distinguished from viruses from Eurasia. The data document that these two populations of virus appear to be evolving separately. However, at the amino acid level, all the influenza genes that have been examined closely seem to be highly conserved between the two populations (Suarez, 2000). The only clear exception is within gulls, whose AI viruses appear to have some distinctive amino acid changes that identify them as a unique lineage (Swayne *et al*., 2008). However, gulls are susceptible to both the gull-specific lineage viruses and the general AI virus lineage. Once AI viruses have jumped species and become established in new hosts, the viruses do start to accumulate amino acid

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changes unique to that lineage, but comparison of different viral lineages from the same species do not show clear patterns or specific mutations that are required for virus adaptation, again demonstrating that adaptation can follow many paths (Suarez, 2008).

The clearest changes that are suggestive of adaptation to gallinaceous birds are in the hemagglutinin and neuraminidase proteins. One change that is thought to occur that has a major effect on virulence is the addition of amino acids, typically the basic amino acids lysine and arginine, at the cleavage site of the hemagglutinin gene of viruses of the H5 and H7 subtype. The mutation or insertion of basic amino acids at the cleavage site allows for different proteases to cleave the hemagglutinin protein into the HA1 and HA2 subunits, which is required for the virus to be infectious (Perdue, 2008). Typically, trypsin-like proteases cleave the hemagglutinin protein extracellularly in the enteric and respiratory tracts, which accounts for the restriction of LPAI viruses to replicate primarily on mucosal surfaces. The change in the cleavage site by substitution to additional basic amino acids or by insertion of additional amino acids allows for the hemagglutinin protein to be cleaved by a wider range of proteases, including ubiquitous proteases. The ubiquitous proteases can be found in the endoplasmic reticulum and the hemagglutinin protein can be cleaved during the translation process, allowing the virus to be infectious before it is released from the cell. This increases the types of cells that the virus can productively infect, allowing both mucosal and systemic replication, which results in an increase in virulence that typically results in the HP phenotype (Suarez, 2008).

HPAI is defined by the ability to infect and kill chickens using a standardized dose given intravenously (World Organization for Animal Health, 2006). This definition has generally been a practical and useful definition, but several examples have been described where the genotype and the phenotype of the virus in chickens have not correlated, including the recent outbreak of H5N2 in Texas in 2004. In this case, the HA cleavage site had four basic amino acids compatible with earlier HP viruses, but the virus did not cause mortality in chickens infected by the standard intravenous pathogenicity test (Lee *et al.*, 2005). The phenotypes of HPAI viruses are even less predictable in other species, although experimental data show some similarity in the pathotype in gallinaceous birds (Perkins and Swayne, 2001). However, most HPAI viruses from chickens are non-pathogenic in domestic ducks. The one major exception is the Asian lineage H5N1 HPAI viruses that were first isolated in China in 1996. The initial viruses that were examined could infect but were non-pathogenic in ducks, but over time these viruses became more and more pathogenic in ducks (Pantin-Jackwood and Swayne, 2007; Pfeiffer *et al.*, 2009). Similar observations have been made with the Asian H5N1 lineage in wild duck species, with many species being infected but with unpredictable virulence. Again the genetic differences accounting for these phenotypic differences are not clear. Finally, some species, like pigeons, appear to be highly refractory to experimental infection at typical challenge doses of 10^5–10^6 EID_{50} of virus (Perkins and Swayne, 2002; Fang *et al.*, 2006; Liu *et al.*, 2007; Werner *et al.*, 2007; Brown *et al.*, 2009a, b).

