Original Article
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Isolation and phylogenetic analysis of hemagglutinin gene of H9N2 influenza viruses from chickens in South China from 2012 to 2013

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As part of our ongoing influenza surveillance program in South China, 19 field strains of H9N2 subtype avian influenza viruses (AIVs) were isolated from dead or diseased chicken flocks in Guangdong province, South China, between 2012 and 2013. Hemagglutinin (HA) genes of these strains were sequenced and analyzed and phylogenic analysis showed that 12 of the 19 isolates belonged to the lineage h9.4.2.5, while the other seven belonged to h9.4.2.6. Specifically, we found that all of the viruses isolated in 2013 belonged to lineage h9.4.2.5. The lineage h9.4.2.5 viruses contained a PSRSSR ↓ GLF motif at HA cleavage site, while the lineage h9.4.2.6 viruses contained a PARSSR ↓ GLF at the same position. Most of the isolates in lineage h9.4.2.5 lost one potential glycosylation site at residues 200–202, and had an additional one at residues 295–297 in HA1. Notably, 19 isolates had an amino acid exchange (Q226L) in the receptor binding site, which indicated that the viruses had potential affinity of binding to human like receptor. The present study shows the importance of continuing surveillance of new H9N2 strains to better prepare for the next epidemic or pandemic outbreak of H9N2 AIV infections in chicken flocks.

Keywords: H9N2 subtype, avian influenza virus, phylogenetic analysis, South China

Introduction

Wild aquatic birds are the most important natural host of influenza A viruses (IAVs). To date, IAVs have been classified into 18 HA subtypes (H1–H18) and 10 neuraminidase (NA) subtypes (N1–N10) according to antigenic differences in the viral surface glycoproteins [2,9,32,33]. H9N2 subtype avian influenza virus (AIV) has circulated worldwide since it was first isolated from turkeys in Wisconsin in 1966 [15]. In mainland China, H9N2 subtype AIV was first isolated from a chicken flock in Guangdong in 1994 [13]. Subsequently, this subtype virus spread extensively and prevailed in other areas of China [4,5,11,35]. Currently, it is one of the predominant AIV circulating in the poultry population in China.

H9N2 AIV infections usually cause mild respiratory symptoms and egg production decline; however, when occurring concurrently with other pathogens such as *Escherichia coli* it can lead to severe infection and moderate mortality in chicken populations [3,18,24]. The wide circulation of H9N2 viruses has caused tremendous economic loss in the poultry industry worldwide [35]. Some H9N2 viruses have acquired receptor binding characteristics typical of human strains (2,6-NeuAcGal) and affected humans occasionally. In addition, H9N2 AIVs may be significant donors of genetic material to emerging human pathogens. Previous studies have demonstrated that H9N2 viruses contributed the internal genes to the human H5N1, H7N9, and H10N8 viruses [10,12,20]. Therefore, the persistence of the H9N2 virus is significant for poultry industries and public health.

Vaccination is the major measure used to control infection by H9N2 AIV in China. Vaccines have been widely applied in chicken flocks since the late 1990s. However, commercial vaccines cannot provide complete protection to prevent the infection of endemic strains [29], and serious diseases caused by H9 AIVs infection in vaccinated chicken flocks have been reported continually in China [4,5]. A previous study showed that the vaccine strains and pandemic H9N2 strains belong to different lineages. The present paper describes the isolation of

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new emerging H9N2 subtype AIVs from chickens between 2012 and 2013 in Guangdong province, South China. Phylogenetic analysis and molecular characterization of the HA gene of the isolates were conducted to provide useful guidance for the control of H9 AIV and vaccine candidate selection.

Materials and Methods

Viruses

Between 2012 and 2013, 19 H9N2 subtype avian influenza viruses were isolated from vaccinated chicken flocks that had been administered A/chicken/Guangdong/SS/94 (H9N2) (SS/94) strain inactivated vaccine in Guangdong province, South China (Table 1). The sick chickens showed typical respiratory illness and moderate mortality. Tracheal swabs and clinical samples (including trachea, spleen, kidney, liver, and other tissue samples) were collected from dead or diseased chickens. Swabs and tissue suspensions prepared in phosphate-buffered saline were filtered through 0.22 μm filters and inoculated into 10-day-old specific pathogen free (SPF) chicken embryos, after which allantoic fluid containing the propagated virus was harvested for further analysis. Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests were conducted to identify the virus subtypes using antisera specific to the reference strains of influenza viruses.

