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## The emergence and diversification of panzootic H5N1 influenza viruses

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### Abstract

The Asian highly pathogenic avian influenza H5N1 virus was first detected in the goose population of Guangdong, China in 1996. The viruses in this lineage are unique in their ecological success, demonstrating an extremely broad host range and becoming established in poultry over much of Asia and in Africa. H5N1 viruses have also diverged into multiple clades and subclades that generally do not cross neutralize, which has greatly confounded control measures in poultry and pre-pandemic vaccine strain selection. Although H5N1 viruses currently cannot transmit efficiently between mammals they exhibit high mortality in humans and recent experimental studies have shown that it is possible to generate an H5N1 virus that is transmissible in mammals. In addition to causing unprecedented economic losses, the long-term presence of the H5N1 virus in poultry and its frequent introductions to humans continue to pose a significant pandemic threat. Here we provide a summary of the genesis, molecular epidemiology and evolution of this H5N1 lineage, particularly the factors that have contributed to the continued diversification and ecological success of H5N1 viruses, with particular reference to the poultry production systems they have emerged from.

### Keywords

highly pathogenic avian influenza; pandemic; ecology; poultry markets

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## 1. Introduction

Southern China has been long-term regarded as an “influenza epicenter” for the genesis and emergence of pandemic viruses (Shortridge, 1982). The ‘bird flu’ incident in Hong Kong in 1997, where highly pathogenic avian influenza (HPAI) H5N1 viruses infected 18 people, reinforced this view while also changing our concept of influenza subtypes that may cause human outbreaks and their emergence pathways. Previously it was assumed that only mammalian viruses, i.e. those with  $\alpha$ -2,6 receptor specificity could infect humans while viruses with avian receptor specificity could not (Webster et al., 1992). The direct avian-to-human transmission of H5N1 in the Hong Kong outbreak presented a lesson that caused a conceptual shift in influenza biology.

Prior to the outbreak of HPAI H5N1 in the goose population in Guangdong in 1996 there was no previous record of HPAI outbreaks in China or the region (Alexander, 2007; Alexander and Brown, 2009; Guo et al., 1998; Xu et al., 1999). The Asian H5N1 lineage viruses were also exceptional in generating multiple genetic variants (Guan et al., 2002a, 2003) and spreading over a vast geographic region, becoming endemic in poultry not only in its original outbreak region but in 63 countries and regions in Eurasia and Africa (Chen et al., 2005; Domenech et al., 2009; FAO, 2012a–c; Hafez et al., 2010; Li et al., 2004; Smith et al., 2006a,b; Wan et al., 2008). The zoonoticity of HPAI H5N1 viruses in birds has led to a major panzootic, particularly in poultry with an estimated 400 million birds killed and economic losses of US\$20 billion (FAO, 2012c), while associated human infections mark H5N1 viruses as a high risk candidate for the next influenza pandemic strain.

Despite the enormous impact of Asian HPAI H5N1 viruses in so many countries, the early understanding of the emergence, ecology and evolution of this virus lineage was largely based on disease outbreak investigations in different countries. While the success of the long-term, systematic influenza surveillance in farms and live-poultry markets in Hong Kong and southern China is internationally recognized, there are still major areas of enzootic H5N1 activity where little or no effective longitudinal surveillance in poultry is conducted. This is of continuing concern as surveillance has contributed immensely to our understanding of factors that have contributed to the unique success of this virus. Detection of viruses through enhanced surveillance encourages implementation of control and prevention strategies, such as stamping out. Characterizing isolated viruses allow the development and implementation of effective control measures such as vaccines. These practices can reduce environmental virus load and reduce transmission.

## 2. Emergence of the Asian HPAI H5N1 lineage

Historically, of the more than 20 HPAI outbreaks recorded since 1959 the majority have occurred in chicken and turkey flocks, with only a few HPAI outbreaks observed in other types of birds (Alexander, 2007; Alexander and Brown, 2009). In Germany in 1979, an H7N7 virus outbreak in commercial chicken farms also infected geese in adjacent farms (Rohm et al., 1996). The isolation of the A/goose/Guangdong/1/1996-like (Gs/GD) H5N1 viruses from sick geese on two farms in Guangdong, China in 1996 was the first detected case of HPAI outbreak that originated in domestic geese (Xu et al., 1999).