Our current understanding is that HPAI viruses are created by the passage of H5 or H7 LPAI viruses in gallinaceous birds such that an unknown selective pressure pushes for the creation of the virulent virus. The virus once created in poultry can then spread to other species including, in some cases, wild birds, although usually only transiently. HPAI viruses are not thought to normally exist in wild birds. This theory is supported by the isolation of thousands of LPAI viruses from wild birds, with only two known exceptions. The first known exception of HPAI virus in wild birds not associated with poultry was in terns in South Africa in 1961. A HPAI virus was isolated during this outbreak associated with mortality in the terns, but the virus did not appear to persist in terns nor was a similar virus found in poultry (Becker, 1966). The second known exception is the Asian lineage H5N1 HPAI virus that was endemic in poultry for almost 9 years before it appeared to jump into and persist in wild birds. The Asian lineage H5N1 HPAI virus was first isolated in domestic geese (Xu *et al.*, 1999), and from 1996 to 2005 it was associated almost exclusively with poultry species (Sims and Brown, 2008). However, in 2005 viruses from this lineage caused a large disease outbreak in wild birds in China (Liu *et al.*, 2005). This specific variant virus then appeared to move large distances, fueling the spread of the virus into Europe and Africa (Sims and Brown, 2008). Although large epidemic outbreaks in wild birds have rarely been seen since 2006, the virus appears to be maintained in the wild bird population, providing opportunities for spread back to domestic poultry (Lvov *et al.*, 2010; Sharshov *et al.*, 2010). To conclude, the changes at the HA cleavage site appear to be primarily a gallinaceous bird adaptation, but the actual sequence for this change is quite variable. In addition the cleavage site changes are not major species barriers that prevent the viruses from spreading to other species of birds or to mammals.

The second recognized adaptation to gallinaceous birds is the presence of stalk deletions in the neuraminidase gene (Matrosovich *et al.*, 1999). The stalk region of the NA protein is typically around 30 amino acids in length, predicted to be hydrophilic, and is between the transmembrane region and the globular head that contains the enzymatic site of the protein. The stalk is extremely variable in sequence between subtypes, and the principal function of the stalk is to extend the enzymatically active site of the protein away from the cell or viral surface. The neuraminidase protein removes sialic acid from both viral and host proteins and is important in the release of the virus from the infected cell, in part by preventing the virus from sticking to other influenza viruses as it exits

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the host cell (Liu *et al.*, 1995). In wild birds, the stalk length within a subtype is highly conserved, but when AI viruses replicate in poultry, stalk deletions often occur. The number of amino acids that are deleted is extremely variable from just a few to over 20. Most commonly the deletion length is 12–20 amino acids. Although the emergence of stalk deletion mutants appears to be the result of positive selection pressure, paradoxically the stalk deletion actually greatly decreases the activity of the protein. The enzymatically active site remains undisturbed, but the shorter stalk reduces the flexibility or reach of the enzyme's receptor site, effectively reducing the enzymatic activity. Experimentally, stalk deletions appear to cause the virus to aggregate on the cell surface, effectively reducing the amount of virus being released from the cell (Colman, 1989; Suzuki, 2005). In one experimental study the introduction of a stalk deletion in a wild duck isolate increased the virulence of the virus for chickens, but the mechanism is still unknown (Munier *et al.*, 2010).

Stalk deletions are often closely aligned to increased numbers of glycosylation sites in the hemagglutinin gene. Increased N-linked glycosylation near the receptor-binding site appears to reduce the affinity of the receptor for sialic acid, essentially making the virus less sticky. The changes in the HA and NA proteins appear to be compensatory changes, where one seems to negate the change of the other. The less sticky virus does not require an efficient NA gene, and a poorly efficient NA gene can increase viral spread with a hemagglutinin protein with lower affinity to sialic acid (Matrosovich *et al.*, 1999; Mitnaul *et al.*, 2000). However, it is unclear what change occurs first or what advantage either change may have for the virus in gallinaceous birds.

For the internal proteins, no clear pattern has emerged for determining whether a virus is adapted to wild birds or poultry. One of the most commonly studied changes is whether there is glutamic acid or lysine at position 627 of the PB2 protein. The presence of lysine appears to strongly correlate with viruses that are adapted to mammals. The marker appears to primarily differentiate adaptation to birds and mammals, but it does not differentiate between birds species (Subbarao *et al.*, 1993). The primary function of the protein appears to be adaptation to temperature, as the lysine at this position allows the virus to replicate at lower temperatures, which correlates with the lower temperature of the respiratory tract in mammals compared to birds (Massin *et al.*, 2001; Hatta *et al.*, 2007). However, in the H5N1 Asian lineage viruses, a subset of viruses with lysine at this position have been isolated from many different species of birds with no apparent effect on the virulence observed in birds (Chen *et al.*, 2006a). Other markers in a number of different internal genes have been identified that separate avian from mammalian species adaptation, but little information is available that separates adaptation of viruses for various avian species.