Reverse transcription polymerase chain reaction (RT-PCR), cloning and sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, then amplified by one-step RT-PCR with a PrimeScript One-Step RT-PCR Kit (Takara Bio, China) as previously described [4]. The specific primer set designed for amplification of the H9N2 HA gene was HA-F: 5'-CAAGATGGAAGTAGTATCACT-3' and HA-R: 5'-TTGCCAATTATATACAAATGT-3'. RT-PCR was conducted by subjecting the samples to 50°C for 30 min, 94°C for 2 min and then 30 cycles of 94°C for 40 sec, 53°C for 40 sec and 72°C for 2 min, followed by final extension at 72°C for 10 min. PCR products were purified using an Axygen AxyPrep DNA Gel Extraction Kit (Corning, USA) according to the manufacturer's instructions, after which the product was cloned into pMD-19T vector (Takara Bio) for sequencing. The purified recombinant plasmids were sequenced by Invitrogen Trading (Shanghai).

Genetic and phylogenetic analysis

Multiple sequence alignments were performed by the MUSCLE program using the MEGA5.05 software. The phylogenetic tree was constructed using the MEGA5.05 software and the neighbor-joining method and bootstrap values were calculated from 1,000 replications [7]. The potential N-glycosylation sites (PGS) of deduced amino acid sequences of HA genes were predicted using the Center for Biological Sequence Analysis online server 1.0 [14].

Table 1. H9N2 avian influenza viruses (AIVs) isolated from chickens in this study

Isolate	Abbreviation	Date of isolation	Accession number
A/chicken/Guangdong/LZL01/2012	LZL	Jan-2012	KJ769000
A/chicken/Guangdong/YYS01/2012	YYS01	Jan-2012	KJ768995
A/chicken/Guangdong/SJR01/2012	SJR01	Jan-2012	KJ768999
A/chicken/Guangdong/LRZ01/2012	LRZ01	Jan-2012	KJ769001
A/chicken/Guangdong/CGH01/2012	CGH01	Feb-2012	KJ768993
A/chicken/Guangdong/WBQ02/2012	WBQ02	Feb-2012	KJ768997
A/chicken/Guangdong/JJM02/2012	JJM02	Feb-2012	KJ768984
A/chicken/Guangdong/CJD02/2012	CJD02	Feb-2012	KJ768991
A/chicken/Guangdong/DSF02/2012	DSF02	Feb-2012	KJ768989
A/chicken/Guangdong/H19/2012	H19	Mar-2012	KJ768986
A/chicken/Guangdong/H36/2012	H36	Mar-2012	KJ768985
A/chicken/Guangdong/ZMJ12/2012	ZMJ12	Dec-2012	KJ768994
A/chicken/Guangdong/CJS01/2013	CJS01	Jan-2013	KJ768990
A/chicken/Guangdong/WSR03/2013	WSR03	Mar-2013	KJ768996
A/chicken/Guangdong/SSF03/2013	SSF03	Mar-2013	KJ768998
A/chicken/Guangdong/DXX03/	DXX03	Mar-2013	KJ768988
A/chicken/Guangdong/CGQ03/2013	CGQ03	Mar-2013	KJ768992
A/chicken/Guangdong/CGC03/2013	CGC03	Mar-2013	KJ769002
A/chicken/Guangdong/H07/2013	H07	Jul-2013	KJ768987

Antigenic analysis

Antigenic analysis was performed by HI tests to evaluate the antigenic relationships between the emerging viruses and vaccine strain SS/94. Polyclonal antibodies against eight randomly selected isolates of H9 subtype AIVs (four in h9.4.2.5 and others in h9.4.2.6) were generated using six week old SPF chickens. Each chicken was injected with 1 mL of oil emulsion-inactivated vaccine derived from the eight tested viruses and SS/94. The sera were collected on day 21 after injection. HI tests were performed as previously described [8].

Results

Homology analysis

The coding sequences of the 19 viral HA genes contained 1683 nucleotides. There were no nucleotide insertions or deletions. Homology analysis was performed to compare the nucleotide sequences of surface protein genes of the tested viruses with H9N2 representative strains, as well as with the vaccine strains China SS/94, A/chicken/Shandong/6/96 (6/96), and A/chicken/Shanghai/F/98 (F/98). The result showed that the HA gene nucleotide sequence and deduced amino acid sequence identities among the isolates ranged from 87.4 to 99.3% and 91.4 to 99.5%, respectively. The HA genes of these isolates were 90.5 to 92.8% identical to the representative virus, A/duck/Hong Kong/Y280/1997 (Y280), at the amino acid level, indicating that they belong phylogenetically to the Y280-like lineage. When compared with the three H9N2 vaccine strains, the nucleotide sequence and deduced amino acid sequence similarities among the 19 isolates ranged from 89.6% to 92.0% and 91.1% to 94.5%, respectively. These results indicated that most of the isolates are genetically distant from the vaccine strains.