From 2000 to 2006, our influenza surveillance of over 22,000 geese in southern China showed that over 90% of the 1,535 influenza strains detected were HPAI H5N1. Over the same period, almost all H5 influenza isolates from domestic ducks in the region were HPAI H5N1 viruses (Duan et al., 2008). These comprised the majority of HPAI H5N1 isolates, with ducks having an isolation rate 5 to 10 times of that in chicken and minor poultry. This surveillance was conducted in apparently healthy birds, indicating that viruses were shed during asymptomatic infections. As China has approximately 65% of the world's domestic duck and 95% of the domestic goose populations (Figure 1 and FAO 2012d), it has become an ideal environment for the virus to become endemic. These studies suggest that HPAI H5N1 is well adapted to domestic geese and ducks, and that both are important hosts in the long-term maintenance of the H5N1 virus.

Experimental studies have shown that H5 and H7 lowly pathogenic avian influenza (LPAI) virus precursors may evolve into HPAI viruses after a series of passages in domestic poultry (Bean et al., 1985; Horimoto and Kawaoka, 1995; Kawaoka et al., 1984; Kawaoka and Webster, 1985; Webster et al., 1986, 1989). The Pennsylvania H5N2/1983 and the Mexico H5N2/1995 outbreaks were also shown to have evolved from LPAI from migratory birds (Bean et al., 1985; Horimoto et al., 1995). Since 1997, several LPAI H5 and H7 outbreaks were observed in domestic poultry in Europe, in which the precursor virus was shown to have originated from migratory birds (Banks et al., 2001; Campitelli et al., 2004, de Marco et al., 2003, 2005, Munster et al., 2005).

Characterization of LPAI H5 viruses isolated from poultry and migratory birds in China and Europe from the 1970s to the 2000s showed that the HPAI Gs/GD virus was derived from LPAI strains resident in migratory birds along the Western Pacific flyway (Duan et al., 2007). However, systematic influenza surveillance in southern China since 2000 has yielded only a single Eurasian gene pool LPAI H5 virus from domestic geese, which contained internal gene segments from viruses lineages established in terrestrial poultry (Duan et al., 2007). Phylogenetic analyses of viruses of other subtypes from domestic geese revealed that all were closely related to viruses resident in domestic ducks (YG unpublished data). However, we are unable to determine whether the Asian HPAI H5N1 virus was initially generated in domestic geese that it was first isolated from.

### 3. Genomic diversification

A distinguishing characteristic of the Asian HPAI H5N1 virus is the diversification of the lineage into over 20 distinct HA clades and the generation of numerous genetic reassortants, or genotypes (Chen et al., 2006; Guan et al., 2002a,b, 2003; Li et al., 2004; Smith et al., 2006a), with a rapid increase of genomic diversity of H5N1 viruses in China from mid-1999 to early-2000 (Vijaykrishna et al., 2008). In contrast, all previous HPAI and LPAI outbreaks contained a single H5 and H7 virus genotype in each outbreak (Donatelli et al., 2001; Fouchier et al., 2004; Horimoto et al., 1995).

Evolutionary analyses indicate that the genomic diversity of H5N1 has been generated within the southern China poultry system, where more than 40 distinct genotypes have been identified (Duan et al., 2008). There is also little evidence of reassortment occurring in other

regions affected by H5N1, with all genotypes detected beyond China having been previously detected there years in advance (de Benedictis et al., 2007; Bragstad et al., 2007; Li et al., 2004; Lipatov et al., 2007; Wang et al., 2008). It therefore appears that the ecology of poultry systems in southern China, which contain a diverse assemblage of aquatic and terrestrial birds that harbor co-circulating endemic populations of H9N2 and H6N1 viruses (Duan et al., 2008; Vijaykrishna et al., 2008), is unique in generating H5N1 reassortant viruses.

Southern China has the largest population of domestic ducks in the world (Figure 1) in which a high diversity of influenza virus subtypes co-circulate throughout the year (Chin et al., 2002; Huang et al., 2010; Li et al., 2003; Shortridge, 1992). Surveillance data has demonstrated that H5N1 viruses are perpetuated primarily in domestic ducks in this region (Li et al., 2004; Smith et al., 2006a). Approximately 11% of influenza A positive samples collected from poultry in Southern China were positive for multiple influenza subtypes, indicating co-infections, with the majority of these samples coming from ducks in farms and markets (Pepin et al., 2012). Furthermore, it has been demonstrated that domestic ducks experience asymptomatic infection to H5N1 viruses and shed virus over extended periods, which was also observed in our surveillance (Sturm-Ramirez et al., 2005). Domestic ducks therefore act as a silent carrier and source for H5N1 infection of terrestrial poultry (Hulse-Post et al., 2005).

