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Molecular characterization and receptor binding specificity of H9N2 avian influenza viruses based on poultry-related environmental surveillance in China between 2013 and 2016

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ABSTRACT

H9N2 avian influenza viruses (AIVs) have become panzootic and caused sporadic human cases since 1998. Based on the poultry-related environmental surveillance data in mainland China from 2013 to 2016, a total of 68 representative environment isolates were selected and further investigated systematically.

Phylogenetic analysis indicated that Y280-like H9N2 viruses have been predominant during 2013–2016 and acquired multiple specific amino acid substitutions that might favor viral transmission from avian to mammalians. Additionally, the viruses have undergone dramatic evolution and reassortment, resulting in an increased genetic diversity or acting as the gene contributors to new avian viruses. Receptor-binding tests indicated that most of the H9N2 isolates bound to human-type receptor, making them easily cross the species barrier and infect human efficiently. Our results suggested that the H9N2 AIVs prevalent in poultry may pose severe public health threat.

1. Introduction

H9N2 subtype avian influenza viruses (AIVs) have widely circulated in the world since its first detection from turkeys in Wisconsin in 1966 (Homme et al., 1970). This subtype virus has caused great economic losses due to reduced egg production or high mortality associated with co-infection with other pathogens (Shanmuganatham et al., 2013; Zhao et al., 2013; Sun et al., 2010). In the mainland China, the first outbreak of H9N2 chicken influenza occurred in the Guangdong province in 1994 (Chen et al., 1994) and their low pathogenic nature to poultry has made them as a low priority for animal disease control. Subsequently, the virus rapidly spread across many different regions of mainland China and became the most prevalent influenza virus circulating in poultry such as chicken, duck, and quail (Li et al., 2005, 2003). Epidemiological and genetic studies revealed that the hemagglutinin (HA) gene of the H9N2 AIVs could be divided into Eurasian avian and American avian lineages. The Eurasian lineage could be further divided into three major sublineages: the A/quail/HongKong/G1/97-like (G1-like) viruses that were enzootic in Southeast and South Asia and the Middle East; the A/chicken/Beijing/1/94-like or A/duck/Hongkong/Y280/97-like (BJ94-like or Y280-like) viruses mainly prevalent in China; and a subgroup of A/duck/HongKong/Y439/97 (Y439-like) viruses that circulate in Korea (Choi et al., 2004; Davidson et al., 2013; Kwon et al., 2006). The broad prevalence of H9N2 AIVs in poultry naturally increased their risk of transmission to mammals, especially swine and humans. BJ94-like viruses were isolated from the domestic pigs in Hong Kong in 1998 (Peiris et al., 2001) and H9N2 virus infection in pig farms was also confirmed in Shandong and several other provinces in mainland China (Xu et al., 2004). Most importantly, several infectious cases in humans presenting with mild respiratory disease have been reported since 1997 in Hong Kong and the mainland China (Peiris et al., 1999; Butt et al., 2005; WHO, 2014 Influenza at the human-animal interface). Although only a small number of H9N2 viruses have been isolated from humans, thus far, and human infections to H9N2 AIVs have also been indicated by serological survey using hemagglutination inhibition (HI) and microneutralization (MN) assays, with positive rates for H9N2 antibodies

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of 1.3–1.4% in the general population and more than 15% in retail poultry workers (Wang et al., 2009; Chen et al., 2011; Huang et al., 2013), indicating that the introduction of H9N2 viruses to humans was not rare.

In addition, H9N2 AIVs have been shown to be donors of internal genes to generate zoonotic influenza viruses with pandemic potential. Prior phylogenetic analysis has revealed that H9N2 has donated its six internal genes to highly pathogenic avian influenza (HPAIV) H5N1 viruses, which caused the Hong Kong H5N1 outbreak in 1997 (Guan et al., 1999). Recent studies indicated that the H9N2 viruses contributed the six internal genes to the newly emerged H7N9, H10N8 and the H5N6 viruses that caused human infections in China (Gao et al., 2013; Chen et al., 2014; Yang et al., 2017). These facts prompted us to assess the biologic properties and pandemic potential of these H9N2 AIVs. In this study, 68 representative H9N2 viruses isolated from environment in mainland China from 2013 to 2016 were fully sequenced, and their genetic characteristics and receptor binding properties were also investigated. Our results suggested that the widely distributed H9N2 AIVs may pose severe public health threat, and particular attention should be paid to H9N2 AIVs to avert any future pandemic in humans in China.

2. Results

2.1. Virus isolation and selection

During the period from January 2013 to December 2016, a total of 2,3264 environmental samples were collected from live poultry markets (LPMs) and poultry farms covering 31 provinces, autonomous regions and municipalities in mainland China. We detected different subtypes of influenza A viruses, including H1, H2, H3, H4, H5, H6, H7, H9, H10, and H11. Of these, a total of 612 H9N2 and 64 mix of H9 with other subtype AIVs were recovered (the H9 isolation rate was 2.9%). To investigate the molecular evolution of the H9N2 viruses from different times and places, we selected 68 representative isolates covering 17 provinces, autonomous regions and municipalities based on the polygenic tree of HA gene, including 16 isolated in 2013, 14 in 2014, 21 in 2015, and 17 in 2016 (Table S1), respectively.