Control of AI virus outbreaks in poultry

The movement of AI viruses from the wild bird reservoir to domestic bird species is not uncommon, but rarely results in viruses becoming endemic in poultry. The direct exposure of poultry to wild birds is the most likely source of introduction, with some of the best documented cases of exposure being in commercial turkeys in Minnesota where multiple outbreaks of AI were observed yearly in the 1980s and early 1990s (Halvorson and Kelleher, 1985). AI viruses of many different HA and NA subtypes were isolated from turkeys in different outbreaks, and typically at times when wild ducks were migrating to or from their summer breeding grounds. During the migratory wild duck season, turkeys were raised outside and the wild birds could fly over or actually land in the turkey pens. During the 1990s the management system was changed so that the turkeys were reared in confinement for their entire lives, and the incidence of AI virus was greatly decreased (Swayne and Suarez, 2005). Other known risks of exposure to AI viruses have been through contaminated drinking water. Outbreaks in the United States, Canada, Chile, and Australia have all been linked to using unpurified surface water where wild ducks have access to the drinking water supply for poultry (Hinshaw *et al.*, 1979; Sivanandan *et al.*, 1991; Suarez *et al.*, 2004; Pasick *et al.*, 2010). Another unusual source of infection of poultry with an HPAI virus is through contaminated meat and offal. Because HPAI is systemic, the virus is found in most if not all the internal organs including skeletal and heart muscle. These tissues experimentally have been shown to be infectious from oral feeding, and a recent case in Germany of feeding uncooked meat and offal scraps from infected domestic ducks to chickens initiated an outbreak of H5N1 in that country (Tumpey *et al.*, 2002; Swayne and Beck, 2005; Harder *et al.*, 2009). Limiting exposure of poultry to wild birds through confinement rearing and other biosecurity measures likely provides the best opportunity to reduce the risk of AI virus introduction from wild birds.

Once a virus has become adapted to and becomes endemic in a poultry population, control becomes difficult. Transmission of infectious virus between farms or different regions can occur in many ways, but the movement of infected poultry is the most common. A standard outbreak control tool is to quarantine infected flocks and control animal movement within an outbreak zone. The outbreak zone is usually a prescribed distance (e.g. 5 or 10 km) from an outbreak site or is defined by specific geographic borders. Outbreaks of HPAI result in restrictions on trade in poultry or poultry products that is usually immediate and can affect the entire country, resulting in serious economic losses for the producer (Swayne and Suarez, 2005). Outbreaks of LPAI virus in poultry may also result in trade restrictions.

In the US and for most countries with a poultry export market, the main control tool for HPAI and for

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Table 2. Notable events in the H5N1 Asian lineage epizootic

Guangdong, China	1996	First report of HPAI Asian H5N1 lineage (HA gene)	Xu *et al.* (1999)
Hong Kong, SAR	1997	H5N1 outbreak in live bird markets in spring with the first human case	Subbarao *et al.* (1998), Suarez *et al.* (1998)
Hong Kong, SAR	1999	H5N1 detected from geese imported from Guangdong	Cauthen *et al.* (2000)
Hanoi, Vietnam	2001	H5N1 isolated from live bird markets	Nguyen *et al.* (2005)
Quarantine station, South Korea	2001	H5N1 isolated from frozen duck meat from China	Tumpey *et al.* (2002)
Hong Kong, SAR	2002	H5N1 on commercial farms. Vaccine used as part of control	Ellis *et al.* (2006)
Hong Kong, SAR	2002	H5N1 causes mortality in wild bird species	Sturm-Ramirez *et al.* (2004)
Indonesia, Thailand, Japan, Vietnam, South Korea, China, Cambodia, Laos	2003–2004	H5N1 spreads rapidly in many nations in Southeast Asia	CDC (2004)
Vietnam	2004	H5N1 detected in humans emphasizing zoonotic risk	Tran *et al.* (2004)
China	2005	H5N1 causes lethal outbreak in wild birds at Qinghai Lake	Chen *et al.* (2005), Liu *et al.* (2005)
Mongolia, Russia, Kazakhstan	2005	H5N1 spread through migratory birds to summer breeding grounds and into poultry	L'Vov *et al.*, (2006), Gilbert *et al.* (2006)
Europe, Middle East, Africa	2006	H5N1 spreads through migratory birds to wintering grounds	Alexander (2007)
Vietnam, Indonesia, Egypt, China	2007	H5N1 officially reported as endemic in three countries and unofficially in China	Domenech *et al.* (2009)