It is also apparent that reassortment between influenza viruses from domestic and migratory birds has contributed to the increased diversity of influenza viruses in Eurasian poultry populations (Duan et al., 2007). Phylogenetic and antigenic analyses demonstrated that multiple H5N1 variants contained some gene segments that were directly derived from the viruses resident in migratory birds (Duan et al., 2007; Smith et al., 2009b). Specifically, different influenza subtypes from wild birds, i.e. the natural gene pool, are introduced into domestic ducks. These viruses then reassort with H5N1 viruses endemic in domestic ducks, and these new variants are transmitted within the populations of ducks and other poultry species, both in farms and live-poultry markets, which results in frequent interspecies transmissions, outbreak events and genetic drift (Vijaykrishna et al., 2008). The co-circulation of multiple influenza subtypes and their establishment in poultry in China appears to be unique. While surveillance for H5N1 viruses has intensified globally since 2003, little information is available regarding other subtypes such as H9N2 and H6N1/N2 viruses that are thought to be endemic in poultry in several countries.

An unusual aspect of the genomic diversity of HPAI H5N1 viruses is the incorporation of gene segments that are most closely related to genes from influenza viruses isolated in the 1970's that have been used extensively in poultry vaccines (Duan et al., 2007; FAO, 2009). Although these 1970's viruses have never been detected during surveillance in China, gene segments of A/turkey/England/N28/1973-like (H5N2), and A/African starling/England-Q/983/1979-like (H7N1) viruses have been detected in contemporary isolates from chicken and tree sparrows (Duan et al., 2007). This phenomenon was first observed in Hubei Province in 1997, with some of these Hubei-like H5N1 viruses detected in surveillance up to 2004 (Duan et al., 2007; Kou et al., 2005). Given that these viruses have not been circulating in poultry, the most parsimonious explanation is that incompletely inactivated vaccines

manufactured with these viruses have led to the introduction of laboratory strains into the field. Indeed, the first commercially available H5 vaccine was named N28 (after HPAI A/turkey/England/N28/73, H5N2), and several subtypes of seed virus strains including H5, H7 and H9 were imported to China for vaccine production as early as in 1995 (Liu et al., 2006) and were used to manufacture inactivated whole virus vaccines or inactivated recombinant vaccines (Bublöt et al., 2007; Qiao et al., 2006; Webster et al., 2006). Attempts to manufacture bi- or trivalent vaccines for H5, H7 and H9 viruses could lead to virus reassortment during manufacture. As was observed in HPAI H5N1 viruses, all H9N2 and H6N1 viruses isolated since 2000 are also multiple genetic reassortant variants (Cheung et al., 2007; Li et al., 2003; Xu et al., 2007a,b).

The majority of the H5N1 reassortant viruses began emerging in late 2000 to early 2001 (Vijaykrishna et al., 2008), and around this time multiple different genotypes of H5N1 viruses were isolated during a single outbreak event and even on the same sampling occasion at the same market (Guan et al., 2002). Whether vaccination was involved in the generation of reassortant H5N1 and H9N2 viruses remains unknown.

#### 4. Virus transmission and outbreaks

Both intra- and inter-regional transmission pathways HPAI H5N1 virus lineages are closely related to poultry movement (van Kerkhove et al., 2009; van den Berg, 2009; Soares Magalhaes et al., 2010a), the introduction of H5N1 virus into Vietnam being a good example (Vijaykrishna et al., 2008; Soares Magalhaes et al., 2010b). In 2001, only sporadic infected birds were detected in Vietnam but no outbreaks were reported (Nguyen et al., 2005; Wan et al., 2008). However, in 2003 H5N1 Clade 1 virus was introduced and caused the first wave of major outbreaks in Vietnam, with genetic analyses showing progenitor viruses were prevalent in poultry in Yunnan, China at least ten months before outbreaks were detected (Smith et al., 2006b; Vijaykrishna et al., 2008; Wang et al., 2008).

Wild birds that directly interact with either back-yard poultry or low level of bio-security poultry farms have also been implicated in local transmission from an infected farm. This pattern was observed in Korea during the 2006–2007 outbreaks and in Japan in 2007, where viruses from outbreaks in poultry were related to those found in wild birds (Lee et al., 2008; Shivakoti et al., 2010). A similar situation has also been observed in Hong Kong where a diverse group of dead wild birds, predominantly passerines and raptors, tested positive for H5N1 viruses despite there being no poultry outbreaks in Hong Kong during the same period (Smith et al., 2009b). Wild birds might also play a role in the dissemination of the virus in some regions of China as sparrows were reported to be positive for the H5N1 virus (Kou et al., 2005; Liu et al., 2010). The repeated detection of H5N1 virus from wild birds, particularly in the absence of outbreaks in poultry at the same time, demonstrates the potential for wild birds to disperse the virus locally over tens or hundreds of kilometers.

