Review

H9N2 Subtype Avian Influenza Viruses in China: Current Advances and Future Perspectives

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Abstract | The H9N2 subtype avian influenza virus (H9N2 AIV), which has been in circulation for two decades, has become the predominant subtype in China, attracting considerable attention due to human infections and the contribution of internal genes to H5N1 and H7N9 influenza viruses. Multiple H9N2 AIV genotypes have been identified from various species, including birds and mammals. H9N2 AIVs could spread through water, air, and live bird markets. Since the first isolate from 1994 in China, H9N2 AIVs have been prevalent over 20 areas of China, especially in South China, such as Guangdong, Guangxi, and Fujian provinces. Vaccination is the predominant strategy to prevent and control H9N2 AIVs. There are three inactivated vaccines of H9N2 AIVs have been used in domestic poultry in China. But H9N2 AIVs occurred frequently and caused severe economic losses to the poultry industries. H9N2 AIVs can evade the pressure of vaccination by genetic evolution and antigen variation. Moreover, the ratio of L226 in the haemagglutin (HA) receptor binding site has increased, which indicates that the H9N2 virus has the increasing potential to infection and cause human epidemics. The threat to the poultry industry and public health should not be ignored. In this review, we summarised the findings of recent studies on H9N2 AIVs epidemics and strategies for their prevention in poultry in China.

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Influenza A viruses can be divided into different subtypes based on the hemagglutinin (HA) and neuraminidase (NA) glycoproteins. Along with the H18N11 subtype influenza virus identified in bats, eighteen HA (H1–H18) and eleven NA (N1–N11) subtypes of influenza A viruses have been identified (Tong et al., 2013). However, the H5N1 and H9N2 avian influenza viruses (AIV) are the predominant subtypes circulating in poultry around the world. Such infections have caused severe economic losses in the poultry industry and represent a threat to public health (Butt et al., 2005a; Chen, 2009). The H9N2 subtype avian influenza virus (H9N2 AIV) has been circulating for two decades in China and has attracted considerable attention due the contribution of internal genes to the highly pathogenic avian influenza (HPAI) H5N1 virus in Hong Kong in 1997 and the novel H7N9 influenza virus in mainland China

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in 2013 (Gao et al., 2013; Lin et al., 2000). There are many reports about the H9N2 AIV reassortant with other subtypes, such as H5N1, H7N9, H10N8, H5N3, H3N8, H4N3, H4N6, H7N7, H10N4, and H14N5 viruses (Chen et al., 2014; Dong et al., 2011; Gao et al., 2013; Monne et al., 2013). The novel reassortant subtypes such as H5N2, and H7N9 have emerged and threaten public health and domestic poultry (Gao et al., 2013; Mi et al., 2013; Zhao et al., 2012). Therefore, the H9N2 subtype avian influenza viruses have been classified as candidate viruses with pandemic potential. Multiple strategies should be implemented for the prevention and control of the circulation of H9N2 AIVs in China. Here, we reviewed the epidemiology, pathogenicity, and strategies for the prevention and control of the H9N2 AIVs.

Epidemiology

The H9N2 AIV subtype was first isolated in the United States in 1966 (Homme and Easterday, 1970). In mainland China, an outbreak of this virus was first reported in poultry in Guangdong Province in 1994 (Guo et al., 2000). Since then, H9N2 AIV infection have extended to other areas rapidly, such as Guangxi, Fujian and Jiangsu provinces (Chen et al., 2012; Jiang et al., 2012; Li et al., 2005; Li et al., 2003; Xu et al., 2007), and become the predominant AIV subtype in China (Li et al., 2005). The host range of H9N2 AIVs is diversity, various birds such as chicken, duck, quail, turkey, pheasant, pigeon, partridge, chukkar, waterfowl, and mammals like pig, dog, and human were reported infected by H9N2 AIVs (Butt et al., 2005a; Dong et al., 2011; Lin et al., 2000; Peiris et al., 1999; Shanmuganatham et al., 2013; Sun et al., 2013; Xie et al., 2012; Xu et al., 2007; Xue et al., 2014; Yu et al., 2008).

