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ORIGINAL ARTICLE

Co-circulation of multiple genotypes of influenza A (H7N9) viruses in eastern China, 2016-2017

Xian Qi[1] · Xiaofei An[2] · Yongjun Jiao[1] · Huiyan Yu[1] · Ke Xu[1] · Lunbiao Cui[1] · Shenjiao Wang[1] · Fei Deng[1] · Xiang Huo[1] · Haodi Huang[1] · Qigang Dai[1] · Changjun Bao[1]

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Abstract

Five epidemic waves of human infection with influenza A (H7N9) virus have emerged in China since spring 2013. We previously described the epidemiological characterization of the fifth wave in Jiangsu province. In this study, 41 H7N9 viruses from patients and live-poultry markets were isolated and sequenced to further elucidate the genetic features of viruses of the fifth wave in Jiangsu province. Phylogenetic analysis revealed substantial genetic diversity in the internal genes, and 18 genotypes were identified from the 41 H7N9 virus strains. Furthermore, our data revealed that 41 isolates from Jiangsu contained the G186V and Q226L/I mutations in their haemagglutinin (HA) protein, which may increase the ability of these viruses to bind the human receptor. Four basic amino acid insertions were not observed in the HA cleavage sites of 167 H7N9 viruses from Jiangsu, which revealed that highly pathogenic avian influenza (HPAI) H7N9 viruses did not spread to Jiangsu province in the fifth wave. These findings revealed that multiple genotypes of H7N9 viruses co-circulated in the fifth wave in Jiangsu province, which indicated that the viruses have undergone ongoing evolution with genetic mutation and reassortment. Our study highlights the need to constantly monitor the evolution of H7N9 viruses and reinforce systematic influenza surveillance of humans, birds, and pigs in China.

Introduction

Human infections with avian influenza viruses frequently have occurred since 18 individuals were first infected with highly pathogenic avian influenza (HPAI) H5N1 viruses in Hong Kong in 1997. Several subtypes of avian influenza viruses have since been reported to infect humans, including H5, H9, H10, H6 and H7 [1–6]. In spring 2013, a novel reassortant H7N9 avian influenza virus causing severe respiratory disease in humans emerged in the Yangtze River Delta region of China [3, 7, 8]. Since then, five epidemic waves of human infection with H7N9 viruses have been observed [9, 10]. As of 26 October 2017, worldwide, 1564 confirmed cases of human infection with H7N9 viruses have been reported to the World Health Organization (WHO), with at least 607 deaths. In the fifth wave (1 October 2016-30 September 2017), the cumulative number of human infection cases was 764, which is markedly higher than in each of the previous four waves (135, 320, 226 and 119, respectively) [10].

Jiangsu province, located in the Yangtze River Delta of China, had the highest cumulative numbers of reported human infection (n = 150) with H7N9 viruses in the fifth wave. We previously described the epidemiological characterization of the current fifth wave in Jiangsu province [11]. In this study, 41 H7N9 viruses from patients and live-poultry markets were isolated and sequenced to further investigate the genetic features of viruses of the fifth wave in Jiangsu province. Compared with the haemagglutinin and neuraminidase genes, the internal genes of the 41 H7N9 viruses showed a higher degree of diversity. Based on phylogenetic analysis, at least 18 genotypes were identified.

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Xian Qi, Xiaofei An and Yongjun Jiao contribute equally to this work.

✉ Xian Qi
qixiansyc@163.com

✉ Changjun Bao
bao2000_cn@163.com

[1] Jiangsu Provincial Center for Disease Control and Prevention, Jiangsu, China

[2] Department of Endocrinology, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, China

The co-existence of multiple genotypes of H7N9 viruses indicated that the viruses have undergone ongoing evolution with genetic mutation and reassortment in the fifth wave in Jiangsu province.

Materials and methods

Virus surveillance and isolation

Since 2013, surveillance for influenza A (H7N9) viruses has been conducted in human and live-poultry markets (LPMs) in Jiangsu province. Samples collected from patients with suspected infection and LPMs were tested for H7N9, virus using real-time PCR, by local municipal centers for disease control and prevention (CDC). All H7N9-positive samples were submitted to Jiangsu CDC for virus isolation. Two hundred μl of each original specimen was inoculated allantoically into 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs for 48 to 72 hours at 37 °C in a biosafety level 3 (BSL-3) facility (BSL-3 Lab of Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China).

Genome sequencing

Viral RNA extraction was performed using an RNeasy Plus Mini Kit (QIAGEN, Germany). The primer Uni12 (5'-AGC GAAAGCAGG-3') was used for reverse transcription [12]. PCR was performed with a set of 28 primer pairs specific for H7N9 influenza virus. All primer sequences are available upon request. PCR products were purified using a QIAamp Gel Extraction Kit (QIAGEN) and sequenced using an ABI 3735 DNA Analyzer (Applied Biosystems, USA) using an ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis

The incoming sequences were compiled using the Lasergene sequence analysis software package (DNAStar, Madison, WI, USA). Nucleotide BLASTn analysis (http://www.ncbi.nlm.nih.gov/BLAST) was used to identify related reference viruses. Whole genome sequences of 54 H7N9 viruses isolated from 2013 to 2017 were included for phylogenetic analysis, all of which were obtained from the GenBank and Global Initiative on Sharing All Influenza Data (GISAID) databases. Among them, four viruses, A/Shanghai/2/2013, A/Anhui/1/2013, A/Hong Kong/125/2017 (A/Hunan/02650/2016-like virus) and A/Guangdong/17SF003/2016) were H7N9 candidate vaccine viruses (CVVs) proposed by WHO. Pairwise sequence alignments were also performed with the MegAlign program (DNASTAR) to investigate nucleotide and amino acid sequence similarities. Phylogenetic analysis of the aligned sequences for eight genomic segments was performed by the maximum composite likelihood method using MEGA6 software [13]. The reliability of the unrooted neighbor-joining tree was assessed by bootstrap analysis with 1,000 replications; only bootstrap values ≥ 70% are shown. Horizontal distances are proportional to genetic distance. Alignments of each influenza virus sequence were created using the program ClustalX 1.83.

Based on the bootstrap value and branch length, each gene segment was classified into different clades. The genotypes of the isolates were identified based on the combination of clades to which their eight gene segments belonged.