In contrast, migratory birds have played a key role in the long-distance transmission of HPAI H5N1 virus. The H5N1 outbreak in geese and gulls at Qinghai Lake, China in 2005 was the first evidence of large-scale death from H5N1 virus in migratory birds (Chen et al., 2005). Closely related Clade 2.2 viruses were subsequently transmitted to Mongolia, Siberia,

Central Asia, East and Western European countries, and Africa (de Benedictis et al., 2007; Bragstad et al., 2007; Chen et al., 2005; Coven, 2006; Gall-Recule et al., 2008; Lipatov et al., 2007; Onishchenko et al., 2006). Virological and serological studies have shown that migratory birds can be naturally infected by HPAI H5N1 viruses and that infected birds could survive and shed the virus during migration (Brown et al., 2006; Goletic et al., 2010; Keawcharoen et al., 2008; Marjuki et al., 2009; Saad et al., 2007). Furthermore, analysis of the spatio-temporal dynamics of H5N1 outbreaks found an association with the seasonal migration of wild birds from 2003 to 2006 that helped to explain the westward spread of the virus (Si et al., 2009).

An unusual aspect of the H5N1 panzootic is the fact that many geographical areas remain unaffected, despite harboring migratory bird populations from Eurasia. This is particularly true for North America and Australia, as both continents have strong connections through the East Atlantic and East Asian Australian flyways (Arzey, 2006; Krauss et al., 2007; Peterson et al., 2007; Winker and Gibson, 2010). Additionally, current knowledge cannot explain why only one-way transmission of HPAI H5N1 viruses has occurred, from Asia to Europe and then to Africa, with no apparent reverse West-East transmission. It is likely that many factors, such as poultry movement, or species specificity of different subtype influenza viruses are involved (Munster et al., 2007; Pepin et al., 2012).

An important consequence of the transmission of H5N1 to different areas is the diversification of geographically isolated populations to form multiple sublineages, with 9 distinct clades now circulating (Figure 2). Antigenic drift within the H5N1 sublineages, which generally do not cross neutralize, has greatly complicated control of H5N1 viruses through vaccination, both in poultry populations and for human pre-pandemic vaccine preparation (Chen and Bu, 2009; WHO, 2012a). In particular, this has created the necessity of continually updating the candidate virus chosen for future H5N1 vaccines with, for example, 23 human pre-pandemic vaccine candidates against 12 distinct H5N1 lineages (WHO, 2012a). Just as reassortment occurs predominantly in China, with little evidence of reassortment occurring in other regions affected by H5N1, all progenitor viruses to all H5N1 clades now described have been detected in China years before their detection in other regions (e.g. Wang et al., 2008). These observations further suggest that there is something unique about the ecology of poultry production systems in China.

## **5. Influenza transmission dynamics and poultry production systems in China**

Southern China has been considered a hypothetical influenza epicenter since the early 1980's as several pandemic influenza viruses emerged from this region and there was frequent influenza activity at the human-animal interface (Shortridge and Stuart-Harris, 1982). This has been attributed to agricultural practices and economic development; in particular the huge number of farmed poultry, poly-culture farming practices and the live-poultry market system combining to form a distinct influenza ecosystem.

In China, approximately 15 billion poultry were produced each year over the last decade (FAO, 2012d). Production of poultry is spread across all sectors, from integrated industrial

with high levels of bio-security to village or backyard production with poly-culture farming, i.e. the production of multiple bird species on a single farm, a common practice. This equates to the largest domestic population of influenza virus natural reservoirs, which has facilitated the interaction of viruses from different sources, such as the natural gene pool, to increase the genetic diversity of influenza viruses in this population (see above). A confounding factor for this interaction between the domestic aquatic and natural gene pools has been the recent establishment of large farming operations in major aggregation sites for migratory birds, such as Poyang, Dongting and Qinghai Lakes (Chen et al., 2006; Huang et al., 2010). This has directly increased the interaction between domestic waterfowl and migratory birds, with our surveillance in sentinel ducks providing direct evidence of transmission of low pathogenic H5, H7 and H8 viruses from migratory birds to domestic ducks, all subtypes that were rarely detected in live-poultry markets (Chen et al., 2006; YG, unpublished).