2.2. Phylogenetic analysis of the HA and NA genes

To better understand the genetic relationship and ecology of the H9N2 viruses in mainland China, we sequenced the full genomes of all the selected 68 representative isolates. The homological analysis showed that only the HA gene of EN/GD/21115/15 and EN/XJ/60401/16 shared a low similarity (81–83%) with others, and the other 66 viruses have a high nucleotide identity among each other (92–100%) at the nucleotide level. Evolutionary relationships of HA and NA nucleotide sequences were determined by comparing the studying H9N2 isolates with the established Eurasian H9N2 lineages: the G1, BJ94-like or Y280 and Korean lineages represented by their respective prototype viruses. All of the HA genes of the viruses from this study except two viruses (EN/GD/21115/15 and EN/XJ/60401/16) belonged to the Y280-like lineage, and they formed two phylogenetic subgroups, I and II (Fig. 1A). Most of the HA genes of the studying H9N2 viruses clustered into subgroup II and were distinct from the virus EN/FJ/08423/13, which grouped into subgroupIwith the early H9N2 isolates. The HA gene of EN/GD/21115/15 and EN/XJ/60401/16 was quite different from others and went to the G1-like lineage (Fig. 1A). No Y439-like HA genes circulating in Korea were found in this study.

The NA genes of all the H9N2 viruses in this study belonged to the Eurasian lineage and formed three main sub-lineages, sharing a nucleotide identity 82–99%. The most NA genes were closely and clustered together with the Y280 lineage strains in one group (the viruses contained a three-amino-acid deletion in the stalk region), and 7 viruses belonged to the G9-like lineage (the viruses did not contain a stalk deletion), which is one of the prototype strains from the Y280 lineage. While the NA genes of EN/GD/21115/15 and EN/XJ/60401/16 were distinct from the others and clustered into the G1-like lineage (Fig. 1B). Similar to HA, none of the NA genes grouped within the Korean-like lineage (Y439-like).

Taken together, our data suggested that most environment strains in mainland China belonged to Y280-like lineage, showing that Y280-like viruses have been predominant in poultry in mainland China during 2013–2016, however, the G1-like lineage viruses were co-circulating with Y280-like lineage viruses but have a very low prevalence.

2.3. Phylogenetic analysis of the internal genes

The six internal genes shared nucleotide identities of 84–99%, 85–99%, 86–100%, 86–99%, 94–100%, 86–100%, with the PB2, PB1, PA, NP, M and NS genes respectively of all the 68 H9N2 viruses. All of the six internal genes of the viruses in the present study belonged to the Eurasian lineage.

The topology of the phylogenetic trees of PB2 and MP were very similar (Fig. 1C and Figure S1-MP). All of the PB2 and MP genes of the viruses in the present study belonged to the G1-like lineage, and they formed two phylogenetic subgroups. All of the viruses except EN/XJ/60401/16 clustered together and belonged to subgroupII, while EN/XJ/60401/16 was distinct from others and grouped into subgroup I with the early H9N2 isolates in China. The phylogenetic tree indicated that the PB1, PA and NS genes of all the viruses except two viruses (EN/FJ/08423/13 and EN/XJ/60401/16) were in the same clade and belonged to the A/chicken/Shanghai/F/98-like lineage (SF98-like) (Zhang et al., 2009), however, the PB1 and NS of EN/FJ/08423/13 were closely related to the Y280-like lineage and the EN/XJ/60401/16 related to the G1-like lineage (Fig. S1). Intriguingly, the PA gene of the two H9N2 viruses belonged to the Y439 lineage (Fig. 1D). All of the NP genes of the viruses except the EN/XJ/60401/16 were belonged to the SF98-like lineage and the viruses formed two subgroups, most of the viruses clustered together and belonged to subgroupII, while one virus (EN/FJ/08423/13) was highly related with SF98 and grouped into subgroup I. However, the EN/XJ/60401/16 was different from others and belonged to the G1 lineage (Figure S1-NP). Phylogenetic analysis of the six internal genes revealed that the H9N2 viruses have undergone dramatic evolution and reassortment in mainland China from 2013 to 2016.

2.4. Genotyping

Based on the phylogenetic analysis of all 8 gene segments of the 68 representative H9N2 AIVs, we assigned the virus genotypes, as previously described (Pu et al., 2015). A total of five H9N2 influenza virus genotypes (G57, G66, G70, G71 and G72) were identified in our isolates, including three novel genotypes that have not been recognized in previous studies and were designated genotypes G70, G71 and G72 (Table S1 and Table 1). The genotype G57 represented by the virus A/chicken/Rizhao/249/2013 including 59 viruses in our surveillance was the predominant genotype in circulation and the remaining 4 genotypes were only occasionally detected from environment in mainland China during 2013–2016 (Table 1). The three novel genotypes all had undergone reassortment two or three times, with gene segments from Y280-like, G9-like, SF98-like, and Y439-like viruses. Although the novel genotypes (G70, G71 and G72) were only occasionally detected in the environment, the number of the genotypes has increased in our study, suggesting that H9N2 influenza viruses have become more and more diversified in mainland China during 2013–2016.