H9N2 AIVs could spread through water, air, and live bird markets. In China, the geographical distribution of H9N2 AIVs was shown in Figure 1. Clearly four provinces, such as Guangdong, Anhui, Henan and Shandong provinces were predominantly prevalent area, in which there were over 170 HA gene sequences of H9N2 available in NCBI Influenza Virus Resource Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) until to 11th June 2014. Dong et al. summarised the distribution of H9N2 influenza viruses in different hosts and regions from 1966 to 2009, showing a wide distribution and multiple hosts of H9N2 influenza viruses in China (Dong et al., 2011). We analysed the numbers of H9N2 AIVs distributed in different hosts and periods in China between 2010 and 2013 according to the haemagglutinin (HA) sequences of H9N2 AIV published in the NCBI Influenza Virus Resource Database until to 11th June, 2014(Table 1). The results suggest that H9N2 AIVs continue to circulate in China, but in recent years has mainly affected chickens, ducks and wild waterfowl (Table 1), which are the most important reservoir of AIV (Chen et al., 2004; Fouchier et al., 2005). Water as an important factor play a key role in the spread of AIV (Zhang et al., 2014). Migration of waterfowl also increases the spread of AIV to other regions, thus representing an even greater challenge to the control of avian influenza in China (Chen, 2009). Recently, 22 H9N2 AIVs were isolated from wild waterfowl in Dongting Lake in China from 2011 to 2012 (Zhu et al., 2014). Large populations of waterfowl are bred in southern China because of abundant water area, most in backyards or small scale farms (Chen, 2009). Waterfowl AIVs can be transferred to other domestic animals, such as chickens and pigs, when contact is made with waterfowl (Chen, 2009).

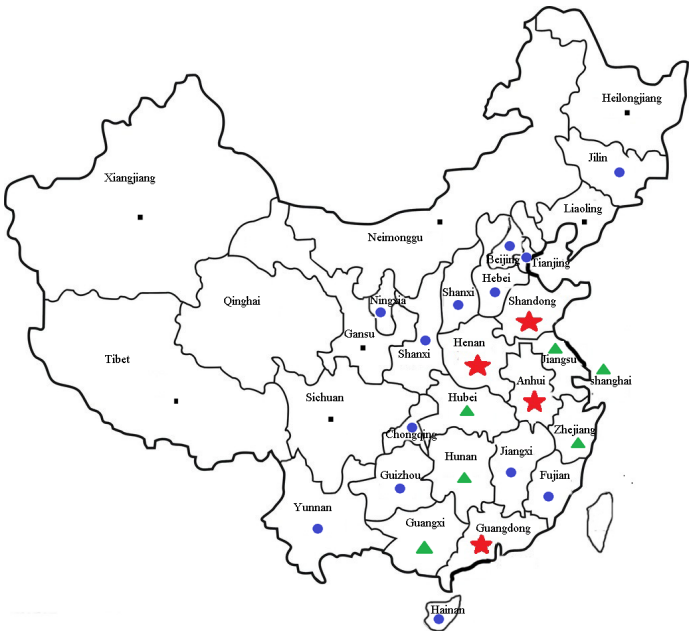

Figure 1. *The geographical distribution of H9 AIVs in China until 11th June, 2014. The red star indicates the amount of H9N2 AIVs HA genes was more than 100 available in NCBI Influenza Virus Resource Database; The green triangle indicates the amount of H9N2 AIVs was between 50 and 100; The blue circle indicates the amount was between 20 and 50; The black square indicates the amount was lower than 20.*

Furthermore, Live bird markets (LBMs), which are the major site of poultry trading in China, are gene pools of the influenza virus, providing an ideal environment for genetic reassortant and interspecies transmission (Ge et al., 2009; Jiao et al., 2012; Nishi et al., 2014; Su et al., 2013; Teng et al., 2012; Teng et al., 2012; Zhang et al., 2012; Zhao et al., 2013). Isolation and serum-surveys of H9N2 AIVs in LBMs have shown that LBMs are important sites for the circulation of H9N2 AIVs (Ge et al., 2009; Su et al., 2014). The antibody level of H9N2 AIVs was high in mammals such as dogs living in close proximity to LBMs (Ge et al., 2009; Su et al., 2014). Therefore, continued surveillance and control of H9N2 AIVs in LBMs is extremely required.