While the majority of poultry are chicken and ducks, another important characteristic of the influenza ecosystem in China is the increase in the variety of different poultry types (termed minor poultry), particularly chukka, Guinea fowl, partridge and pheasant. All of these relatively new members of the poultry population in China are susceptible to different avian influenza viruses including HPAI H5N1 viruses (Cheung et al., 2007; Humberd et al., 2006; Xu et al. 2007a,b). Minor poultry can act as intermediate hosts in avian to mammal interspecies transmission or function as mixing vessels to facilitate reassortment events for viruses from different hosts (Humberd et al., 2006; Liu et al., 2003; Makarova et al., 2003; Matrosovich et al., 2001; Perez et al., 2003a,b). At least two subtypes of influenza viruses have become established in minor poultry and genetic analyses indicates that those viruses are directly involved in reassortment of H5N1 viruses with other subtypes (Cheung et al., 2007; Guan et al., 1999; Xu et al., 2007a,b). The introduction of increasing numbers of species into the poultry production system in China over the past two decades has therefore created a more complex influenza ecosystem.

Poultry trade in China relies principally on networks of wholesale and retail live-poultry markets that are present in almost each city and small town across the country. Within this system, multiple species of live poultry, both aquatic and terrestrial, are kept in close proximity and often in conditions of poor hygiene. Poultry in these markets can come from local or remote producers, and not just from large industrial operations but also from backyard farms (Martin et al., 2011). Birds are mixed together regardless of origin and infected poultry from low bio-security farms could therefore transmit the virus to naïve birds from high-level bio-security farms. This system of markets leads to a highly connected and large population of poultry (Martin et al., 2011), which is known to favor the emergence and persistence of infectious diseases (May et al., 2001). A further compounding factor is that the virus may spread to previously unaffected areas via contaminated cages or other environmental articles (Fiebig et al., 2009; Martin et al., 2011; Webster, 2004). The structure of the live-poultry market system generates a huge network of poultry movement that greatly increases the chance of interaction among poultry from different regions and origins, vastly reducing the efficacy of control efforts. Even if H5N1 virus is eradicated by adopting comprehensive control measures in an affected region, the virus could be transmitted back

by poultry movement or contaminated cages in a short period of time, as was observed in the live-poultry markets in Hong Kong in early 2002 (Sims et al., 2003).

## 6. Vaccination

Depopulation and vaccination programs are the major control measures applied to HPAI outbreaks and avian virus vaccine development has become a substantial endeavor (Kapczynski and Swayne, 2009). Most epidemics are generally controlled by the culling of infected poultry, however, vaccination has been combined with culling in some instances (FAO, 2007; Swayne and Kapczynski, 2008; Swayne et al., 2011). Vaccination reduces poultry mortality due to H5N1 HPAI, lessening economic losses and minimizing the removal of an important protein source for the human population during outbreaks (Ellis et al., 2004; Marangon et al., 2004; Villarreal, 2009). Experimental data and field studies also suggest that vaccination can effectively protect naïve birds from H5N1 infection and also reduce or block transmission (Ellis et al., 2004; Poetri et al., 2009; Rudolf et al., 2009; Soares Magalhaes et al., 2010b; van der Goot et al., 2008). However, when the different control strategies are compared, outbreaks controlled by culling poultry are short-lived with eradication achieved within 1 year, while HPAI outbreaks that utilize culling combined with vaccination campaigns have not been eradicated as swiftly (Swayne and Kapczynski, 2008). Despite the instigation of large-scale vaccination programs in a number of countries in Asia, H5N1 viruses continue to circulate in those poultry populations with outbreaks frequently recorded (FAO, 2012a). For this review, we focus on the utility of vaccination as a control strategy at the virus population level, with particular reference to the poultry production systems that these vaccination programs are applied within.

Since 2004, large-scale vaccination has become the preferred tool to eliminate or manage H5N1 virus infection in poultry in China, with over 35 billion doses of vaccine administered from 2004–2007 (Chen and Bu, 2009). However, H5N1 viruses in China are still prevalent, with systematic surveillance indicating that H5N1 activity and associated cases of sporadic human infection had increased, particularly during the year 2005 and 2006 (Smith et al., 2006a; WHO, 2011). Possible reasons for these observations may be that the efficacy of vaccination is low, or that the H5N1 virus still replicates in vaccinated domestic ducks without apparent mortality and ducks may therefore act as silent carriers of the virus (Hulse-Post et al., 2005; Savill et al., 2006; Songserm et al., 2006)

Several critical points may have a substantial negative impact on the efficacy of a vaccination program. First, there is a significant correlation between the coverage of the bird population being vaccinated and the efficacy of vaccination, which has been demonstrated in a number of countries (Peyre et al., 2009a,b). As mentioned above, China has an annual estimated annual production of approximately 15 billion poultry, with over 70% of that standing population reared in Sectors 3 and 4 – i.e. small-scale farms or backyards (Sims, 2007; FAO, 2012d). While the reported vaccination numbers are impressive, it is therefore impossible to achieve full coverage for an annual production of 15 billion domestic poultry, even with the use of vaccines providing protective immunity (Chen and Bu, 2009; Tian et al, 2005), although extended shedding from vaccine-challenge studies in chickens have also been observed (Pfeiffer et al., 2010). These large numbers of unvaccinated poultry would



therefore remain capable of spreading the virus through poultry movement or at live-poultry markets.