LPAI of the H5 and H7 subtypes is the identification and euthanasia of infected birds. This 'stamping out' approach, although costly, can result in rapid control of an outbreak when the infrastructure for diagnosis, indemnity, euthanasia, and disposal of infected birds is readily available, as occurred in the H5N2 outbreak in Texas in 2004 (Pelzel *et al.*, 2006). The stamping out approach has not been as effective in countries with poor veterinary infrastructure or where the virus is widespread in the country when it was first diagnosed.

One tool for the control of AI that is getting more attention is the vaccination of susceptible poultry. Proper vaccination for AI virus can prevent clinical disease, reduce virus shedding if vaccinated birds become infected with the virus, and can increase the amount of virus required to infect vaccinated birds (Lee and Suarez, 2005). Vaccination needs to be part of an integrated approach that includes intensive surveillance, quarantine, animal movement controls, increased biosecurity, and education. Additionally, the availability of well-matched and efficacious vaccines to provide optimal protection is also important (Lee and Suarez, 2005).

For most outbreaks of HPAI in poultry, quarantine, animal movement controls, increased biosecurity, stamping, and in rare cases the addition of vaccination have been sufficient to eradicate the virus. However, the Asian lineage H5N1 HPAI virus has not been controlled, and has become the most widespread and devastating HPAI outbreak reported. Why this lineage of virus has resulted in such a different result will be discussed further.

Asian lineage H5N1 HPAI

The Asian lineage H5N1 HPAI virus was first isolated in domestic geese in 1996 from Guangdong China (Xu *et al.*, 1999). The virus appeared to primarily circulate in southern China for almost 7 years before it began an unprecedented spread across Asia and eventually into Europe and Africa, where it has subsequently been reported from over 60 countries and has become endemic in poultry in at least four countries. This lineage of virus has a number of unusual features that has made it difficult to control. Key events in the history of this virus lineage are listed in Table 2. These events help us to understand why the H5N1 appears to be a unique event in the history of infectious disease in veterinary medicine (Sims and Brown, 2008). Some of these factors will be discussed in more detail in the remainder of this paper.

Viral variation within the Asian H5N1 HPAI lineage

The Asian H5N1 HPAI lineage has extensive viral variation in every gene, and the only constant in this viral lineage is the hemagglutinin gene. The first reported isolation of this lineage of virus was in domestic geese in Guangdong, China in 2006. This outbreak had clinical disease with some mortality in the affected goose flocks, but there was no report of involvement of chickens in this original outbreak. This virus had a hemagglutinin gene of Eurasian origin, had an insertion of multiple basic

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amino acids at the cleavage site consistent with a HPAI virus, and experimentally caused mortality in chickens. The virus had a neuraminidase gene with a full length stalk, which is suggestive of a virus not well adapted to gallinaceous poultry (Xu *et al.*, 1999). Even the origin of this first reported case is unusual because most HPAI outbreaks have been associated with outbreaks in turkeys or chickens, although secondary spread to domestic waterfowl has been reported previously (Capua and Mutinelli, 2001). The origin of HPAI is generally believed to follow the model of transmission of LPAI from wild birds to gallinaceous poultry, possibly through intermediate species, and then mutation in gallinaceous poultry to the HP form of the virus. This case leaves two distinct possibilities. First, with no known association of the Goose/Guangdong HPAI virus in gallinaceous poultry, consideration must be given that LPAI viruses can mutate to HPAI in other species. Alternatively, the goose outbreak in 1996 was not the first or only H5N1 outbreak in the region, but it was the only one reported. The second possibility has at least some support because the Chinese government did not report to the World Organization of Animal Health (OIE) that an HPAI outbreak occurred in the country at this time and a published report from 1997 stated that no HPAI had ever been diagnosed in China (Sun, 1997). The incomplete or non-reporting of AI outbreaks is not unique to China, because many countries have restricted information about the disease because of the trade implications, but the early history of H5N1 will likely never be well understood because of the lack of transparency in the region.

