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Epidemiological and genetic characteristics of the fifth avian influenza A(H7N9) wave in Suzhou, China, from October 2016 to April 2017

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Abstract
Human infections with H7N9 viruses can cause severe pneumonia and even death. To characterize the epidemiology and genetics of the H7N9 viruses circulating during from October 2016 to April 2017 in Suzhou, China, all pharyngeal swab samples were collected from severe acute respiratory infections (SARI) cases during this fifth wave of infection, and we amplified the H7N9 H7 and N9 genes using a real-time polymerase chain reaction (PCR). Positive samples were subjected to virus isolation and gene sequencing to analyze the evolution and variation of the H7N9 strains. The epidemiological features of H7N9 patients have not changed and there were no significant mutations in the key sites of the hemagglutinin (HA) gene sequence, but we identified the K526R and E627 K substitutions in the PB2 protein. In the neuraminidase (NA) protein, drug-resistant mutations (R294 K and H276Y) occurred in a few strains. Most of the H7N9 viruses isolated from Suzhou had no drug resistance mutations, but it is necessary to closely monitor and analyze the probable emergence of mutations and the spread of resistant strains. The reduction of the *N*-glycosylation site at position 42 of NA was observed in some strains.

Keywords H7N9 viruses · Real-time PCR · Hemagglutinin · Mutation · *N*-glycosylation

Introduction

In Mach 2013, human cases infected with a novel avian influenza A(H7N9), which is a multiple re-assortment virus, were first reported in eastern China [1]. Since the first outbreak, the H7N9 virus has been a public health concern, and five seasonal epidemic waves have been documented in China, especially in southern areas [2]. As of August 1, 2017, 1557 laboratory-confirmed cases, including 601 deaths, of human infections with H7N9 virus in mainland China have been reported to the World Health Organization by the National Health and Family Planning Commission of China (http://www.who.int/csr/don/07-august-2017-ah7n9-china/en/). The general fatality rate was 38.6% (601/1557). Cases with H7N9 virus infections were distributed in more than 20 provinces, and some limited human-to-human transmissions have been identified [2–4]. Currently, the fifth epidemic wave is ongoing, and compared with previous epidemics, the number of confirmed cases has increased significantly [5], which has resulted in widespread social concern.

Suzhou has approximately 10.6 million permanent residents. It is located in the Yangtze River Delta region and borders Shanghai, the largest city in China. From 2013 to 2017, H7N9 virus epidemics have occurred annually, but to different degrees, during spring and winter in Suzhou. To describe the epidemiological and genetic characteristics of the H7N9 infections in Suzhou, we identified hospitalized patients who were suspected of having such infections, and we analyzed the genetic characteristics of the corresponding strains.

Materials and methods

Ethical statement

This study was approved by Ethics Committee of the Suzhou Municipal Center for Disease Control and Prevention, and

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all experiments were conducted in accordance with relevant guidelines and regulations. Data collection and sample testing were conducted under the guidance of the procedure document approved by the China Center for Disease Control and Prevention. The data from the H7N9 cases were collected only after receiving the informed consent of the cases or their families. Anonymous data for analysis was obtained only by authorized personnel.

Sample collection and detection

Throat-swab samples were collected from all SARI cases with severe pneumonia, and the patient's name, age, and disease information, such as the date of onset of illness and clinical symptoms, were obtained simultaneously. SARI case is defined as the patient had the following 4 clinical manifestations: (1) acute onset; (2) axillary temperature ≥ 38 °C; (3) cough or sore throat; (4) shortness of breath (respiratory frequency ≥ 25 times/min), Blood oxygen saturation < 90%, or breathing difficulties.

All specimens were detected within 24 h and then stored immediately at − 70 °C for virus isolation. Viral RNA was extracted from the 200 ul original swabs sample in viral transport medium using the QIAsymphony Viral/Bacteria Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Respectively, the Influenza A/B, H1N1(2009), H3, H5N1, and H7N9 Real-time PCR kits (Bioperfectus Technologies, Jiangsu, China) were used to analyze every sample using the ABI7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Sequencing and analysis

H7N9 viruses were isolated and sequenced at Biosafety Level-3 laboratory of the Chinese national influenza center (CNIC). The *N*-glycosylation sites of the hemagglutinin (HA) and neuraminidase (NA) proteins were predicted by the online software NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). DNASTAR software was used to analyze the gene sequences homology matrix. ClustalX2.1 software was used for a multiple sequence alignment. A phylogenetic tree was generated by MEGA v6.06 software, using the neighbor-joining method with 1000 bootstraps. Representative H7N9 virus sequences of different geographical regions of China were obtained from the Global Initiative on Sharing Avian Influenza Data(GISAID).

Results

Epidemiological characteristics of confirmed cases

From October 2016 to April 30, 2017, there were 54 cases of laboratory-confirmed H7N9 virus infections, including 21 deaths, during the fifth epidemic wave in Suzhou. Some epidemiological features of the patients are summarized in Table 1. The fatality rate of all the reported cases was 38.9% (21/54), and this did not differ significantly ($p > 0.05$) compared with that at the national level (38.6%). More than 85% of the reported cases had contacted live poultry or were exposed to a live poultry market 10 days before the onset of illness. Of these reported cases, most were males, with a gender ratio of 2.6:1. The age distribution of the H7N9 cases was not uniform, and the median age was 58 years (range 24–89). The occupations of these confirmed cases included retirees, office clerks, and farm workers. All the cases suffered from fever (range 37.8–42 °C with a median of 39.4 °C). Cough was another common symptom. As shown in Fig. 1, the H7N9 virus epidemics occurred during winter and spring.