2.5. Molecular characterization

All of the HA genes contain 1742 nucleotides and most of the H9N2 viruses retained a conserved amino acid sequence of PSRSSR↓GL at the cleavage site (arrow) between HA1 and HA2 except for four viruses,

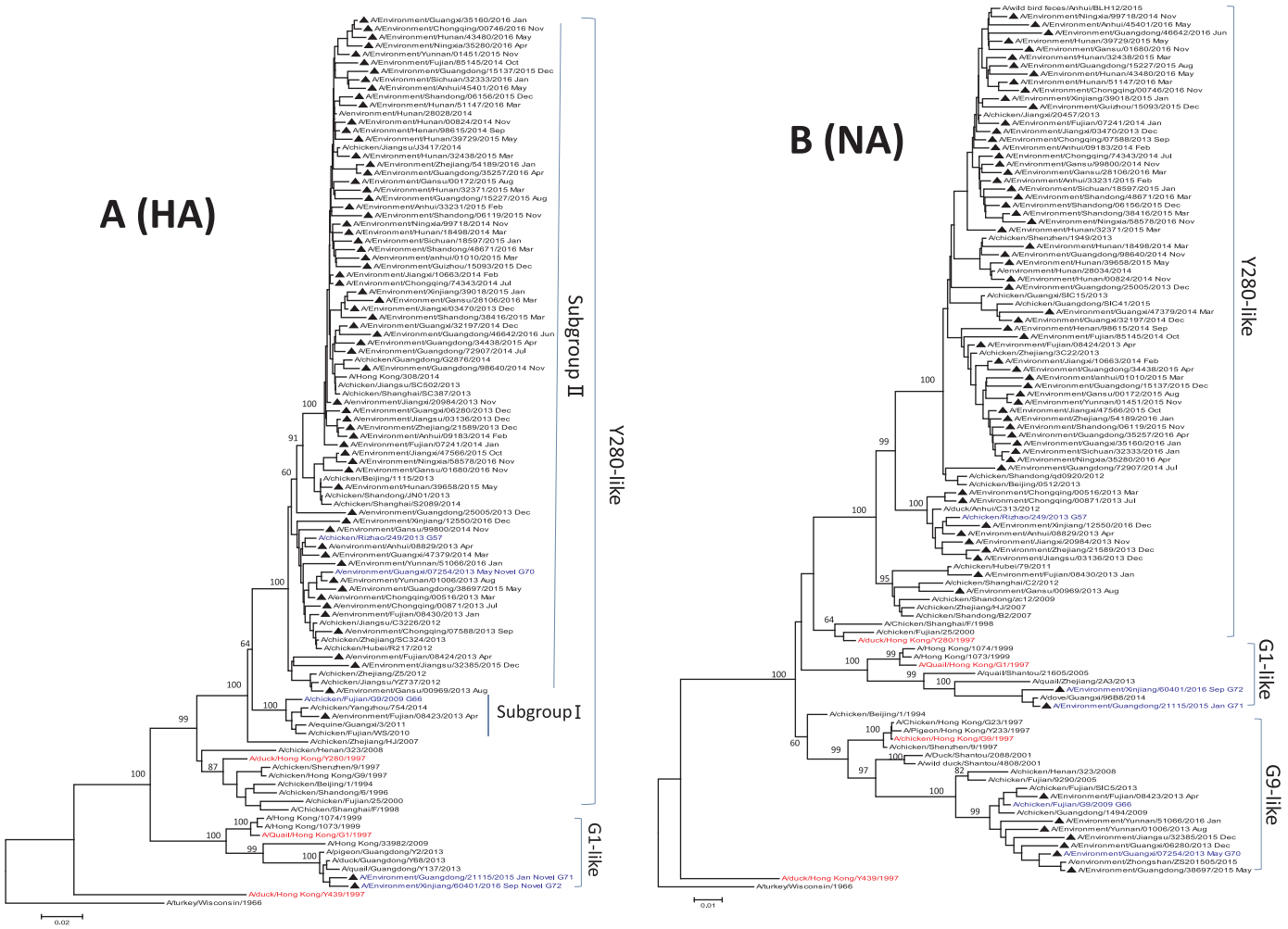

Fig. 1. Phylogenetic trees of HA (A), NA (B), PB2 (C) and PA (D) genes of H9N2 subtype AIVs. Full-length sequences with complete open reading frames were used for the phylogenetic analyses and neighbor-joining (NJ) trees were generated using MEGA 6.06. Estimates of the phylogenies were calculated by performing 1000 neighbor-joining bootstrap replicates. The phylogenetic trees of the genes were rooted to A/turkey/Wisconsin/1966 (A to D). The representative genotype strains were shown in blue and the representative strains of each lineage were shown in red. Our 68 isolates were highlighted by triangles. The bootstrap values ≥ 60 were shown.

which was a typical feature of land avian influenza viruses (Webster et al., 1992). The virus EN/FJ/08423/13 isolated in 2013 had a PSKSSR↓GL motif, and the strain EN/HN/32371/15 isolated in 2015 had PSRSIR↓GL at the HA cleavage site. Two H9N2 viruses isolated in 2015 and 2016 (EN/GD/21115/15 and EN/XJ/60401/16) had a PARSSR↓GL motif, which is identical to those of early H9N2 isolates before 2006 (Table 2). The sequences of the cleavage site of all these strains were associated with low pathogenicity in chickens (Steinhauer, 1999). It has been well documented that the receptor binding site motif of HA was critical for cellular receptor specificity and determining virus host range (Gambaryan et al., 2002; Ha et al., 2001). We also analyzed the amino acids at receptor binding sites of HA, especially 101(Y), 153(W), 155(T), 183(N/H), 190(T/A/V), 194(L), and 195(Y) (as summarized in Table 2, H3 numbering). The amino acids at positions L226 and G228 (H3 numbering) were similar in all viruses except EN/GD/21115/15 and EN/XJ/60401/16, suggesting that the all the H9N2 viruses except the two viruses would preferentially bind to the α2,6-linked sialic acid (SA) receptors that are predominant in humans (Matrosovich et al., 2001), and there was a potential cause of reported human infection (Butt et al., 2005; Matrosovich et al., 2004). However, position Q226 and G228 of HA was a typical avian virus signature, suggesting that the two viruses can bind to α2,3-linked SA (avian receptors) (Wan et al., 2007; Ha et al., 2001).