Results

Virus isolation

Between October 2016 and March 2017, a total of 152 H7N9 viruses were isolated from human samples (n = 103) or environmental samples (n = 34) in a BSL-3 facility. Eight gene segments of 41 H7N9 viruses (human, n = 35, environment, n = 6) isolated from December 2016 to February 2017 were sequenced. All 35 human cases were admitted to hospital, and 16 (46%) died. Of the 35 cases, male patients accounted for 74% (n = 26), and the median age was 56 years (23-89).

Phylogenetic analysis

Pairwise alignment of all 41 H7N9 isolates showed that the lowest nucleotide sequence identity in the eight genes ranged from 93.5% to 98.9% (PB2, 97.7%; PB1, 94.7%; PA, 95.2%; HA, 95.9%; NP, 93.5%; NA, 98.8%; M, 96.3%; NS, 97.2%). Among the four CVVs, the HA segments of 41 viruses shared the highest nucleotide sequence similarity with A/Hunan/02650/2016 (97.4%-98.7% identity). For the HA genes, A/Xuzhou/550/ 2017 shared low nucleotide sequence similarity with the other 40 viruses (95.9%-96.5% identity), while the other 40 viruses shared 97.9%-99.2% nucleotide sequence identity. These findings showed that all of the H7N9 viruses were very similar, although their genes showed different levels of diversity (Table 1).

The Yangtze River Delta region (including Jiangsu province, the city of Shanghai and Zhejiang province) has been identified as the original source of H7N9 viruses, and since the second H7N9 epidemic wave, the Pearl River Delta region (mainly Guangdong province) has been identified as an additional H7N9 outbreak source [14]. In the fifth wave, HA genes of H7N9 viruses formed two distinct lineages: the Yangtze River Delta lineage and the Pearl River Delta lineage [9]. In this study, HA genes of all 41 H7N9 isolates

Table 1 Nucleotide sequence identity between 41 isolates and four H7N9 candidate vaccine viruses

Gene segment	Identity (%)				
	41 isolates	SH/2	AH/1	GD/17SF003	HN/02650
HA	95.9-100	96.9-97.5	97.0-97.6	96.3-96.8	98.3-98.7
NA	98.8-100	97.6-98.0	97.6-98.0	96.7-97.1	97.4-97.9
PB2	97.9-100	96.6-97.2	96.7-97.2	96.5-97.0	94.3-94.8
PB1	94.7-100	95.9-98.9	95.9-98.9	95.0-97.2	94.8-97.4
PA	95.2-100	96.7-98.5	96.5-98.5	96.0-96.7	95.9-99.4
NP	93.5-100	95.1-98.7	95.2-98.7	94.1-98.3	93.8-99.5
M	96.2-100	96.6-97.6	96.6-97.6	96.3-98.9	96.8-99.7
NS	97.2-100	98.3-99.0	98.3-99.1	97.7-98.5	97.4-98.4

Nucleotide sequence identity values are based on comparisons of the nucleotide sequences of PB2 (28-2307), PB1 (25-2298), PA (25-2175), HA (33-1733), NP (46-1542), NA (21-1430), M (26-1007) and NS (27-864). A/Shanghai/2/2013, SH/2; A/Anhui/1/2013, AH/1; A/Hunan/02650/2016, HN/02650; A/Guangdong/17SF003/2016, GD/17SF003

from Jiangsu province belonged to the former (Fig. 1). In the Yangtze River Delta lineage of the HA tree, 40 out of 41 viruses from Jiangsu province clustered in one clade (clade 1.1). However, A/Xuzhou/550/ 2017 formed an independent clade (clade 1.4). The NA tree exhibited a topology similar to that of the HA tree, with viruses also forming the Yangtze River Delta lineage and Pearl River Delta lineage, and 41 H7N9 isolates from Jiangsu clustered in clade 1.1 (Fig. 1). Interestingly, A/Xuzhou/550/2017 was located at the root of clade 1.1. Together with the low sequence identities from pairwise alignment, we could deduce that A/Xuzhou/550/ 2017 was separated from the other 40 viruses. Six internal genes of 41 H7N9 viruses could be classified into more than one clade, with one major clade and at least one minor clade (Fig. 1). In all 41 H7N9 viruses, the PB2 genes clustered in four clades, and the PB1, PA and M genes were grouped into three clades. The NP and NS genes were divided into two clades. These results illustrate the genetic diversity of the H7N9 viruses circulating during this outbreak in Jiangsu, and this mainly resulted from the high level of genetic heterogeneity of the internal genes of the H7N9 viruses.

Genotype identification

Based on phylogenetic analysis of the eight genes, we classified the 41 H7N9 viruses into 18 genotypes, of which G2 (n = 7) and G8 (n = 5) were the most frequent (Table 2). Except for G4 and G9, all of the genotypes were detected in H7N9 isolates from humans. Geographically, 13 genotypes co-circulated in the southern part of Jiangsu Province (including Suzhou, Wuxi, Changzhou and Nanjing city). In contrast, only five genotypes co-circulated in the northern part of Jiangsu (including Taizhou, Yancheng, Huaian and Xuzhou city). The co-existence of multiple genotypes of H7N9 viruses in Jiangsu province indicated that the viruses have undergone ongoing evolution with genetic mutation and reassortment in the fifth wave.

Molecular characterization

Molecular markers associated with host adaptation, virulence, and drug resistance were analyzed (Table 3). HA proteins of all 41 viruses contained the mutations G186V and Q226L/I (H3 numbering), which suggested that they may have dual receptor affinity for both human-type 2,6-linked sialic acid and avian-type 2,6-linked sialic acid receptors [15–17]. Multiple basic amino acids were not observed in the HA cleavage sites of any of the 41 viruses, which is a molecular feature of low-pathogenic avian influenza (LPAI) viruses. More than half of human-origin H7N9 viruses (n = 18) possessed the PB2-E27K substitution, which may increase viral replication and pathogenicity in mammalian hosts [18, 19]. Some mutations in the NA protein, such as E119V, R152K, I222K/R, and R292K, can confer resistance to neuraminidase inhibitors (NAIs) [20, 21]. In this study, the isolate A/Suzhou/88/2016 possessed the NA-R292K mutation, implying that it has lost sensitivity to NAIs. All 41 isolates had a five-amino-acid deletion in the NA stalk, which may be associated with adaptation to territory-based poultry. The substitution S31N in the M2 protein suggests resistance to adamantine antiviral drugs.