Influenza virus commonly spreads around the world in seasonal epidemics. Previous studies have shown high prevalence of H9N2 AIVs in winter and spring rather than in summer in chicken farms (Chen et al., 2012; Xu et al., 2007; Zhao et al., 2013). However, the rate of H9N2 isolation has no relationship with the season in live poultry markets and the infection rate in healthy chicken flocks is unknown (Ge et al., 2009).

Pathogenicity

Generally, H9N2 AIVs are non-pathogenic to chickens under experimental conditions (Choi et al., 2004; Guo et al., 2000; Li et al., 2005; Zhang et al., 2008). However, poultry in the field infected with H9N2 subtype AIVs caused mild-to-moderate respiratory disease, high morbidity, and reduced egg production when co-infected with other viruses or bacteria such as infectious bronchitis virus, *Escherichia coli* and *Mycoplasma gallisepticum* (Nili and Asasi, 2002; Zhang et al., 2008). In contrast to chickens, H9N2 AIV shows different pathogenicity in mice. G9 (A/chicken/Hongkong/G9/97) and G1 (A/Quail/HongKong/G1/1997) viruses have been shown to replicate systemically and to be lethal in mice (Choi et al., 2004; Guo et al., 2000). However, another study demonstrated that the G9- and G1-like H9N2 AIVs replicate only in the respiratory system of mice and were not lethal (Lu et al., 2001). Previous studies showed that H9N2 AIVs isolated in mainland China exhibited diverse replication phenotypes in mice without prior adaptation (Li et al., 2005; Lin et al., 2014). H9N2 AIV was also shown to adapt well in mice after serial passage in mouse lung and was highly pathogenic in mice (Liu et al., 2014), which indicated that H9N2 AIVs are a potential threat to public health following adaptation to humans and other mammals (Liu et al., 2014). So the pathogencity of H9N2 AIVs need to be paid close attention in China.

Table 1. *Distribution of H9N2 influenza viruses in different hosts in China from 2010 to 3013.*

Host	Numbers of H9N2 AIV in different hosts and period				
	1966-2009[a]	2010	2011	2012	2013
Chicken	206	187	583	41	49
Duck	41	18	8		
Silkie chicken	15				9
Pheasant	24				
Chukkar	13				
Pigeon	3		1		1
Partridge	32				
Guinea fowl	8				
Human	4				
Swine	12				
Quail	77	1			
Spot-billed duck			5		
Mallard			6		
Goose			1		
Wild waterfowl			7	15	
Wild duck	2				
Baikal teal			1		
Brambling				1	
Egret				1	
Canine			1		
Equine			1		
Turtledove					1
Environment					5
Bird	5				
Total	442	206	614	58	65

[a]Refer to Dong et al., 2011.

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Virus Evolution

The evolution of H9N2 AIVs was through antigen drift, antigen shift and reasortanment to generate antigenic diversity. However, persistent reassortment among H9N2 AIVs or other subtypes has generated novel genotypes. The antigenic evolution of H9N2 has certain regularity. Epidemiological and phylogenetic studies indicate that three distinct sublineages of H9N2 AIVs have been established: Ck/Bj/94-like (represented by A/Chicken/Beijing/1/94), G1-like and Korea-like (representated by A/chicken/Y439/1997 or A/chicken/Korea/383490-p96323/1996) (Dong et al., 2011; Sun et al., 2010; Xu et al., 2007). Since the first H9N2 AIV was isolated in 1994, Ck/Bei/94-like has become dominant in China. Reassortment of Ck/Bei/94-like with G1-like, G9-like, F98-like (represented by A/chicken/Shanghai/F/98) and TY/WI/66-like (represented by A/turkey/Wisconsin/1/1966) viruses, generated nine genotypes (A-I), which were isolated from chickens and ducks during 1996 and 2002 (Li et al., 2005). Subsequently, novel genotypes were reported to be circulating in domestic poultry (Xu et al., 2007). Both G1 and Ck/Bei/94-like viruses have circulated in quail since 2000 in southern China (Xu et al., 2007). And more genotypes, such as B62-B65, were detected in poultry in China (Bi et al., 2011). In mainland China, Ck/Bei-like and G1-like were the predominant viruses circulating in domestic poultry (Li et al., 2005; Sun et al., 2010; Xu et al., 2007; Xu et al., 2007). Because of antigen variation, the H9N2 vaccine, CK/SD/6/96, was heterologous with some of the prevailing viruses and could not protect against shedding virus (Li et al., 2005).