Phylogenetic analysis

Phylogenetic analysis based on the HA genes showed that the

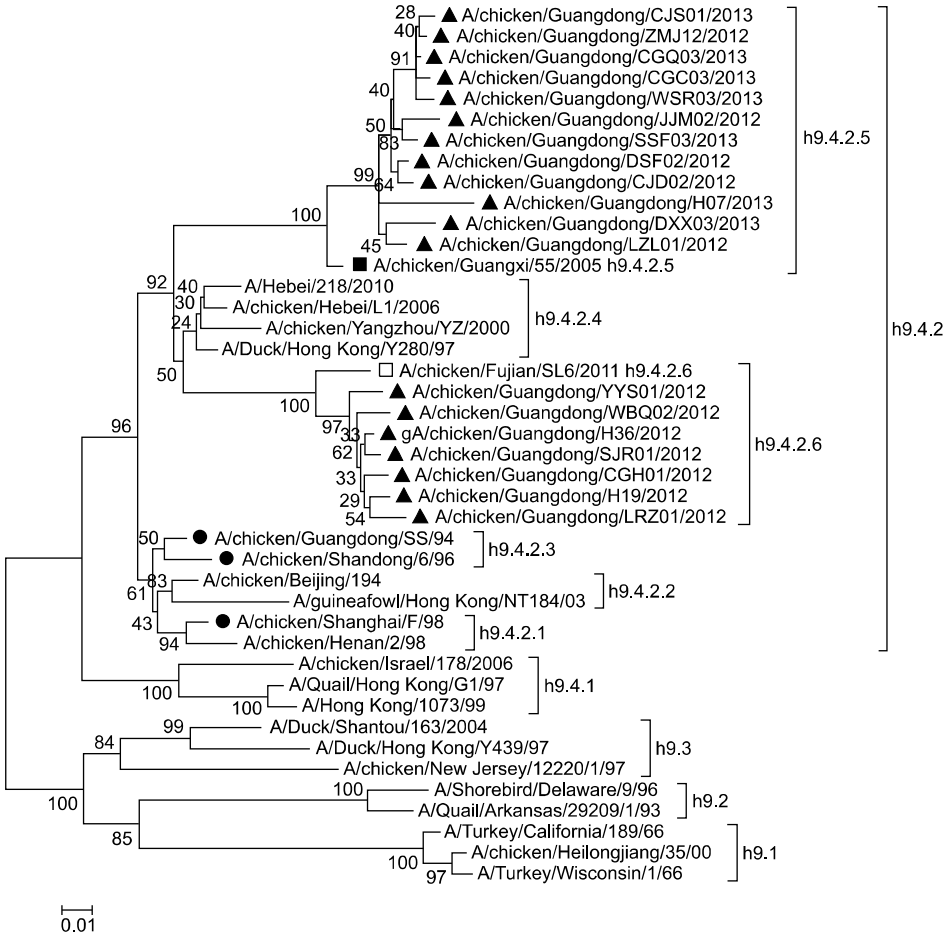

Fig. 1. Phylogenetic tree of H9 avian influenza viruses isolated in Guangdong from 2012 to 2013 based on the viral HA gene sequences. The isolates from Guangdong were marked with squares and the vaccine strains with circles. Solid and hollow squares indicate reference to lineage h9.4.2.5 and h9.4.2.6, respectively. The second and tertiary lineages of the viruses referred to previous nomenclature systems [16,21].

19 H9N2 strains isolated in the present study belonged to lineage h9.4.2 represented by Y280 or BJ194 (Fig. 1), which has been the predominant strain in China in recent years. In the tertiary lineages, the19 isolates were clustered into two lineages. Seven isolates fell into lineage h9.4.2.6, which was isolated from January to March, 2012. The other 12 isolates, which were isolated from January, 2012 to July, 2013, belonged to lineage h9.4.2.5. Notably, by the end of 2012 few of lineage h9.4.2.6 viruses were detected in flocks, and the lineage h9.4.2.5 viruses became predominant in Guangdong province. Vaccine strains SS/94 and 6/96 were clustered in lineage h9.4.2.3, and strain F/98 belonged to h9.4.2.1. All of these strains had a very different genetic distance from the newly emerging h9.4.2.5 viruses (Fig. 1).