Discussion

Jiangsu province was one of the most strongly impacted regions in the fifth wave of human infections with H7N9, and most of the infections in Jiangsu occurred from December 2016 to February 2017 (n = 124). In this study, phylogenetic analysis of 41 H7N9 viruses showed that at least 18 genotypes co-circulated in the fifth wave in Jiangsu,

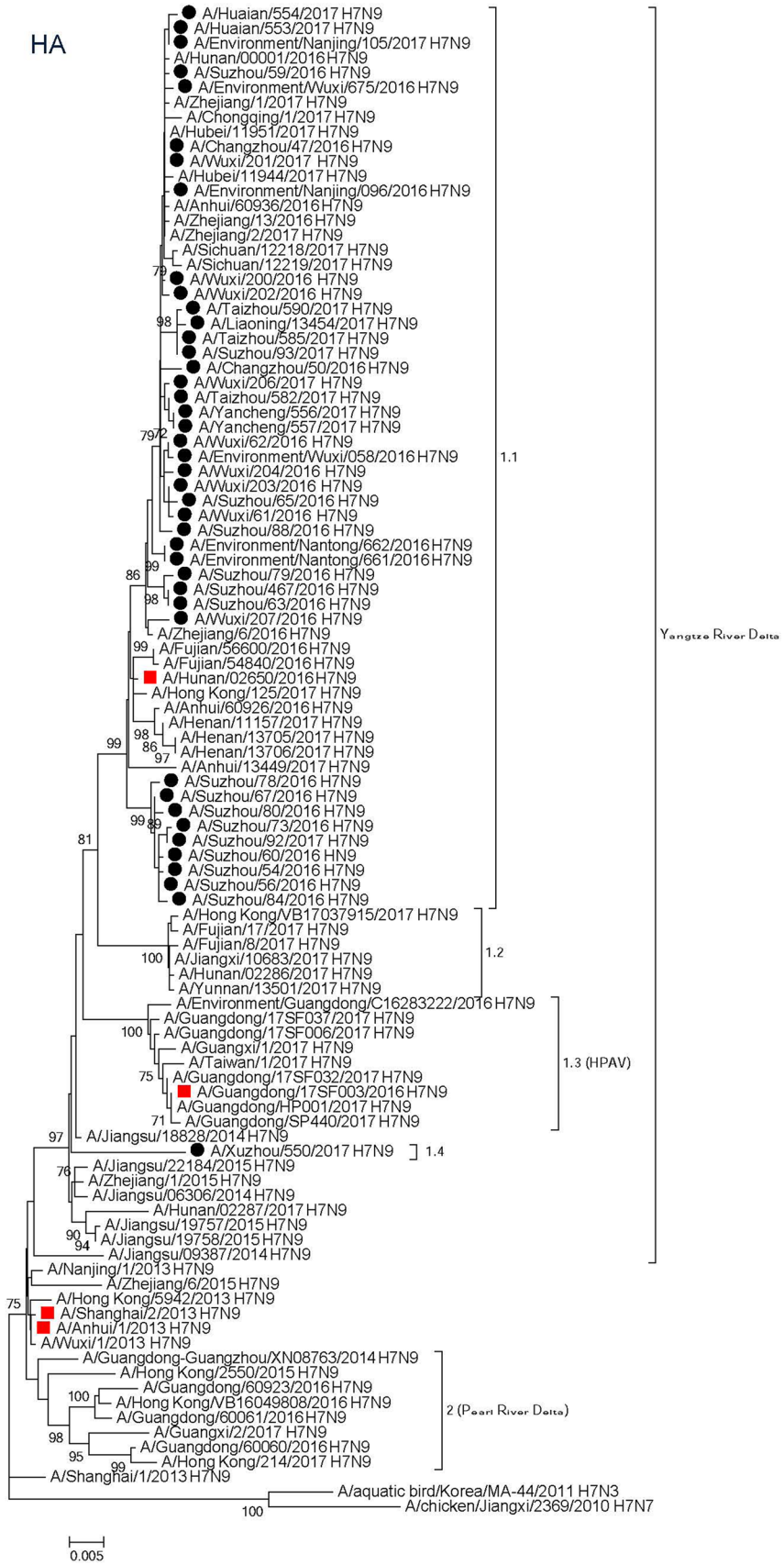
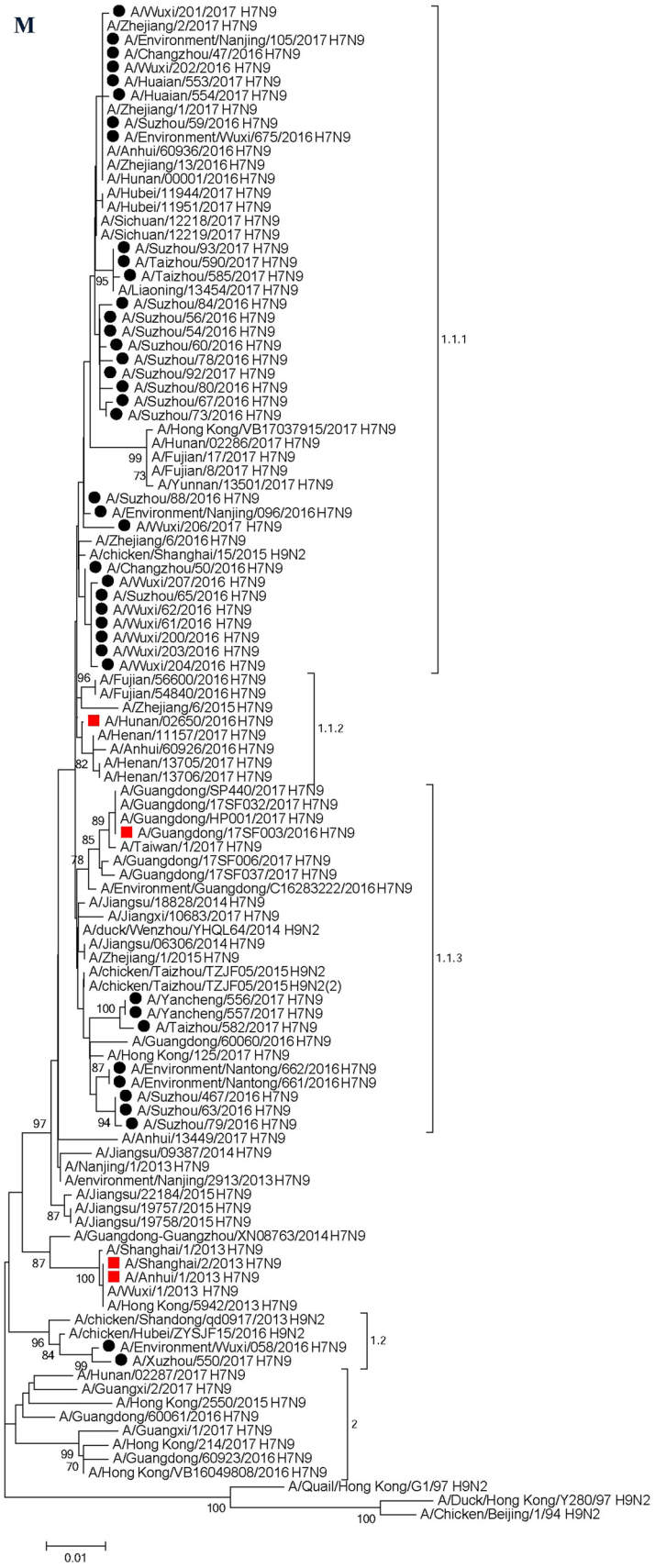