Eight N-linked potential glycosylation sites (PGS) with the N-X-T/S motif (in which X may be any amino acid except Proline) were highly conserved and present in HA protein of most the studying viruses (positions: 29, 82, 141, 298, 305, 313, 492, 551), which were the same as most previous reported H9N2 viruses (Li et al., 2005). However, the amino acid changes of L to T at position 107 led to an additional PGS at position 105 in EN/GD/21115/15 and EN/XJ/60401/16 viruses, and S to P mutation at position 315 led to the loss of the PGS at position 313.

With the exception of the 7 viruses which belonged to the G9-like lineage , the NA proteins of the other 61 isolates had the amino acid deletion in the stalk region. All the Y280-like lineage viruses had the 3-amino acid deletion at positions 63–65, which was consistent with the sequence of poultry H9N2 viruses prevalent in mainland China (Zhang et al., 2009; Ge et al., 2009). Additionally, two amino acids (sites 38–39) were deleted at the NA stalk region of EN/GD/21115/15 and EN/XJ/60401/16, which was similar to G1-like viruses, as shown in Table 2. It has been reported that this amino acid deletion might be necessary for virus adaptation from wild birds to poultry (chicken) (Matrosovich et al., 1999). H274Y substitution was not detected in the NA protein, suggesting the isolates would be sensitive to neuraminidase inhibitors such as oseltamivir (Hurt et al., 2009). The S31N mutation responsible for amantadine resistance was found in M2 protein of all H9N2 strains, suggesting that a wide spread of amantadine and

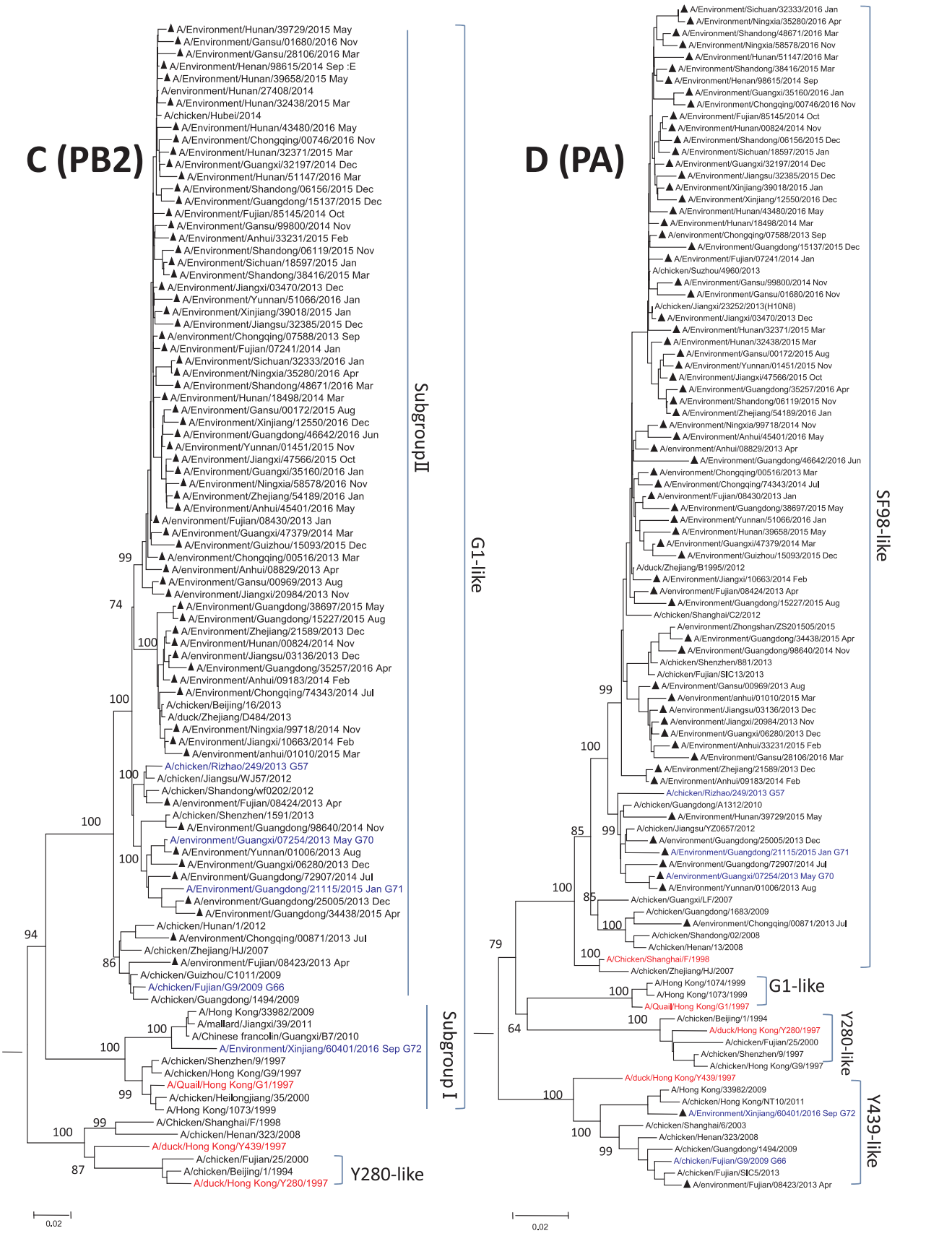

Fig. 1. (*continued*)

Table 1
Gene constellation of H9N2 viruses isolated from environment between 2013 and 2016.