In addition, phylogenetic analysis of 22 H9N2 AIVs, which were isolated from wild waterfowl in Dongting Lake in China from 2011 to 2012 indicated that these viruses were generated by the reassortment of American and Eurasian lineage viruses (Zhu et al., 2014). The HA genes of these viruses have shown to belong to American lineage II (Zhu et al., 2014), which suggested expansion of the gene pool of H9N2 AIVs in China. Although the American lineage mainly infects turkeys and wild birds, the introduction of this lineage has added complexity to the prevalence and evolution of H9N2 AIVs in China. Ongoing surveillance of wild birds and domestic poultry should be conducted in China.

In addition, H9N2 AIVs also could reassort with other subtype viruses to donate their internal and/or NA genes to other subtype viruses. The pathogencity of H9N2 AIV after reassortant with other subtype influenza virus will be enhanced. For example, a novel H5N2 viruse generated from the reassortment of H5N1 and H9N2 AIVs are highly pathogenic in poultry (Mi et al., 2013; NISHI et al., 2014; Ogata et al., 2008; Zhao et al., 2012). In China, the A/chicken/Hebei/1102/2010(H5N2) contains an H9N2-like NA gene and six internal genes, while the HA gene belongs to clade 7 H5N1. And the A/chicken/Jiangsu/1001/2013(H5N2) contains six H9N2-like genes except for the HA and M genes. Especially, the ressortant H7N9 virus, which internal genes was contributed by an H9N2 AIV, was responsible for an outbreak in China and lethal to humans (Gao et al., 2013). The H9N2 AIV has also been reported to donate internal genes to the H10N8 virus that infects humans, causing the death of a 73 year-old woman in China (Chen et al., 2014). These results suggest that the reassortant of H9N2 AIVs continue to occur in China and that the H9N2 AIVs act as an "enabler" of AIV infection of humans.

From 1966 to 2013, the phylogenetic relationships of all H9N2 AIVs in GenBank were analysed. The H9N2 AIVs variation is complicated. To unify the nomenclature of genotypes, Dong et al. conducted an analysis of 571 genomes of H9N2 AIVs isolated from 1966 to 2009 (Dong et al., 2011), in which 74 lineages and 98 genotypes were identified that were further divided into seven series (A–G) (Dong et al., 2011). Similarly, Jiang et al. has analysed the phylogenetic relationships among the HA genes of 745 H9 AIVs during 2008 to 2011 in China (Jiang et al., 2012). Another 113 viruses isolated between 2011 and 2012 have also been analyzed (Chen et al., 2013). The results showed that most of the H9N2 AIVs in China were belong to Ck/Bei-like (lineage h9.4.2). Based on the viral HA gene, the lineage h9.4.2 was divided into six lineages (lineages h9.4.2.1–h9.4.2.6) with time pass (Jiang et al., 2012; Liu et al., 2009). The H9N2 AIVs of lineages h9.4.2.1–h9.4.2.4 were mainly isolated before 2007 in China. Lineage h9.4.2.5 has been epidemic since 2007.Lineage h9.4.2.6 emerged in southern China in 2010 and was transmitted to northern China (Chen et al., 2013; Jiang et al., 2012). Here, we analysed the phylogenetic relationships of the 66 HA genes isolated in China from 2013 to 2014, the results