Fig. 1 Phylogenetic trees for the HA, NA, NP, M, NS, PB2, PB1, and PA gene segments of 41 H7N9 and related reference viruses. The unrooted neighbor-joining phylogenetic trees were generated by the maximum composite likelihood model in MEGA 6 software. The reliability of the tree was assessed by bootstrap analysis with 1,000 replications. Bootstrap values are shown for selected nodes (only for those with a frequency greater than 70%). Horizontal distances are proportional to genetic distance. The 41 H7N9 viruses from Jiangsu in 2016-2017 are indicated by a black circle (●), and the four H7N9 candidate vaccine viruses are indicated by a square (■)

Fig. 1 (continued)



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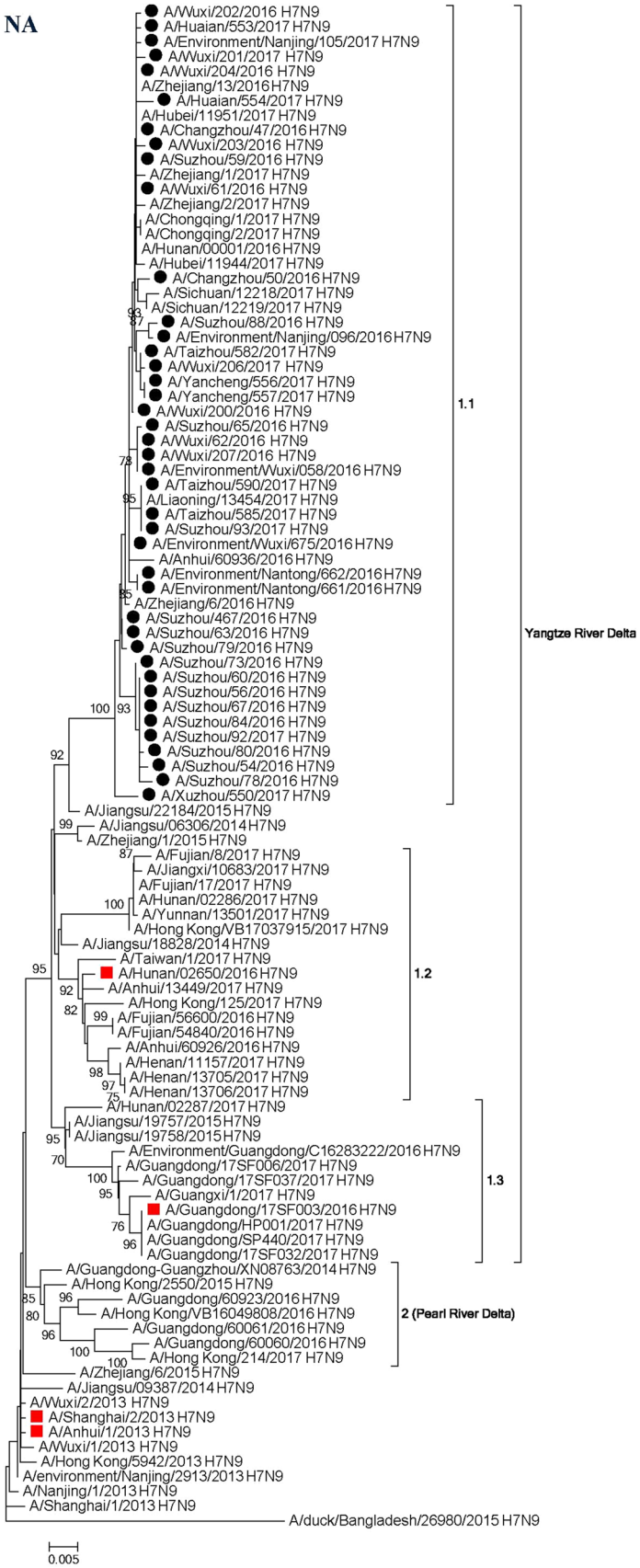