Representative Virus[a]	Lineage[b] of the gene segment								Genotypes[d]	No. (%) of viruses
	HA	NA	PB2	PB1	PA	NP	MP	NS		
A/chicken/Rizhao/249/2013	Y280 (clade[c] 9)	Y280 (clade 1)	G1 (clade 6)	SF98 (clade 3)	SF98 (clade 3)	SF98 (clade 4)	G1 (clade 2)	SF98 (clade 7)	G57	59 (86.7)
A/chicken/Fujian/G9/2009	Y280 (clade 9)	G9 (clade 0)	G1 (clade 6)	Y280 (clade 0)	Y439 (clade 2)	SF98 (clade 4)	G1 (clade 2)	Y280 (clade 6)	G66	1 (1.5)
EN/GX/07254/13	Y280 (clade 9)	G9 (clade 0)	G1 (clade 6)	SF98 (clade 3)	SF98 (clade 3)	SF98 (clade 4)	G1 (clade 2)	SF98 (clade 7)	G70	6 (8.8)
EN/GD/21115/15	G1	G1	G1 (clade 6)	SF98 (clade 3)	SF98 (clade 3)	SF98 (clade 4)	G1 (clade 2)	SF98 (clade 7)	G71	1 (1.5)
EN/XJ/60401/16	G1	G1	G1 (clade 5)	G1 (clade 2)	Y439 (clade 2)	G1 (clade 1)	G1 (clade 2)	G1 (clade 2)	G72	1 (1.5)

[a] Genotypes of representative isolates were assigned as described previously (reference Pu et al., 2015). The full names of the viruses were listed in Table S1.
[b] Lineage of representative isolates were assigned as described previously (reference Choi et al., 2004; Davidson et al., 2013; Kwon et al., 2006, Zhang et al., 2009).
[c] The clade of each viral gene segment was assigned as described previously (reference Pu et al., 2015).
[d] Genotypes were established in light of the phylogenetic relationships and were determined by the combination of clade assignments of each of the eight segments.

Table 2
Key molecular markers of H9N2 viruses in this study.

Gene	Function	Mutation	Amino acid(s)	No. (%) of viruses
HA[a]	Alters receptor specificity	Y101	Y	68 (100)
		W153	W	68 (100)
		I155T	T	68 (100)
		H183N	H	2 (2.9)
		H183N	N	66 (97.1)
		A190T/V	A	19 (27.9)
		A190T/V	T	47 (69.1)
		A190T/V	V	2 (3.0)
		L194	L	68 (100)
		Y195	Y	68 (100)
		Q226L	Q	2 (2.9)
		Q226L	L	66 (97.1)
		M227	M	68 (100)
		G228S	G	68 (100)
	Cleavage peptides		PSRSSR↓GL[b]	64 (94.1)
			PSKSSR↓GL	1 (1.5)
			PARSSR↓GL	2 (2.9)
			PSRSIR↓GL	1 (1.5)
NA	Increases virus adaptation from wild birds to poultry	Amino acid deletion	Yes (63–65)	59 (86.8)
			Yes (38–39)	2 (2.9)
			No	7 (10.3)
PB2	Reduces drug sensitivity	H274Y	H	68 (100)
	Enhances virulence in mice	Q591K	Q	68 (100)
	Increases virulence in mammalian models	E627K	E	68 (100)
		D701N	D	67 (98.5)
		D701N	E	1 (1.5)
PB1	Increases transmission in ferrets	I368V	V	68 (100)
M2	Reduces susceptibility to licensed anti-influenza virus medications	V27A	V	68 (100)
		S31N	N	68 (100)
NS1	Increases virulence in mammalian models	D92E	D	68 (100)

[a] Position numbers are according to H3 numbering.
[b] The downward-pointing arrows indicate the cleavage site.

rimantadine resistant H9N2 AIVs (Suzuki et al., 2003).

The gene PB2 has been known to be critical for the pathogenicity of the virus. Therefore, the role of the PB2 segment must be viewed as potential causes, and zoonotic H9N2 possibly resulting in future human to human transmission (Gabriel et al., 2005). The combination mutations that confer adaption to mammals at PB2 E627K and D701N were not found in all the H9N2 isolates, while one virus EN/GD/46642/16 contained D701E mutation. Wang et al. demonstrated that the Q591K mutation in H9N2 viruses enhanced the polymerase activity and the pathogenicity in mammalian host (Wang et al., 2016), and none of our H9 viruses have the Q591K amino acid change. The D92E substitution of NS1 protein could enhance virus resistance (Seo et al., 2002), and it was not present in any of the studying H9N2 isolates (Table 2).