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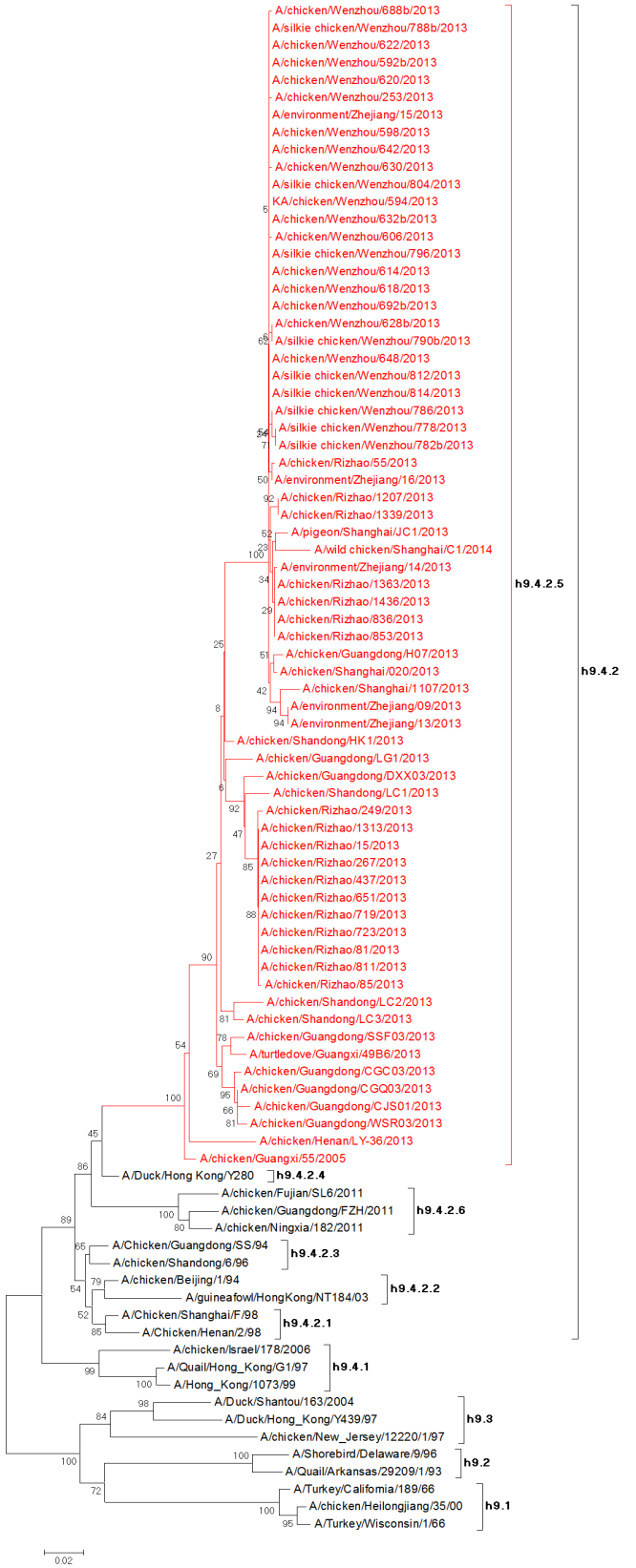

Figure 2. *Phylogenetic relationships of the H9N2 AIV HA genes in China during 2013 to 2014. The red strain indicates H9N2 AIV belonging to lineage h9.4.2.5. The tree was constructed using the neighbour-joining method with MEGA5.05 (www.megasoftware.net/) and 1,000 bootstrap replicates*

showed that all the viruses are belong to lineage h9.4.2.5 (Figure 2), but no lineage h9.4.2.6 was found. These results illustrate the complexity of the prevalence of H9N2 AIVs in China, the underlying mechanism of which merits further investigation.