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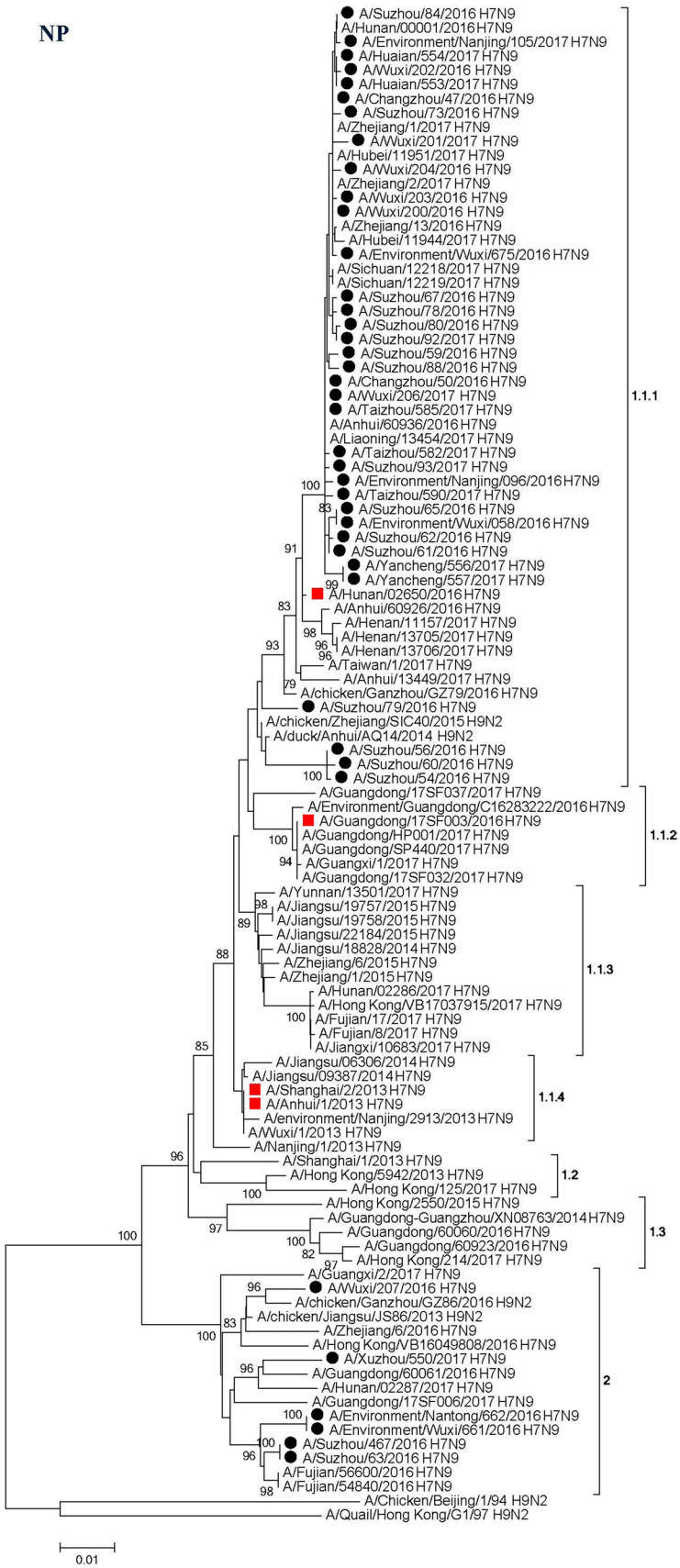

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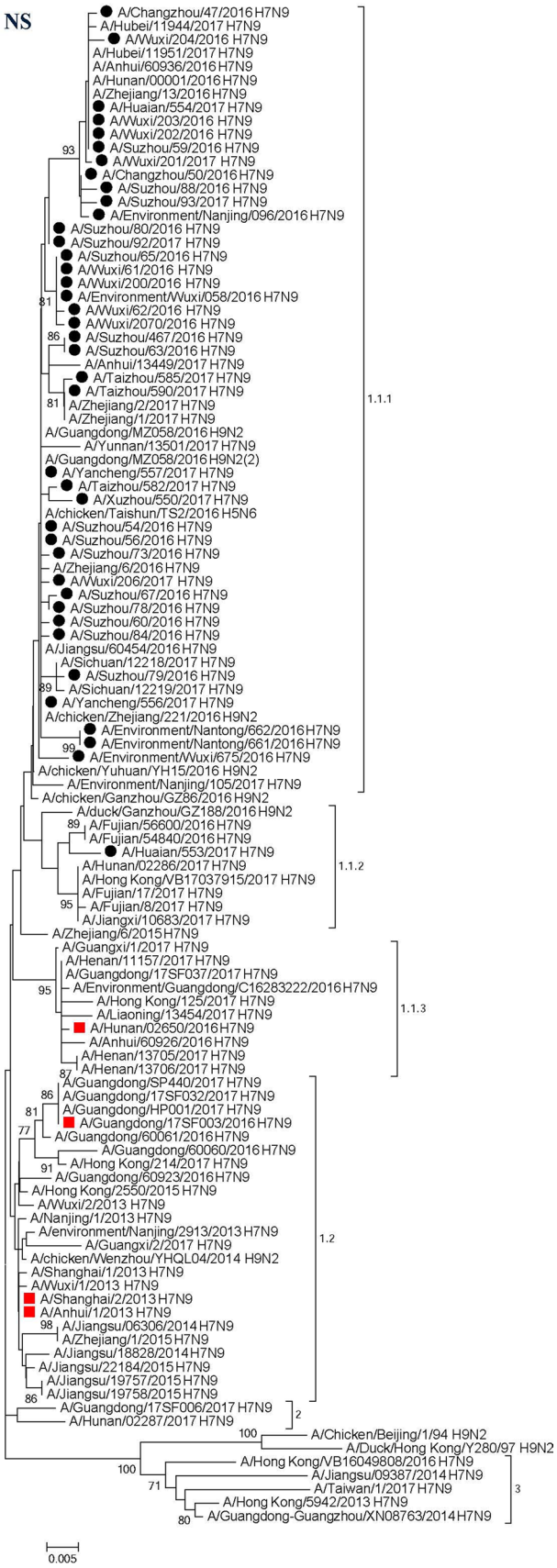