The first isolate in 1996 appears to be the source of the hemagglutinin gene for all the subsequent outbreaks of this lineage of virus, but all the seven other influenza gene segments appear to come from different viral origins depending on the isolate. This variation of the internal genes occurs by reassortment of gene segments between different AI viruses that circulate in the region. One of the best studied cases is the Hong Kong H5N1 outbreak in 1997. This outbreak was centered at the LBMs in the city with the first reported outbreaks occurring in early 1997. The virus remained in the LBMs through the fall of the year, and it was not until 18 human cases occurred that intense international interest resulted in extreme control measures. The decision was made to depopulate all the poultry in Hong Kong in an attempt to eradicate the virus; this action was successful in eradicating this variant of the virus (Sims and Brown, 2008). When the viruses from Hong Kong were sequenced, the H5 gene was similar to the Goose/Guandong/96 virus, but all the other genes were different (Xu *et al.*, 1999). Because of the surveillance occurring in the LBMs, several LPAI viruses were isolated, including H6N1 and H9N2 viruses. The internal genes from some of these viruses appeared to be closely related to the Hong Kong H5N1 virus, and it is speculated that a reassortment event occurred between the Goose/Guangdong/96-like virus and one or both of these LPAI viruses in the LBMs of Hong Kong (Guan *et al.*, 1999; Hoffmann *et al.*, 2000). The Hong Kong 1997 H5N1 viruses appear to have a unique constellation of gene segments, and Hong Kong/97-like viruses have not been seen since the eradication program in Hong Kong at the end of 1997.

Changes were made in the Hong Kong LBM system to reduce the risk of the LBMs harboring AI, but because almost all the poultry coming into Hong Kong originated in southern China, the prevention program was not completely successful. The next reported occurrence of H5N1 was again in Hong Kong in 1999. Indeed, much of the early history of H5N1 seems to be centered in Hong Kong because of the commendable transparency of veterinary officials in Hong Kong for disease reporting in the city, and Hong Kong became a unique window into what was occurring in southern China. As part of the testing and surveillance of birds coming into Hong Kong from China, a consignment of geese were tested for antibodies to H5 influenza and were positive. However, by the time serology results were available, the geese had already been slaughtered, but environmental samples from the cages of the geese were tested and H5N1 was isolated. These viruses were sequenced and characterized biologically. Sequence similarity showed the environmental (Goose)/Hong Kong/99 viruses to have an H5 gene closely associated with the earlier Hong Kong and Guangdong viruses. However, the other viral genes were most closely associated with the Goose/Guangdong/96 virus, including the N1 gene with a full-length stalk. Biologically, the Hong Kong/99 viruses were highly pathogenic for chickens with 100% mortality, but only moderately pathogenic in geese with 50% mortality (Cauthen *et al.*, 2000; Webster *et al.*, 2002). Additional reports of H5N1 viruses being isolated in Hong Kong from 2000 to 2002, either in the LBMs or as part of the surveillance efforts of flocks from China, documented the continued circulation of H5N1 in mainland China. In addition, evidence of viruses with different combinations of internal genes from reassortment was observed (Guan *et al.*, 2002a, b). Reports of H5N1 circulating in apparently healthy ducks in China from 1999 to 2002 were eventually published in the scientific literature (Chen *et al.*, 2004).