2.6. Receptor-binding preference of the H9N2 influenza viruses

Receptor-binding preference has important implication for influenza virus replication and transmission (Herfst et al., 2012; Imai et al., 2012). Receptor binding is the first step for the adaptation of AIVs to humans, and the change of receptor-binding preference from α2,3-linked SA to α2,6-linked SA is thought to be a prerequisite for AIVs to transmit from human to human (Horimoto et al., 2001). In this study, a

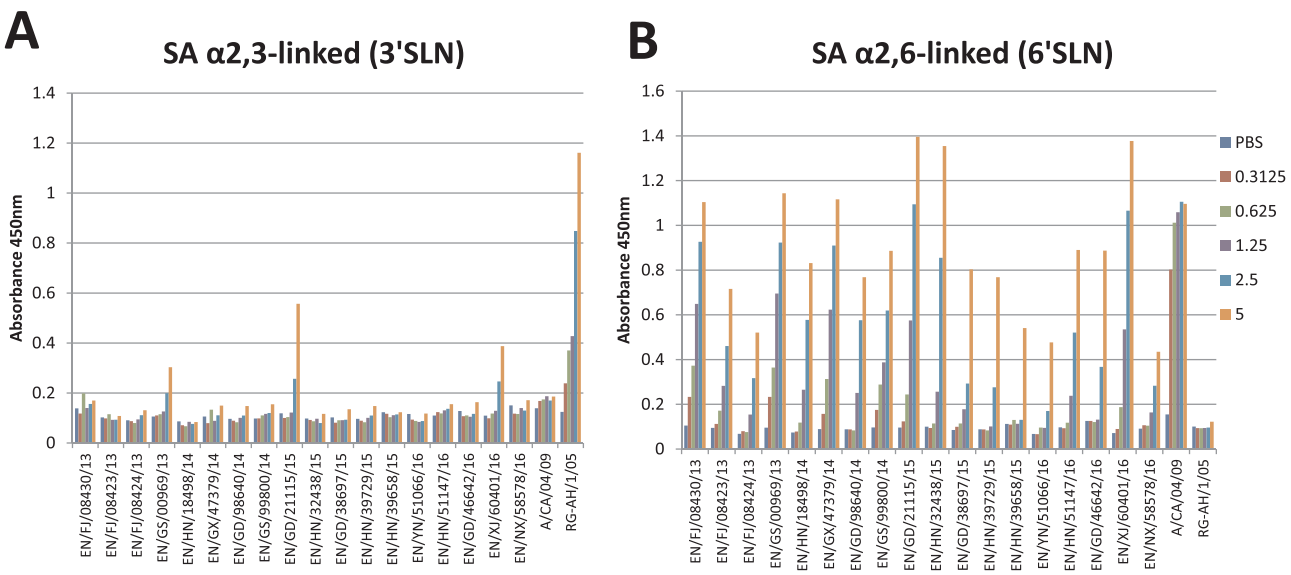

Fig. 2. Characterization of the receptor-binding properties of H9N2 viruses. Binding affinity of the viruses to two different biotinylated glycans (3′-Sialyl-N-acetyllactosamine, 3'SLN; 6'-Sialyl-N-acetyllactosamine, 6'SLN) was tested. 3'SLN was used to represent as α2,3-linked sialic acid and 6′SLN was used to represent as α2,6-linked sialic acid. The H9N2 viruses to both α2,3 (A) and α2,6 (B) glycans at the concentration of 0, 0.3125, 0.625, 1.25, 2.5 and 5 μg/ml. The data shown were the means of two separate assays performed induplicate and absorbance was read at 450 nm. The pandemic H1N1 (A/California/04/2009) and H5N1 (RG-A/Anhui/01/2005) were used as binding control

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solid-phase binding assay using biotinylated glycans containing either α2,3-linked or α2,6-linked SA was performed to determine the receptor binding specificity of 18 representative H9N2 viruses which were selected based on the molecular characterization of HA genes (Fig. 2).

The results showed that 16 H9N2 isolates which carried the combination of four key amino acid residues at the RBS of the HA molecule (183 N, 190 A/T/V, 226 L and 228 G, H3 numbering) (see the molecular characterization above) exclusively bound to the SA α2,6-linked (human receptor) but not to α2,3-linked (avian receptor) (Fig. 2A and B), thus, these viruses displaying a typical human virus-like receptor specificity, as was observed with human influenza viruses. Notably, four viruses with 190 A (EN/FJ/08424/13, EN/HN/39658/15, EN/YN/51066/16 and EN/NX/58578/16) displayed a lower binding affinity for SA α2,6-linked than other viruses. However, the EN/GD/21115/15 and EN/XJ/60401/16 with 183 H, 190 A, 226Q and 228 G (H3 numbering) showed distinguishable affinity. The two viruses could bind to both α2,3-linked and α2,6-linked SA, and their affinity to α2,6-linked SA was much higher than that to α2,3-linked SA (Fig. 2A and B). These results indicated that all environment H9N2 isolates could bind human-type receptor and most of the isolates have acquired the ability to exclusively bound to the human-type receptor, similar to the widely circulating human influenza viruses.

3. Discussion

The crucial role of H9N2 viruses at the animal-human interface might be due to the wide host range, adaptation in both poultry and mammalian, and extensive gene reassortment. Thus, the prevalence of H9N2 viruses throughout Asia, along with their repeated emergence in the human population, puts them high on the list of influenza viruses with pandemic potential (Peiris et al., 1999; Butt et al., 2010; Huang et al., 2015; Yuan et al., 2017; Xu et al., 2018). Therefore, H9N2 AIVs raise a persistent threat to public health and continued surveillance is becoming essential. The aim of this study was to aid in understanding the evolution and properties of the H9N2 strains, which constantly circulated in poultry flocks in mainland China between 2013 and 2016.