Second, there is substantial evidence that the efficacy of H5N1 vaccines in waterfowl is reduced compared to that seen in chickens, particularly as prolonged viral shedding in ducks following vaccination is typically observed (e.g. Kim et al. 2008; Pfeiffer et al., 2010; Tian et al., 2005; Webster et al., 2006). This may be associated with differences in the immune system between chickens and ducks (Swayne and Kapczynski, 2008), and indeed, animal experiments have demonstrated that ducks did not generate significant levels of anti-influenza neutralizing antibodies during the first five weeks of their life (Kida et al., 1980). However, protective immunity from vaccination has been reported for ducks in China from a laboratory study wherein the ducks were immunized at 3-weeks and challenged at 6-weeks (Tian et al., 2005). The same study also tested vaccine efficacy in the field with vaccination occurring at 4-weeks, a boost at 14-weeks and challenge occurring at 51-weeks (Tian et al., 2005).

Yet the usual production cycle of domestic ducks in China is 12 weeks (FAO, 2010), questioning the applicability of these experiments to the populations being vaccinated. Taken together the evidence suggests that, even with a two shot vaccination program, vaccination would not eliminate H5N1 virus from duck populations. Rather, it is probable that the partial protection afforded from vaccination of domestic ducks could facilitate the prolonged shedding virus into the environment with subsequent spread to other species (Hulse-Post et al., 2005; Pfeiffer et al., 2010; Sturm-Ramirez et al., 2005).

Another aspect of vaccination programs that is seldom discussed is the impact of vaccination on the evolution of H5N1 HPAI virus. As mentioned above, Asian HPAI H5N1 is unique in having multiple antigenically divergent HA sublineages emerge regularly since 2004, first in China, then Indonesia and more recently in Egypt, all countries that have used vaccination extensively (Chen et al., 2006; WHO/OIE/FAO, 2008, 2009, 2012). In contrast, countries that did not apply blanket vaccination for H5N1 control, such as Japan and Korea, have not had the virus establish endemically (Sugiura et al., 2009). The impact of vaccination on virus evolution has been observed during H5N2 outbreaks in Mexico, where 2 billion doses of vaccine were administered from 1995–2008 (Eggert et al., 2010). Despite this vaccination program LPAI H5N2 viruses have continued to circulate in Mexico for almost 20 years, with spread to neighboring countries (Escorcia et al., 2010; Lee et al., 2004). It is thought that the long-term use of H5N2 vaccines in poultry has led to antigenic drift and diversification of LPAI H5N2 viruses away from the vaccine strain, with challenge experiments showing that the vaccine was unable to prevent shedding of LPAI H5N2 virus (Escorcia et al., 2010; Lee et al., 2004). There are therefore striking parallels with the HPAI H5N1 situation, and it is therefore possible that the large-scale vaccination programs in China, Egypt and Indonesia may have contributed to the antigenic drift of circulating viruses.

## 7. Pandemic risk

Pandemic preparedness was focused largely on the Asian HPAI H5N1 viruses prior to the H1N1/2009 influenza pandemic (Hamilton, 2009). The H1N1/2009 virus emerged in Mexico and the United States in March and early April 2009, and spread rapidly to 74 countries when the WHO declared a pandemic on 11 June 2009 (WHO, 2009). Genetic analyses revealed that the H1N1/2009 virus was generated by reassortment between different swine virus lineages that had been prevalent in pigs for over 10 years (Garten et al., 2009; Hamilton, 2009; Smith et al., 2009a; Trifonov et al., 2009), providing direct evidence for pigs as mixing vessels in the genesis of human influenza pandemic strains as had long been hypothesized (Scholtissek et al., 1990; Shorridge et al., 1977).

Even though the Asian HPAI H5N1 virus has been prevalent in poultry for at least 16 years resulting in more than 600 human infections in 15 countries (WHO, 2011), there is still no evidence of sustained human-to-human transmission. While there are a number of molecular markers that have been associated with the ability of avian influenza viruses to transmit to and replicate in mammals, such as residues in the receptor binding site (Ha et al., 2001; Rogers et al., 1983), we lack the ability to predict which viruses will develop into a pandemic strain. While the mechanism for H5N1 viruses to be transmitted within humans is still unknown, recent experimental work has demonstrated that reassortment with human strains or repeated transmission in ferrets could adapt H5N1 virus to transmit between mammals (Herfst et al., 2012; Imai et al., 2012).