Critical site analysis of deduced amino acid sequences

The cleavage sites of precursor HA protein of the H9N2 isolates showed various motifs. The lineage h9.4.2.5 viruses had the cleavage site motif PSRSSR ↓ GLF, while all of the lineage h9.4.2.6 viruses (except strain CGH01) harbored PAKSSR ↓ GLF at the cleavage sites. The remaining lineage had the motif PARSSR ↓ GLF (Table 2). These two motifs differed from those of vaccine strain SS/94, which contained the PAGSSR ↓ GLF motif at the cleavage sites. There were no groups of basic residues at the cleavage sites.

The amino acid residues at receptor binding site (RBS) of the HA protein of the 19 isolates detected in this study were conserved except for those at position 190, which had a V190A (or T) substitution (Table 2). On the left-edge of the receptor-binding pocket, lineage h9.4.2.5 isolates had methionine (M) at position 227, while lineage h9.4.2.6 viruses and the vaccine strain SS/94 had glutamine (Q) at the same position. In addition, all 19 isolates had leucine (L) at position 226, which showed that these viruses preferentially bind to NeuAca2,6-Gal linkage. The amino acids in the antigenic sites of most (18/19) isolates were quite well conserved, and only two substitutions (T135I and N193D) were found in isolate H07.

Potential N-glycosylation sites (PGS) of HAs were predicted using the Center for Biological Sequence Analysis online server 1.0. The results showed that there were seven PGSs (positions 11−13, 123−125, 280−282, 287−289, and 295−297 in HA1, and positions 154−156 and 213−215 in HA2) of the lineage h9.4.2.5 viruses (Table 3). An exception was found in the LZL01 strain, which carried an additional PGS at position

Table 2. Receptor-binding pocket, cleavage site and antigenic site of H9 subtype AIV isolates

Isolate/strain	Lineage	Receptor-binding pocket			Cleavage site	Antigenic site	
		Binding site	Left-edge	Right-edge		Site I	Site II
LZL	h9.4.2.5	PWTNVLY*	NGLMGR†	GTSKA‡	PSRSSR ↓ GLF	TKP§	DNL‖
YYS01	h9.4.2.6	PWTNVLY	NGLQGR	GTSKA	PARSSR ↓ GLF	TKP	DNL
SJR01	h9.4.2.6	PWTNTLY	NGLQGR	GTSKA	PARSSR ↓ GLF	TKP	DNL
LRZ01	h9.4.2.6	PWTNALY	NGLQGR	GTSKA	PARSSR ↓ GLF	TKP	DNL
CGH01	h9.4.2.6	PWTNALY	NGLQGR	GTSKA	PARSRR ↓ GLF	TKP	DNL
WBQ02	h9.4.2.6	PWTNTLY	NGLQGR	GTSKA	PARSSR ↓ GLF	TKP	DNL
JJM02	h9.4.2.5	PWTNVLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
CJD02	h9.4.2.5	PWTNVLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
DSF02	h9.4.2.5	PWTNTLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
H19	h9.4.2.6	PWTNALY	NGLQGR	GTSKA	PARSSR ↓ GIF	TKP	DNL
H36	h9.4.2.6	PWTNALY	NGLQGR	GTSKA	PARSSR ↓ GIF	TKP	DNL
ZMJ12	h9.4.2.5	PWTNVLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
CJS01	h9.4.2.5	PWTNVLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
WSR03	h9.4.2.5	PWTNVLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
SSF03	h9.4.2.5	PWTNALY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
DXX03	h9.4.2.5	PWTNVLY	NGLMGR	GTSRA	PSRSSR ↓ GLF	TKP	DNL
CGQ03	h9.4.2.5	PWTNVLY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
CGC03	h9.4.2.5	PWTNALY	NGLMGR	GTSKA	PSRSSR ↓ GLF	TKP	DNL
H07	h9.4.2.5	PWTNTLY	NGLMGR	GISKA	PSRSSR ↓ GLF	IKP	DDL
SS94	h9.4.2.3	PWTNALY	NGQQGR	GTSKA	PAGSSR ↓ GLF	TKP	DNQ
Y280	h9.4.2.4	TWTNTLY	NGLQGR	GTSKA	PARSSR ↓ GLF	TKP	DNL

*Amino acid residues at position 98 (H3 numbering), 153, 155, 183, 190, 194, and 195, respectively. †Amino acid residues at position 224−229. ‡Amino acid residues at position 134−138. §Amino acid residues at position 135, 157, and 162, respectively. ‖Amino acid residues at position 145, 193, and 226, respectively.