Phylogenetic analysis of the surface genes indicated that the Y280-like viruses have been predominant in poultry in mainland China during 2013–2016. Previous studies reported that Y280 and G1 were the predominate lineages in China over the past few years and the viruses have been occasionally reported in humans (Peiris et al., 1999; Butt et al., 2010; Jiang et al., 2012; World Health Organization, 2016). In our study, the G1-lineage virus was co-circulating with Y280- lineage viruses but has a very low prevalence. Recently, Genetic analysis of H9N2 viruses indicated that these viruses have undergone dramatic evolution. Pu et al. reported 69 different genotypes of H9N2 viruses in China from 1994 through 2013 (Pu et al., 2015). Zhang et al. demonstrated that the BJ94–like H9N2 viruses circulating in poultry in eastern China before 1998 have been gradually replaced by SF98–like H9N2 viruses, whose genotypes differ from those of viruses isolated in southern China (Zhang et al., 2009). In our study, phylogenetic analysis of the internal genes revealed that all Y280-like H9N2 viruses contained PB2 and M genes from G1-like viruses, implying the viruses that contain the internal genes of G1-like viruses may still circulate in China. The PB1, PA, NP and NS genes of 66 H9N2 viruses in the present study were closely related with the SF98-like viruses, however, some of them contained a different NA gene. Our phylogenetic analysis suggested that the reassortments had happened not independently and H9N2 viruses had undergo extensive reassortments to generate multiple genotypes from different lineages in poultry. The cocirculation of multiple genotypes resulted in the H9N2 viruses becoming more and more diversified in China. The viruses in the same clade isolated from different province, implying that the viruses were not restricted in the areas where they appeared, but were spread very quickly to the neighbor provinces or the provinces far away. H9N2 viruses have infected humans, but there has been little evidence of human-to-human transmission. Although we do not know which viral factors are necessary for successful human-to-human spread, reassortment is certain to increase the chance that a transmissible virus will be generated. Therefore, pay particular attention to H9N2 AIVs and diligent surveillance is becoming more and more essential.

It is generally accepted that HA receptor-binding preference to α-2,6-linked (human-type receptors) sialylatedglycans is the initial key step for a novel influenza-virus-causing pandemic (Horimoto et al., 2001; Matrosovich et al., 2000). Infections of mammals such as humans and pigs with an H9N2 virus have been well documented (Xu et al.,

2004; Peiris et al., 1999; Cong et al., 2007). In the present study, except two viruses (EN/GD/21115/15 and EN/XJ/60401/16), all the H9N2 strains with the combination of HA molecule (183 N, 190 A/T/V, 226 L and 228 G, H3 numbering) bound exclusively to α-2,6-linked SA, indicating the viruses have acquired the ability to bind human-type receptors during their circulation in avian species. Previous surveillance data showed that the incidence of the Q226L substitution in HA gene exceeded 75% of the H9N2 AIVs that currently in mainland China (Jiang et al., 2012). Li X et al. reported that the mutations I155T and Q226L played important roles in H9N2 virus binding to the human-type receptor (Li et al., 2012) and our result was consistent with the previous studying. In our study, the 155T (H3 numbering) were conserved in all of the tested H9N2 strains. Wan et al. reported that HA-Q226L increased virus binding to SA α2–6 Gal and replication in human airway epithelial cells and the possibility of human infection (Wan et al., 2007). In addition, this receptor-binding site substitution enhanced virus replication and direct contact transmission among ferrets in experimental conditions (Li et al., 2012). On the other hand, previous study reported that the HA-190V significantly enhanced HA affinity to human cells and virus attachment to mouse and human lung tissues (Teng et al., 2016). In our study, the amino acid at 190 was not conserved and molecular analysis demonstrated two of the viruses with 190 V (EN/FJ/08430/13, EN/GD/25005/13) and 47 isolates with 190 T, 19 viruses with 190 A. Our results showed that H9N2 viruses harboring HA-190V/T/A still specifically bound to human type SA-2,6 receptors, and our results were consistent with the previous reports (Li et al., 2012; Teng et al., 2016). Noticeably, the H9N2 viruses with 190 T/V had higher binding affinity with α-2,6-sialylglycopolymers than the viruses with 190 A. We do not know which amino acid(s) commits to synergetic effects with the HA-190T/V and this will be investigated in future studies. Additionally, two viruses, EN/GD/21115/15 and EN/XJ/60401/16, with 183 H, 190 A 226Q and 228 G (H3 numbering) could bind to both α-2,3 and α-2,6-linked SA and showed distinguishable affinity. The molecular determinants of the receptor binding preference of influenza viruses are not fully understood. We speculated that some amino acid changes in HA, such as I155T, promoted the binding affinity of the two viruses for human-type receptors.

Both the genes PB2 and HA are known to be critical for the pathogenicity of the virus (Gabriel et al., 2005). Therefore, the role of the PB2 segment must be viewed as potential causes of zoonotic H9N2 possibly resulting in future human to human transmission. Two amino acid changes in PB2, including at position 627 (E627K) and 701 (D701N), were important for the virulence and transmission of H5N1 viruses in mammals (Gao et al., 2009), and were also frequently presented in the H7N9 viruses isolated from humans (Zhang et al., 2013; Chen et al., 2013). Previous study reported that some H9N2 viruses readily acquired the E627K or D701N mutation in their PB2 gene upon infection of ferrets, further enhancing their virulence and transmission in mammals (Li et al., 2012). In our study, all of the H9N2 viruses contained avian type related residues 627E and 701D except EN/GD/46642/16, which contained D701E mutation. So, the infection by the EN/GD/46642/16 should be the focus of increasing attention, and the significance of the PB2 D701E mutation should be assessed further by reverse genetics and animal experiments.