HPAI H5N1 viruses have been rarely isolated from pigs and serological surveys of pig populations in areas where H5N1 is endemic have indicated no or an extremely low prevalence of H5N1 infection (Choi et al., 2005; Jung et al., 2007; Nidom et al., 2010; Song et al., 2010; Zhu et al., 2008). Experimental infection of pigs with avian H5N1 viruses has resulted in infection that is asymptomatic or causes mild disease symptoms, but no transmission to contact pigs has been observed (Choi et al., 2005; Lipatov et al., 2008). Following the pandemic there have been numerous reports of H1N1/2009 transmission to pigs where the H1N1/2009 virus reassorted with swine viruses (e.g. Vijaykrishna et al., 2010), raising the concern that the pandemic virus may reassort with H5N1 viruses either in pigs or humans. Sustained transmission of H5N1 in pigs would increase the likelihood for reassortment of H5N1 viruses, possibly making the pig an intermediary host for mammalian adapted H5N1 viruses. Introduction of the HPAI H5N1 virus into pigs could lead to a pandemic candidate virus and the threat of this should not be underestimated.

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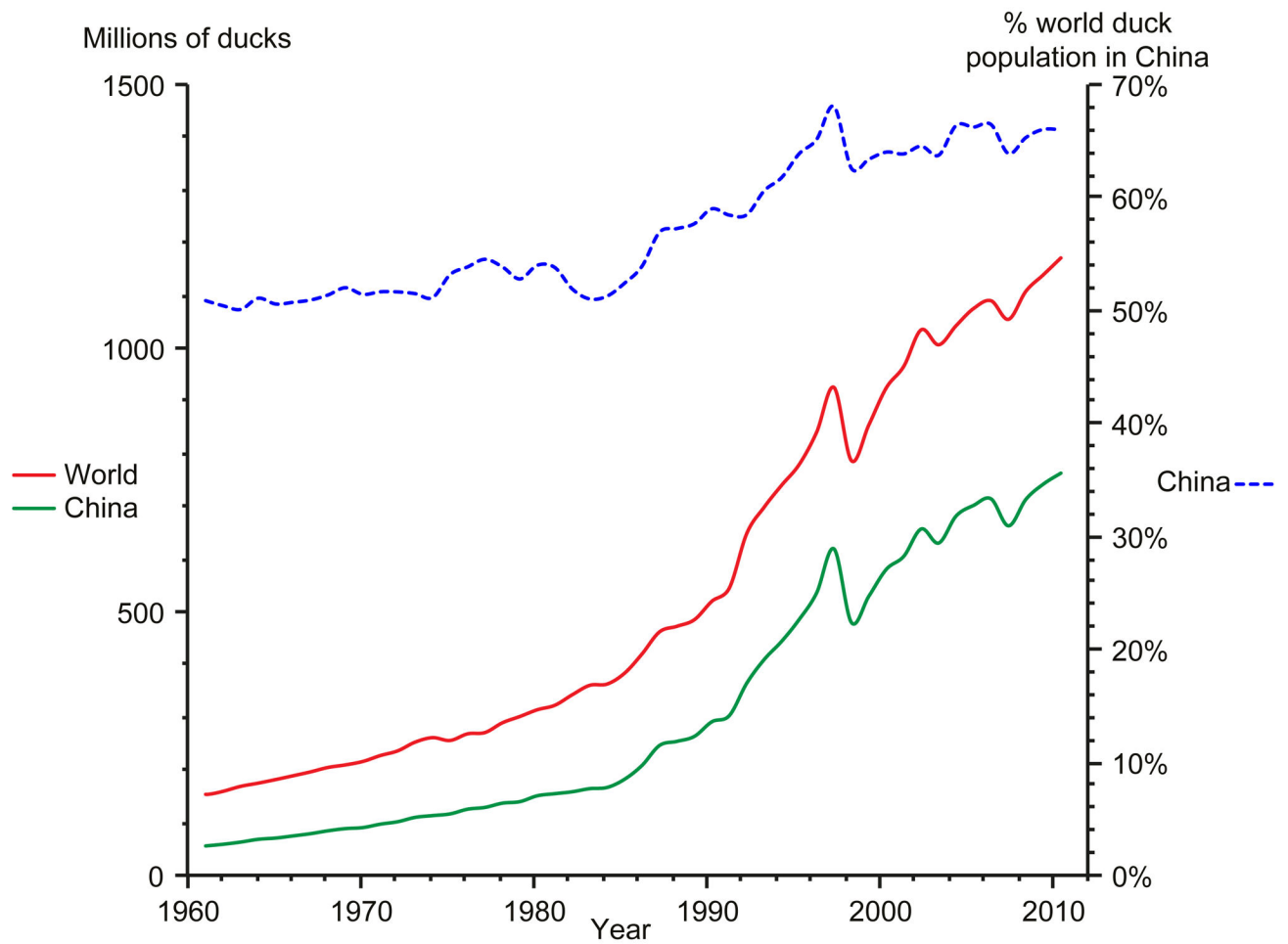
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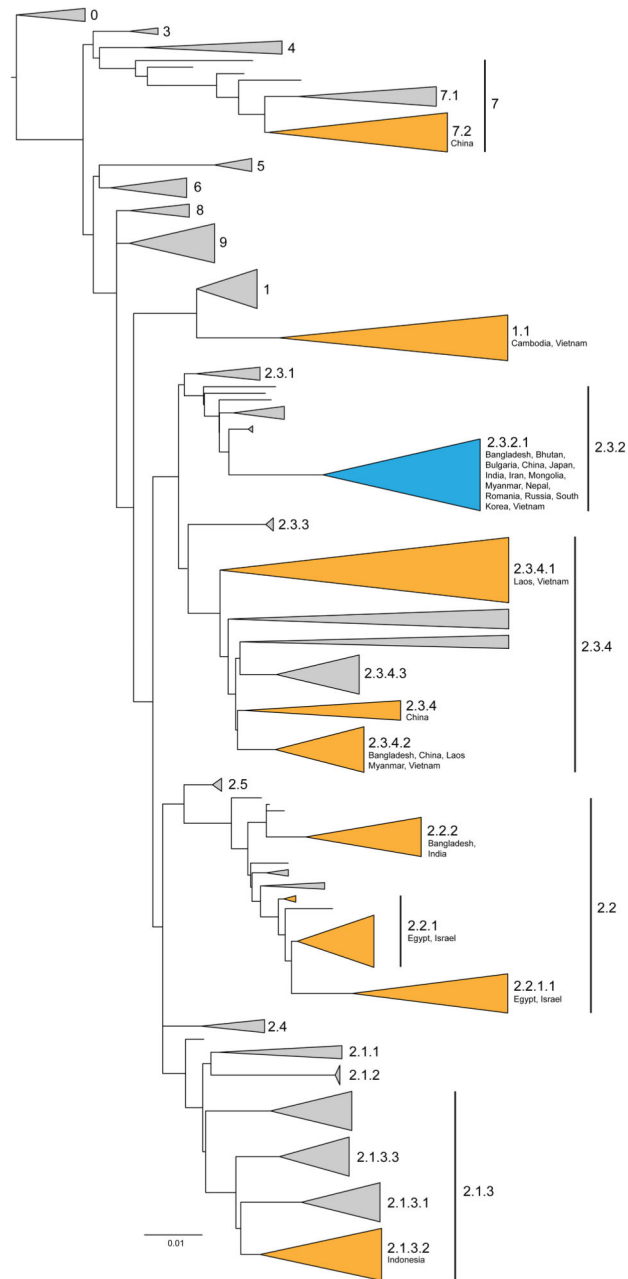
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### Highlights

- A review on the ecology and evolution of H5N1 viruses is presented
- We describe the emergence of H5N1 viruses in southern China
- Trace the genomic diversification of the virus through reassortment with wild bird viruses
- Describe the virus ecology in the poultry production system in China and the use of vaccines and their impact on H5N1 evolution
- Discuss the potential for pandemic emergence of H5N1 virus



**Figure 1.** Changes in world domestic duck population from 1959 to 2010. Green and red solid lines indicate millions of ducks in China and world total, while dashed blue line shows percentage of world duck population in China (from FAO, 2012d).



**Figure 2.**

A stylized phylogenetic tree of H5N1 virus haemagglutinin genes showing the evolutionary relationships among virus clades. Colored triangles indicate virus lineages that have been isolated predominantly from poultry only (orange) or poultry and wild birds (blue) since January 2010. Country names indicate areas that the virus lineages have been detected. Grey triangles indicate virus lineages that have not been detected since January 2010. Compiled from references FAO (2012a–c) and WHO (2012b and 2013). The phylogenetic tree was generated in PAUP\* (Swofford, 2003) using the alignment provided as supplementary information in WHO/OIE/FAO (2012).