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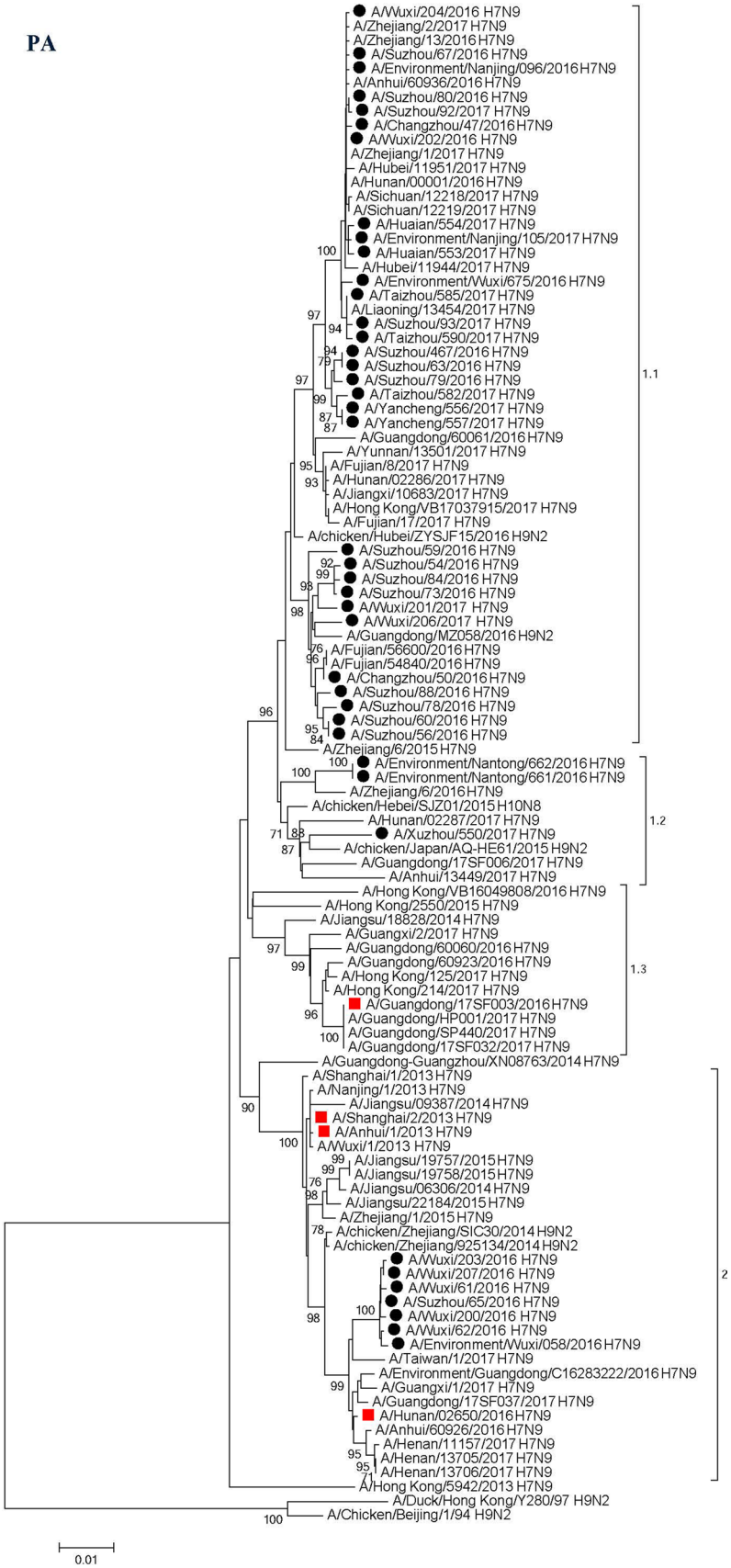

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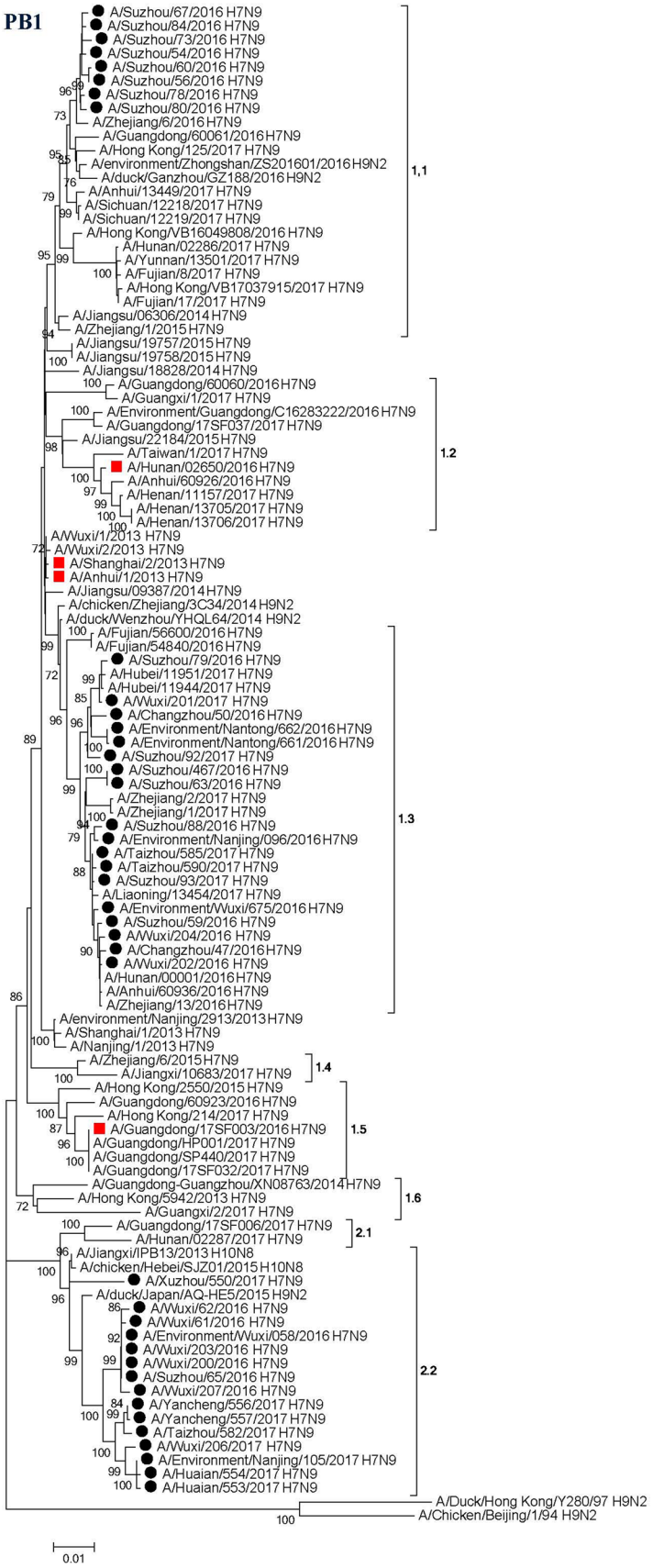