4. Conclusion

In summary, the present study systemically analyzed the H9N2 environment isolates in mainland China from 2013–2016. Phylogenetic analysis indicated that Y280-like H9N2 viruses have been predominant during 2013–2016 and revealed that the viruses had acquired multiple specific amino acid substitutions that might favor viral transmission from avian to mammalians. Additionally, the viruses have undergone dramatic evolution and reassortment, resulting in an increased genetic diversity. Receptor-binding tests indicated that most of the H9N2 isolates bound to human-type receptor, making them easily cross the species barrier and infect human efficiently. Therefore, urgent attention to H9N2 AIVs and diligent surveillance is becoming more and more essential.

5. Materials and methods

5.1. Virus isolation and identification

The H9N2 isolates used in this study were isolated from environmental samples of LPMs or poultry farms in China according to China CDC's guideline of AIVs surveillance (Table S1). Viruses were isolated in specific pathogen free (SPF) chicken embryonated eggs at 37 °C. HA assay with 1% turkey red blood cells (TRBCs) was used to identify the influenza isolates in allantoic fluid of eggs. The HA positive samples were further subtyped by RT-PCR using 16 sets of HA (H1–H16) primers and 9 sets of NA (N1–N9) primers designed by the Chinese National Influenza Center (CNIC).

5.2. Viral RNA extraction and RT-PCR

Virus RNA was extracted from allantoic fluid using RNeasy Mini kit (Qiagen), and was transcribed into cDNA using the Uni12 primer (5′-AGCAAAAGCAGG-3′) and SuperRT cDNA kit (CWBIO, China) according to the manufacturers' recommendations. cDNAs of eight segments were then amplified with gene specific primers designed by CNIC (primer sequences are available upon request). The PCR reaction contained 2 μl cDNA, 1 μl forward primer and reverse primer respectively, 12.5 μl 2 × EsTaq MasterMix (CWBIO), and 8.5 μl RNase-free water in a final volume of 25 μl. A single PCR program was used for all primers, i.e., initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min and 30 s.

5.3. Genome sequencing and phylogenetic analysis

PCR products were visualized by agarose gel electrophoresis, and then purified with QIAquick PCR purification kit (Qiagen). The purified PCR products were then sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (AB) and analyzed with an ABI 3730 DNA analyzer, according to the manufactures' instructions. Full genome sequences of the viruses were deposited in the Global Initiative on Sharing Avian Influenza Data (GISAID) database (the Isolate ID and Accession No see the supplementary materials Table S1). Phylogenetic trees were generated by using neighbor-joining method with MEGA software (version 6.06), and the bootstrap value was tested with 1000 replications for each gene.

5.4. Genotype definition

Virus genotypes were defined in the 68 representative H9N2 isolates based on gene phylogenic analysis. Isolates were genotyped G01 to G72 if sequences and clade assignments were available for all eight segments, as previously described (Pu et al., 2015). The analyzed sequences were derived from viruses isolated in this study or were downloaded from GenBank.

5.5. Direct receptor-binding analysis with synthetic sialylglycopolymers

The receptor preference of each virus was analyzed use of a solid-phase direct binding assay as described previously with minor modifications (Chandrasekaran et al., 2008), using two different synthetic sialylglycopolymers containing either α2,3-linked sialic acid (3'-Sialyl-N-acetyllactosamine: Neu5Ac2–3Gal1–4GlcNAc-PAA) (3′SLN) or α2,6-linked sialic acid (6'-Sialyl-N-acetyllactosamine: Neu5Ac2–6-Gal1–4GlcNAc-PAA) (6′SLN) (GlycoTech Corporation, USA). Briefly , clear microtitre plates (costar 3590) were incubated with serial two-

fold dilutions of sialyglycopolymers in phosphate-buffered saline (PBS) at 4 ℃ overnight, then the plates were exposed to UV light (254 nm) for 10 min. After the glycopolymer solution was removed, the plates were washed three times with the ice-cold PBST (PBS containing 0.1% Twen-20). Following the plates were inoculated with 32 HA units of H9N2 AIVs at 4 ℃ overnight. The plates were washed five times with the PBST and then incubated with the monoclonal antibody against HA of group I influenza virus (Zoonogen, China) at 37 ℃ for 1 h. After being washed for a further five times with PBST, the plates were incubated with the horseradish peroxidase (HRP)-conjugated goat-anti-Human IgG antibody (ZSGB-BIO, China) at 37 ℃ for 1 h. Plates were washed 5 times and incubated with tetramethylbenzidine (TMB, Sigma-Aldrich, St. Louis, MO), and then the reaction was stopped with 0.2 M H2SO4. Each sample was determined in duplicates and the optical density was determined at 490 nm using the Epoch Microplate Spectrophotometer (Bio Tek, Beijing, China). The pdm09 H1N1 virus A/California/04/2009 (H1N1) and RG-A/Anhui/1/2005(H5N1) were used as binding controls.

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Competing interests

The authors declare no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.01.002.

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