The next unusual isolation of H5N1 was from a shipment of duck meat at a quarantine station in South Korea. Because of concerns about HP H5N1, poultry products from China were being tested for AI, and in May 2001, a virus was isolated from duck meat coming from Shanghai, China. The virus had H5 and N1 genes that were clearly in the Goose/Guangdong/96 lineage, but the N1 gene had a 20-amino-acid deletion in the stalk, suggestive of gallinaceous poultry adaptation. Some of the internal gene segments were most closely related to the Goose/Guangdong/96 lineage, but several genes were unrelated and the virus was clearly a reassortant virus. The virus was highly pathogenic in chickens. In Pekin ducks, the virus could infect the ducks, but they

showed no clinical signs. However, immunohistochemistry and virus isolation tests demonstrated that the virus replicated inside skeletal muscle, which corroborates the finding of the virus in the frozen duck meat (Tumpey *et al.*, 2002). This case demonstrated for the first time that apparently clinically healthy birds could be a source of infectious virus that could have public health implications. In addition, as was later shown in Germany, infected poultry products can be a direct source of infection for poultry (Harder *et al.*, 2009).

Evidence of H5N1 infection continued to mount in China, associated with circulation of the virus primarily in domestic ducks and geese, but some outbreaks in chickens were also observed (Guan *et al.*, 2002a, b; Sims *et al.*, 2003; Chen *et al.*, 2004). However, an outbreak of H5N1 in late 2002 in Hong Kong in a zoological collection as well as in a small number of wild birds demonstrated a different pattern. This outbreak was associated with mortality in a variety of avian species and was one of the first well-documented cases of wild birds being infected and having mortality with the H5N1 virus (Guan *et al.*, 2004). The viruses from this period also had considerable variability in the combinations of internal genes found in the different viruses, but a dominant constellation of genes referred to as the Z genotype began to emerge (Li *et al.*, 2004). The non-structural gene with an unusual genotype, a 5-amino-acid deletion in the NS1 protein, was first identified in 2000, but quickly became the most commonly seen segment associated with the H5N1 lineage. This deletion in the NS1 protein was reported to enhance the virulence of the virus in chickens (Long *et al.*, 2008). This plethora of gene combinations has made it extremely difficult to correlate the differences in phenotype to the genotype.

The stage was set in late 2003 for the unprecedented spread of H5N1 across Asia. The first country to officially report H5N1 HPAI to OIE was South Korea in December 2003, and a number of countries followed with reports in the first few months of 2004. However, retrospective analysis shows that the virus had been spreading in some Asian countries much earlier and, in some cases, was diagnosed but purposely not reported for trade reasons. One of the most egregious offenders was Thailand. Thailand was a major exporter of fresh poultry meat to the European Union, and they continued to deny that they had H5N1 in their country despite evidence to the contrary until the first human case was reported from Thailand in 2004. This denial of H5N1 in the poultry sector had serious ramifications as it undermined the country's credibility with trading partners and seriously damaged their long-term poultry exports (McLeod, 2008).

Antigenic drift and the emergence of clades

The hemagglutinin protein of AI from the beginning had some genetic and antigenic drift. However, these differences became more prominent when the virus began spreading across Asia in 2004 and it became apparent that multiple sublineages of the hemagglutinin gene were appearing. So, although all the viruses had a similar origin, at least for the hemagglutinin gene, the viruses were becoming more genetically and phenotypically different. Under the current classification, ten different viral lineages, referred to as clades, have been defined. The clades are labeled from 0 to 9, with the clade 0 group having the Goose/Guangdong/96 virus as the origin (World Health Organization Global Influenza Program Surveillance Network, 2005). The WHO/OIE/FAO H5N1 Evolution working group (http://h5n1.flugenome.org/method.php) defines a clade as having pairwise sequence distance of >1.5% in the hemagglutinin gene. A clade should contain a minimum of representative isolates that cluster together with <1.5% pairwise sequence difference within the group. A clade can also be further divided into subclades if they also meet these criteria. Ideally, antigenic properties, as measured in cross hemagglutination inhibition (HI) titers, should be considered in the identification of clades. There is good evidence that genetic clades do correlate with antigenic differences, but not all clades have been adequately antigenically characterized. This is due, in large part, to the fact that there is not widespread access to AI viruses from many parts of the world, including China that appears to be the origin of most of the defined clades (Chen *et al.*, 2006b; Wallace and Fitch, 2008; Wang *et al.*, 2008).