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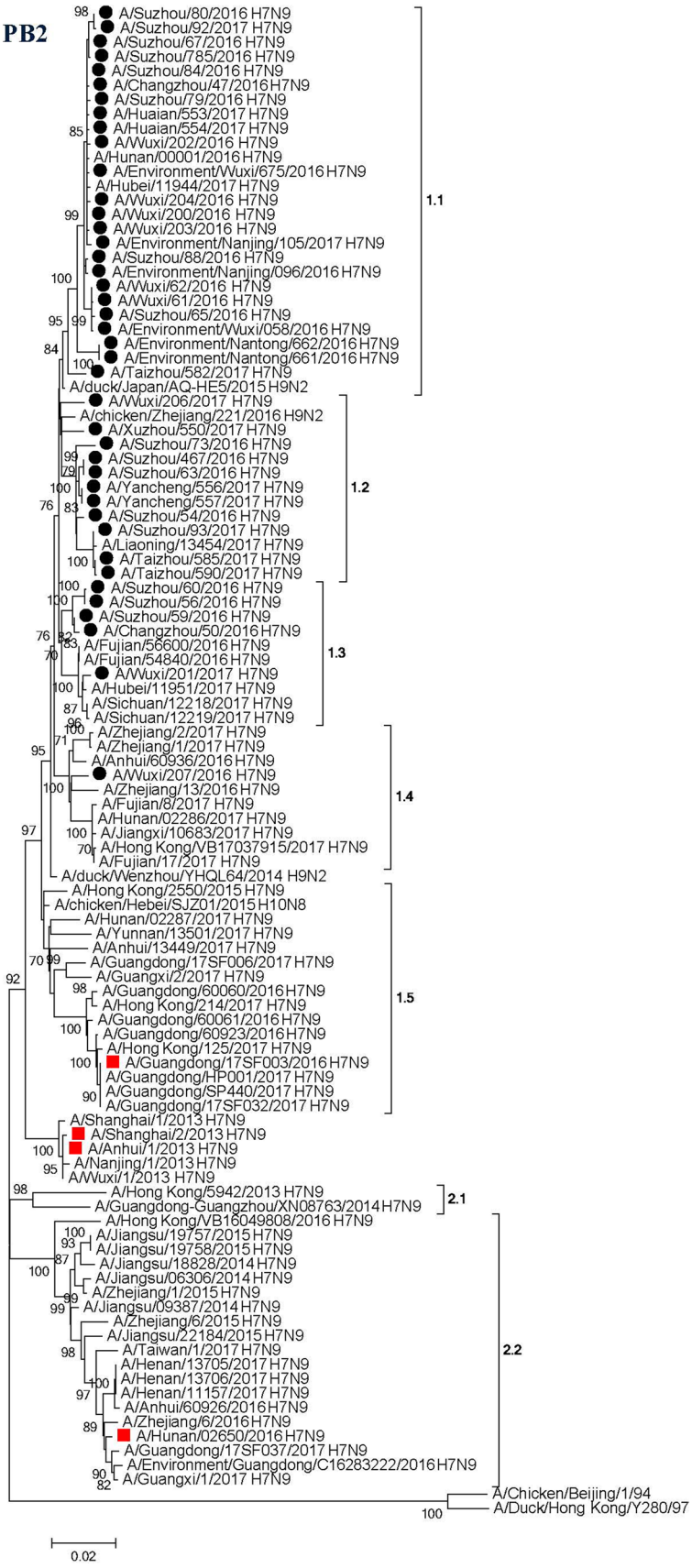

Table 2 Gene constellation of 41 H7N9 influenza A viruses from Jiangsu province, 2016-2017

Virus	Genotype	Host	Lineage							
			HA	NA	PB2	PB1	PA	NP	M	NS
A/Suzhou/80/2016	G1	Human	1.1	1.1	1.1	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/67/2016	G1	Human	1.1	1.1	1.1	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/78/2016	G1	Human	1.1	1.1	1.1	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/84/2016	G1	Human	1.1	1.1	1.1	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Wuxi/202/2016	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Environment/Wuxi/675/2016	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Wuxi/204/2016	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/88/2016	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Environment/Nanjing/096/2016	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/92/2017	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Changzhou/47/2016	G2	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/79/2016	G3	Human	1.1	1.1	1.1	1.3	1.1	1.1.1	1.1.3	1.1.1
A/Environment/Nantong/662/2016	G4	Environment	1.1	1.1	1.1	1.3	1.2	2	1.1.3	1.1.1
A/Environment/Nantong/661/2016	G4	Environment	1.1	1.1	1.1	1.3	1.2	2	1.1.3	1.1.1
A/Huaian/553/2017	G5	Human	1.1	1.1	1.1	2.2	1.1	1.1.1	1.1.1	1.1.2
A/Environment/Nanjing/105/2017	G6	Environment	1.1	1.1	1.1	2.2	1.1	1.1.1	1.1.1	1.1.1
A/Huaian/554/2017	G6	Human	1.1	1.1	1.1	2.2	1.1	1.1.1	1.1.1	1.1.1
A/Taizhou/582/2017	G7	Human	1.1	1.1	1.1	2.2	1.1	1.1.1	1.1.3	1.1.1
A/Wuxi/200/2016	G8	Human	1.1	1.1	1.1	2.2	2	1.1.1	1.1.1	1.1.1
A/Wuxi/203/2016	G8	Human	1.1	1.1	1.1	2.2	2	1.1.1	1.1.1	1.1.1
A/Wuxi/62/2016	G8	Human	1.1	1.1	1.1	2.2	2	1.1.1	1.1.1	1.1.1
A/Wuxi/61/2016	G8	Human	1.1	1.1	1.1	2.2	2	1.1.1	1.1.1	1.1.1
A/Suzhou/65/2016	G8	Human	1.1	1.1	1.1	2.2	2	1.1.1	1.1.1	1.1.1
A/Environment/Wuxi/058/2016	G9	Environment	1.1	1.1	1.1	2.2	2	1.1.1	1.2	1.1.1
A/Suzhou/54/2016	G10	Human	1.1	1.1	1.2	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/73/2016	G10	Human	1.1	1.1	1.2	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/93/2017	G11	Human	1.1	1.1	1.2	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Taizhou/585/2017	G11	Human	1.1	1.1	1.2	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Taizhou/590/2017	G11	Human	1.1	1.1	1.2	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/467/2016	G12	Human	1.1	1.1	1.2	1.3	1.1	2	1.1.3	1.1.1
A/Suzhou/63/2016	G12	Human	1.1	1.1	1.2	1.3	1.1	2	1.1.3	1.1.1
A/Wuxi/206/2017	G13	Human	1.1	1.1	1.2	2.2	1.1	1.1.1	1.1.1	1.1.1
A/Yancheng/556/2017	G14	Human	1.1	1.1	1.2	2.2	1.1	1.1.1	1.1.3	1.1.1
A/Yancheng/557/2017	G14	Human	1.1	1.1	1.2	2.2	1.1	1.1.1	1.1.3	1.1.1
A/Suzhou/60/2016	G15	Human	1.1	1.1	1.3	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/56/2016	G15	Human	1.1	1.1	1.3	1.1	1.1	1.1.1	1.1.1	1.1.1
A/Suzhou/59/2016	G16	Human	1.1	1.1	1.3	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Wuxi/201/2017	G16	Human	1.1	1.1	1.3	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Changzhou/50/2016	G16	Human	1.1	1.1	1.3	1.3	1.1	1.1.1	1.1.1	1.1.1
A/Wuxi/207/2016	G17	Human	1.1	1.1	1.4	2.2	2	2	1.1.1	1.1.1
A/Xuzhou/550/2017	G18	Human	1. 4	1.1	1.2	2.2	1. 2	2	1.2	1.1.1