Table 3. Potential N-glycosylation sites analysis of HA amino acid sequences of H9 AIV isolates

Isolates/strain	Potential N-glycosylation sites							
	HA1						HA2	
	11–13	123–125	200–202	280–282	287–289	295–297	154–156	213–215
LZL	NST	NVS	NRT	NTT	NVS	NCS	NGT	NGS
YYS01	+*	+	+	+	+	−	+	+
SJR01	+	+	NRI	+	+	−	+	+
LRZ01	+	+	+	+	+	−	+	+
CGH01	+	+	+	+	+	−	+	+
WBQ02	+	+	+	+	+	−	+	+
JJM02	+	+	NRI	+	+	+	+	+
CJD02	+	+	NRN	+	+	+	+	+
DSF02	+	+	NRI	+	+	+	+	+
H19	+	+	+	+	+	−	+	+
H36	+	+	+	+	+	−	+	+
ZMJ12	+	+	NRI	+	+	+	+	+
CJS01	+	+	NRI	+	+	+	+	+
WSR03	+	+	NRI	+	+	+	+	+
SSF03	+	+	NRI	+	+	+	+	+
DXX03	+	+	NRI	+	+	+	NRT	+
CGQ03	+	+	NRI	+	+	+	+	+
CGC03	+	+	NRI	+	+	+	+	+
H07	+	+	NRV	+	+	+	+	+
SS94	+	+	+	+	+	−	+	+
Y280	+	+	+	+	+	−	+	+

*Positive (+) indicates the same as A/chicken/Guangdong/LZL01/2012. †Negative (−) indicates the absence of N-glycosylation sites.

200–202 in HA1 (Table 3). When compared with the lineage h9.4.2.5 viruses, the lineage h9.4.2.6 viruses lacked PGS at position 295–297 and had an additional PGS at position 200–202 in HA1, as well as seven PGSs in the HA protein. Vaccine strain SS/94 was similar to lineage h9.4.2.6 viruses that lacked PGS at position 295–297 when compared with lineage h9.4.2.5 viruses. An exception was virus SJR01, which lost one PGS at 200–202.

Antigenic analysis

To evaluate the antigenic relationship between the emerging viruses and current commercial vaccine strain, eight viruses and vaccine strain SS/94 were investigated with antiserum by cross HI tests. As shown in Table 4, antisera against h9.4.2.5 viruses CJS01 and JJM02 reacted well with the emerging viruses (HI tier $\geq$ 1280), and the four antisera against the h9.4.2.6 viruses reacted well with the emerging viruses (HI tier $\geq$ 1280), but none of the antisera against emerging viruses reacted well with the SS/94. Moreover, the SS/94 antisera showed low HI titer ($\leq$ 320) against the emerging H9N2 viruses. These results suggest that the SS/94 strain was antigenic different when compared to the H9N2 viruses circulating in Guangdong Province.

Discussion

H9N2 subtype virus can infect various poultry species for long periods of time without showing symptoms. When this subtype co-infects with other subtypes, such as H5N1 and H7N9, new subtypes of viruses might emerge [36]. Vaccination is considered to be a cost-effective measure for avian influenza control compared to stamping-out policy [19]. In China, inactivated H9N2 vaccines have been use since the 1990s. However, in the present study, 19 H9N2 strains were isolated from vaccinated chicken flocks in Guangdong during 2012–2013, which revealed that the vaccine did not provide complete protection against infection.

Previous studies have reported that the prevalent strain in China belonged to clade h9.4.2 [4,5,16,17,21]. Jiang *et al*. [17] studied the phylogenic relationships of the HA gene of H9 AIV that circulated in China from 2008 to 2011, and showed that lineage h9.4.2.5 and emerging lineage h9.4.2.6 were dominant. The emerging lineage h9.4.2.6 emerged first in southern China in 2010 and subsequently extended to northern China [5,17].