The spread of H5N1 in 2004 included two genetically distinct groups of viruses, clades 1 and 2. The clade 1 viruses spread to Vietnam, Cambodia, Thailand, Laos, Malaysia, and Hong Kong. The clade 2 viruses spread to South Korea and Indonesia (Wang *et al.*, 2008). After this initial introduction of virus, a variety of outcomes was possible. One outcome was that the virus could be eradicated relatively quickly, as occurred in South Korea. Other possibilities were that the virus could be eradicated after lengthy circulation of the virus (Thailand), or it could persist and become endemic (Vietnam and Indonesia). With all these scenarios, the potential for introduction of new viruses was also possible. Once a virus was introduced into a country, if the virus was allowed to persist, then the geographic separation of the viruses allowed them to independently evolve compared to viruses in other countries. For example, a single introduction of virus of the Clade 2 type appears to have occurred in Indonesia (Wang *et al.*, 2008). This virus, although from a single source or closely related group of viruses, quickly spread to a number of Indonesia islands. As control measures were increased, the virus within Indonesia became separated geographically, both domestically and internationally, and the virus started to evolve independently from the Clade 2 viruses found in China and other countries (Wallace and Fitch, 2008). The viruses that originally started the outbreak in Indonesia were

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identified as Clade 2.1 viruses to acknowledge the differences in sequence of viruses found in other countries. However, the Clade 2.1 viruses continue to evolve in several independent lineages within Indonesia, and further division into subclades was necessary so that clades 2.1.1, 2.1.2, and 2.1.3 were observed (data not shown). Again there are genetic and antigenic differences between these sublineages that can affect available control measures, specifically vaccination.

Other countries like Vietnam have a different story. In Vietnam, clade 1, 2 and 7 viruses have been identified (Dung Nguyen *et al.*, 2008; Wang *et al.*, 2008; Nguyen *et al.*, 2009). The clade 1 viruses appear to have been introduced first and were found in both the northern and southern parts of the country. However, additional introduction of virus, both clades 2.3.2 and 2.3.4, from China occurred in the north in late 2005 (Pfeiffer *et al.*, 2009). Although vaccination and other control measures are being applied in Vietnam, there is rampant illegal movement of poultry from China, which continues to allow the introduction of new viruses, with clade 7 being the latest (Nguyen *et al.*, 2009). Currently, multiple clades continue to circulate in Vietnam, but a pattern has emerged of clade 1 viruses being endemic in the south in the Mekong River delta and clade 2 viruses being endemic in the north around the Red River delta, but with the introduction of some clade 7 viruses on the Chinese border (Pfeiffer *et al.*, 2009). This correlation with the major river systems likely reflects how important these rivers are for moving poultry within the country.

Currently, H5N1 is known to be endemic in Vietnam, Egypt, Indonesia, and China (Domenech *et al.*, 2009). Other countries including Bangladesh are suspected of having endemic HP H5N1. Unfortunately, accurate and transparent reporting of AI outbreaks continues to be the exception rather than the rule, and true distribution of the virus in poultry is not known. Over 60 countries have had HP H5N1 and most have been able to control and eliminate the infection. The countries that have been successful in eliminating the virus have typically had a better veterinary infrastructure that allows earlier diagnosis and more rapid controls. However, reintroduction is common for countries that border these endemic countries. Often this is because of illegal movement of poultry between countries. Countries like China, Indonesia, Egypt, and Vietnam, partly for economic reasons, used vaccination as the primary control tool, and unfortunately this approach did not eradicate the virus from the country.