Table 3 Amino acid mutations of 41 H7N9 isolates and four candidate vaccine viruses

Protein	Biological function	Mutation	41 isolates	A/Shanghai/2/2013	A/Anhui/1/2013	A/Guangdong/17SF003/2016	A/Hunan/02650/2016
HA	Receptor-binding sites	G186V	V (41)	V	V	V	V
	Receptor-binding sites	Q226L/I	L (41)	L	L	G	L
	Cleavage site		PEIPKGR↓G	PEIPKGR↓G	PEIPKGR↓G	PEVPK**RKRTA**R↓G	PEIPKGR↓G
NA	Stalk region	69-73	Deletion	Deletion	Deletion	Deletion	Deletion
	Antiviral resistance	E119V	E (41)	E	E	E	E
	Antiviral resistance	R152K	R (41)	R	R	R	R
	Antiviral resistance	I222K/R	I (41)	I	I	I	I
	Antiviral resistance	R292K	R (40) K (1)	R	R	R	R
PB2	Increased virulence in mice	Q591K	Q (41)	Q	Q	Q	Q
	Mammalian adaptation	E627K	E (21), K (18), V (2)	K	K	E	K
	Increased virulence in mice	D701N	D (41)	D	D	D	D
PB1	Increased transmission in ferrets	I368V	I (7), V (34)	V	V	V	V
PB1-F2	Increased virulence in mammals	87-90 aa	25 aa (1), 34 (15), 90 (25)	90 aa	90 aa	87 aa	90 aa
PA	Host signature	V100A	V (24), A (7)	A	A	V	A
	Host signature	S409N	N (41)	N	N	N	N
M2	Antiviral resistance	S31N	N (41)	N	N	N	N
NS1	C-terminal PED motif	227-300 aa	217 aa, deletion (41)	Deletion	Deletion	Deletion	Deletion
	Increased virulence in mice	D92E	D (41)	D	D	D	D
	Increased virulence in mice	P42S	S (41)	S	S	S	S

illustrating the genetic diversity of these viruses and their continuous evolution. Compared to the prior four waves, the H7N9 viruses in the fifth wave displayed antigenic divergence, and WHO therefore proposed two new CVVs, A/Hong Kong/125/2017 and A/Guangdong/17SF003/2016 [9]. In this study, all of the viruses from Jiangsu province, except for the virus A/Xuzhou/550/ 2017, clustered in one clade together with A/Hunan/02650/2016-like CVVs in the HA tree. However, the antigenicity of the 41 H7N9 viruses needs further investigation.

The marked increase in the number of human infections with H7N9 virus in the fifth wave appears to be due to the extensive geographic spread and high prevalence of H7N9 viruses in poultry [9, 10, 22], and there is little virological evidence that the viruses themselves have a higher transmissibility to humans. Our sequence data revealed that the 41 isolates from Jiangsu had G186V and Q226L/I mutations in their HA proteins, which means they may possess dual-receptor-binding properties. However, the receptor-binding specificity of the 41 viruses needs to be further tested in binding assays using sialylglycopolymers and glycan arrays. For H7N9 influenza prevention and control strategies, it is necessary to further monitor the genetic changes known to be associated with antigenic mutation, host adaption, virulence, and transmissibility based on viral whole-genomic sequencing.

In December 2016, highly pathogenic avian influenza (HPAI) H7N9 viruses were detected in humans and poultry in Guangdong province that contained four amino acid insertions in the HA cleavage site [9, 17, 22]. Since then,

at least 12 provinces in China have reported HPAI H7N9 viruses in poultry. Phylogenetic analysis showed that the HPAI H7N9 virus is likely to have emerged in late May 2016, and their ancestor virus originated from LPAI H7N9 viruses introduced from the Yangtze Delta region [22]. Because of their increased virulence, HPAI H7N9 viruses pose a greater threat to public health and poultry health than LPAI H7N9 viruses. In this study, in addition to the 41 isolates for which the full genome was sequenced, the HA genes of other H7N9 viruses from humans (n = 91) and the environment (n = 32) were also sequenced (data not shown). Four-basic-amino-acid insertions were not observed in the HA cleavage sites of any of the 167 viruses, which revealed that HPAI H7N9 viruses did not spread to Jiangsu province in the fifth wave.

Since the late 1990s, H5, H9, H6, H7 and H10 viruses of multiple subtypes/genotypes have become enzootic in poultry (including chickens, ducks, quail, etc.) in China [23–26], which provide an abundant gene pools for further inter- or intro-subtype reassortment [27]. Furthermore, the co-circulation of these viruses in southern China, along with their ability to infect humans and their potential for future reassortment with human H3N2 and/or H1N1 viruses clearly raises concern about their pandemic potential [28, 29]. Our study highlights the need to constantly monitor the evolution of H7N9 virus and reinforce systematic influenza surveillance in humans, birds and pigs in China.

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Compliance with ethical standards

Conflict of interest The authors declare that we have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of Jiangsu Provincial Center for Disease Control and Prevention.

Informed consent Informed consent was obtained from all individual participants included in this study.

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