Potential Risk to Public Health

There were reported that some avian influenza viruses could infect human, including H5N1, H9N2, H7N7, H7N2 and H7N3 (2007; Butt et al., 2005b; Fouchier et al., 2004; Hirst et al., 2004; Yuen et al., 1998). There was no H7 subtype virus infected human before 2013 in China. Human infections with H9N2 AIVs in China, Hong Kong, and Bangladesh have been reported since 1998 (Butt et al., 2005a; Lin et al., 2000; Peiris et al., 1999; Shanmuganatham et al., 2013). It has also been reported that H9N2 AIVs infect mice and ferrets directly without pre-adaptation in an intermediate host, and are transmitted by direct contact between ferrets (Guo et al., 2000; Lin et al., 2014; Liu et al., 2014; Wan et al., 2008). Although H9N2 AIVs circulate mainly in domestic poultry and there is no evidence of person-to-person transmission of this virus (Uyeki et al., 2002), the potential health risk to public health cannot be overlooked. The HA 226 position has been reported to influence the transmission ability in ferrets and cell tropism (Wan and Perez, 2007; Wan et al., 2008). Previous studies have demonstrated that the ratio of L226 in the HA receptor binding site (RBS) has become higher than that of Q226 in the past few years in China, which might increase the pandemic risk (Chen et al., 2012; Chen et al., 2013; Zhang et al., 2012). Thus, poultry workers are at increased risk of H9N2 AIV infection (Huang et al., 2013). Moreover, the feral dogs in live poultry markets may infect H9N2 AIV and increase the risk of influenza viruses to public health (Su et al., 2014). Hence, continued surveillance and control of H9N2 AIV is of vital importance.

Prevention and Control of H9N2 AIVs

Vaccination is an effective and economical strategy for the prevention and control of influenza viruses. Three viruses (A/chicken/Guangdong/SS/1994, A/chicken/Shandong/6/1996, and A/chicken/Shanghai/F/98), belonging to the Ck/Bei/94-like subtlineage, have been used commercially to produce inactivated vaccines in China. These vaccines have been used to prevent H9N2 AIV infections in chickens

since the late 1990s. But the antiserum induced by these vaccines did not react efficiently with the prevalent H9N2 AIVs. Huge numbers of H9N2 AIVs evaded the protection of these vaccines, which resulted in the wide prevalence of H9N2 AIVs in China (Jiang et al., 2012; Li et al., 2005; Xue et al., 2014). In order to reduce the prevalence of H9N2 AIVs, the vaccines should be updated based on surveillance data and the local epidemic virus. Moreover, antigen mapping can be used to monitor the antigenic differences among vaccines and circulating strains and predict the effects of vaccination (Smith et al., 2004). In addition, it is important that the selected viruses are compatible with the embryonated chickens' egg culture technique for the production of high levels of antigen.

In addition to inactivated vaccines, virus-like particles (VLPs) vaccines are in development. The viral surface glycoprotein HA is the major antigenic protein, the recombinant VLP vaccines have been widely researched in different influenza virus subtypes (Lee et al., 2013; Lee et al., 2011; Nakayama et al., 2013; Pushko et al., 2011; Smith et al., 2013; Tretyakova et al., 2013). Furthermore, VPLs can contain various HA subtypes within to provide protection against multiple subtypes (Pushko et al., 2011; Tretyakova et al., 2013).

Bio-security is as important as vaccination for the prevention of H9N2 AIV infections in poultry farms. Breeding only one species of poultry to reduce the interspecies transmission of the virus and adopting an "all in, all out" policy to avoid circulation in a single farm are important strategies for the prevention of H9N2 AIV infections. Moreover, reducing other pathogenic infections could influence the pathogenicity of H9N2 AIVs.

Future Perspectives

Although large-scale phylogenetic analyses have been conducted to determine the genetic evolution of H9N2 AIVs, the mechanism by which a genotype or gene contributes to the prevalence of H9N2 AIVs and the function of the H9N2 internal genes in other subtypes need to be further clarified. Compared to the H5N1 virus vaccine, the pre-clinical and clinical research into H9N2 AIVs is lagging far behind in China. Until a more effective vaccine is developed to control epidemics, the H9N2 AIV will continue to circulate in multiple poultry populations. Therefore, long-term surveillance and the implementation of control strategies should be continued in domestic poultry colonies and LBMs in China. Furthermore, further studies should be conducted to predict potential H9N2 AIV epidemics in humans.

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