Wild bird involvement in the epidemiology of H5N1

As previously described, HPAI viruses as a general rule do not persist in wild bird populations. However, the H5N1 lineage of virus no longer follows this rule. During the period 1996–2001, there was little evidence of wild bird involvement with H5N1. The first example showing that wild birds might be important in the spread of the virus was the previously mentioned outbreak in a zoological collection in late 2002 in Hong Kong (Guan *et al.*, 2004). With the emergence of H5N1 in a large number of countries in 2004, some speculation of wild bird involvement was raised as a possible explanation for the rapid spread of the virus, but with little scientific support. Expert opinion felt the spread of the virus was still primarily through movement of infected poultry or poultry products (Sims and Brown, 2008). However, in April 2005, a paradigm shift occurred when a large outbreak of H5N1 with high mortality occurred in variety of wild ducks and gulls in Qinghai Lake, China (Liu *et al.*, 2005; Chen *et al.*, 2005, 2006a). Subsequently, strong evidence of wild bird infection and movement of the virus over large geographic areas resulted in the spread of HPAI virus into Europe and Africa (Chen *et al.*, 2006b; L'Vov *et al.*, 2006). In particular, during the severe winter of 2006, a large number of wild birds were shown to be infected in Europe (Sims and Brown, 2008). The current evidence suggests that the virus is still present in wild birds, but at a lower level than was seen in 2006 (Lvov *et al.*, 2010; Sharshov *et al.*, 2010).

With the important role of wild birds in the epidemiology of H5N1 HPAI viruses, the control and eradication of the virus was greatly complicated. Most previous outbreaks of HPAI have been eradicated, in part because the virus was maintained only in poultry. Previous experience with LPAI viruses shows that you can reduce exposure to wild birds, but you cannot completely prevent exposure to wild birds or to the viruses they excrete. Additional preventive measures were instituted in Europe to try to reduce the risk of infection, including increased biosecurity and involved confinement rearing of poultry. However, confinement rearing for backyard and small holder flocks is difficult and expensive to maintain (Pittman and Laddomada, 2008). An additional difficulty is the increased susceptibility of poultry to the H5N1 HPAI lineage of viruses as measured by the low MID_{50} in chickens (Table 1) (Subbarao *et al.*, 2003; Swayne and Beck, 2005; Swayne and Slemons, 2008). Therefore, poultry flocks exposed to only small amounts of virus may be infected. The true prevalence and persistence of H5N1 HPAI in wild birds is still not known. Although the surveillance of wild birds has increased on a massive scale, there is a lack of understanding of the ecology of HPAI, including what species are infected and how long they can shed (Feare, 2010). Unfortunately, because the virus continues to change and the limited research done with one viral strain may not apply to other viral strains, a complete understanding of the virus in wild birds is not likely to be attained. With the apparent reduction of H5N1 HPAI in wild birds, at least based on reported mass mortality events in wild birds, it is unclear whether the virus will be maintained in wild birds.

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Conclusions

AI viruses remain one of the most important challenges in veterinary medicine, and long-term control appears unlikely because of the wild bird reservoir of LPAI virus. Over the last 20 years, the incidence of new HPAI outbreaks appears to be increasing, suggesting that current poultry-rearing practices may increase the opportunities for outbreaks. Lastly, the emergence and spread of H5N1 HPAI in Asia, Europe, and Africa has greatly increased the stakes for both veterinary and public health communities. AI continues to be a global health issue, and control efforts need to be coordinated before the virus can be eliminated. As an example, the veterinary officials in Hong Kong have instituted the most rigorous controls and have maintained a high level of surveillance for over 13 years, but they continue to face H5N1 and have to respond to outbreaks almost every year. AI also continues to affect a disproportionate number of smallholder and backyard poultry farmers, which results in economic and food security concerns for the poorest people.

The last 13 years have resulted in a huge influx of resources into the veterinary community, both for control and research, and although progress has been made, we still remain far from our goal. An increased understanding of our adversary and innovative approaches for control and eradication is still needed. The most obvious control tools are vaccines, and the next generation of viral-vectored vaccines may have distinct advantages over our current vaccines. However, AI, like other influenza viruses, suffers from antigenic drift, and vaccines will need to be targeted for the best results. Other technologies may include the use of transgenic animals that are resistant to influenza infection. These technologies are on the near horizon, but are currently out of reach. International cooperation, virus sharing, and transparency in disease reporting are all necessary first steps to achieve the goal of eradication of H5N1 HPAI and the prevention of outbreaks with either LP or HPAI viruses.

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