Table 4. Homologous and heterogeneous hemagglutination inhibition titers of some H9 isolates

Antigen	Antisera*								
	CJS01	JJM02	LZL01	H07	H19	LRZ01	WBQ02	H36	SS/94
CJS0 (h9.4.2.5)	10240†	2560	640	1280	1280	5120	640	1280	320
JJM02 (h9.4.2.5)	2560	10240†	1280	2560	1280	5120	1280	2560	160
LZL01 (h9.4.2.5)	2560	5120	2560†	1280	1280	5120	1280	2560	320
H07 (h9.4.2.5)	5120	10240	1280	5120†	1280	5120	1280	2560	320
H19 (h9.4.2.6)	2560	5120	640	1280	5120†	5120	2560	2560	160
LRZ01 (h9.4.2.6)	5120	5120	1280	2560	2560	10240†	1280	2560	160
WBQ02 (h9.4.2.6)	5120	10240	1280	2560	5120	5120	5120†	2560	320
H36 (h9.4.2.6)	1280	1280	320	320	1280	5120	1280	5120†	160
SS/94 (h9.4.2.3)	320	320	40	160	160	320	160	40	1280†

*Antisera were diluted tenfold. †Homologous titers are shown.

Phylogenic analysis of this study showed that lineage h9.4.2.5 and h9.4.2.6 were the predominant clades in Guangdong province from 2012 to 2013. Notably, all viruses isolated in 2013 belonged to lineage h9.4.2.5, while no h9.4.2.6 virus was detected, indicating that H9N2 viruses circulated persistently in domestic chickens and were genetically diverse. Large numbers of chicken are transported to Guangdong province from other areas, which may explain the genetic diversity of H9 AIV in Guangdong.

Glycosylation of viral envelope proteins, which is responsible for viral immune evasion and persistence, is used by several enveloped viruses to escape, block, or minimize the virus-neutralizing antibody response [1]. Generally, H9N2 AIVs vary in the number of PGSs on HA; however, no single specific glycosylation site has been found to correspond to adaptation to land-based poultry [25]. The lineage h9.4.2.5 of the H9 subtype AIVs isolated from 2010 to 2012 usually contain eight PGSs in the HA [5]. In this study, most isolates (11/12) of lineage h9.4.2.5 lost one PGS at residues 200−202 in HA1, causing by the substitution of threonine (T) to another residue. The loss of PGS in HA has been reported to increase the affinity to human-like receptors and virulence in mice in the H5N1 and H1N1 virus, respectively [26,28]. Accordingly, further study to determine whether the loss of PGS appeared in the glucoprotein of the new emerging H9N2 virus enhanced the pathogenicity is warranted.

The receptor specificity of HA is considered to be a factor of host range restriction of influenza viruses [31]. Human-like influenza with L226 (H3 numbering) at HA protein preferentially binds to the α-2,6 sialyl glycan of the receptor, whereas avian-like influenza with Q (glutamine) 226 prefer to bind to α-2,3 sialyl glycan [27,34]. Viruses with mutation at position 226 in HA RBS have the potential to adapt to new hosts [23]. Substitution of Q226L and G228S at HA in H5N1 has been shown to change the viral receptor binding specificity from binding avian-like receptor to human-like receptor [6,22]. In the present study, we found that all isolates in Guangdong province had 226 L from 2012 to 2013, indicating that they had the ability to cross species to infect humans. No G228S was found in this study. Amino acid at position 341 (H3 numbering) in combination with short stalk NA was reported to increase HA cleavage efficiency [30]. All isolates of lineage h9.4.2.5 in this study contained serine (S), whereas lineage h9.4.2.6 contained alanine at this position.

Vaccination is the predominant strategy to prevent and control H9N2 virus. Vaccine strain SS/94 was used to control H9N2 virus in Guangdong province. Antigenic analysis showed that the emerging viruses circulating in Guangdong were antigenically distinct from SS/94. Accordingly, H9N2 vaccine candidates from current circulating strains that reacted well with the emerging strains should be used to update the H9N2 vaccine in the Guangdong area.

In the present study, we found that H9N2 AIVs circulate continually in chicken flocks in Guangdong province. The results also showed that the lineage h9.4.2.5 viruses losing one PGS at position 200−202 would become the prevalent strains in this area. In addition, genetic and phylogenetic analysis results indicated that the prevalent strains and vaccine strain belonged to different clades and are only distantly related. Furthermore, all of the isolates contain human-like receptor and might have the potential to cross species to infect humans. Accordingly, it is recommended that continual surveillance of the circulation of H9N2 in Guangdong and Update vaccine be implemented to prevent and control H9N2 AIV.

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Conflict of Interest

There is no conflict of interest.

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