Table 1 Epidemiological features of the H7N9 cases in Suzhou from October 2016–April 2017

Item	Value
Age	
Median age	58 (Range 24–89 years)
Subgroup—no.	
0–35	7
36–50	10
51–60	14
≥ 60	23
Sex	
Male—proportion (%)	72.2% (39/54)
Gender ratio	2.6:1
Occupation—no.	
Unemployed or retiree	29
Farm worker	8
Office clerk	3
Attendant	13
Catering worker	1
Exposure to live poultry or potentially contaminated environment—proportion (%)	85.2% (46/54)
Mean days of illness onset to hospitalized	5
Days of illness onset to confirmed	Median: 6 (range 1 to 10)
Fever(median, range)	All (39.4 °C, 37.8 to 42 °C)
Cough	All
Fatality rate	38.9% (21/54)

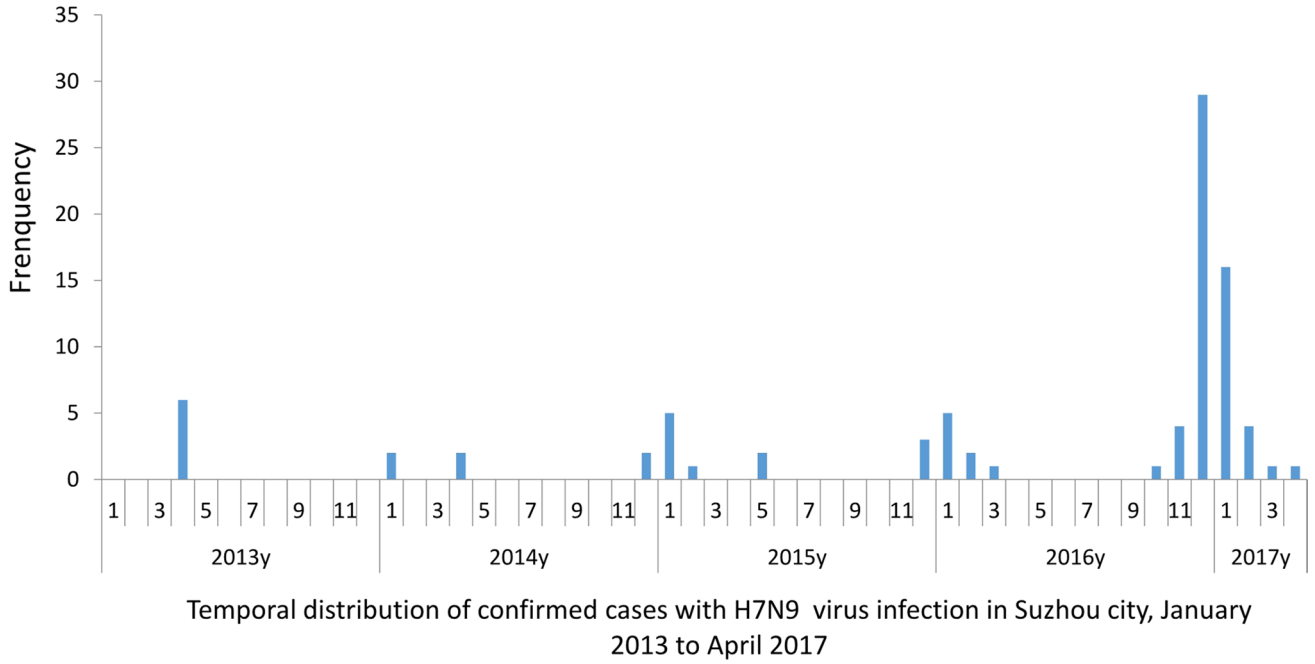


Fig. 1 Temporal distribution of confirmed cases with H7N9 virus infection in Suzhou city, Jan 2013–April 2017

One family-clustered epidemic was identified in Suzhou in December 2016. The two confirmed cases (A/Suzhou/56/2016 and A/Suzhou/60/2016), which were detected successively in the same family, were a father (who died) and a daughter (who survived). The initial case (the father, A/Suzhou/56/2016) had a clear history of exposure to live poultry markets before the onset of illness. The secondary case (the daughter, A/Suzhou/60/2016) had never contacted live poultry or visited a live poultry market before her symptoms occurred, but she contacted respiratory secretions of the initial case without any protection while taking care of her father in the hospital. The father was confirmed to be infected with the H7N9 virus on December 2, 2016, and he died 10 days later. The daughter's symptoms occurred on December 6, and then she was confirmed to have an H7N9 infection.

Phylogenetic analysis

Of the 54 patients, 26 virus strains were successfully isolated and sequenced. According to the results of the HA gene sequences analysis, the H7N9 viruses formed two distinct evolutionary branches. Notably, the Suzhou isolates fell within the Yangtze River Delta branch, and the HA gene of the H7N9 viruses was genetically close to sequences isolated from eastern China in 2016–2017. As shown in Fig. 2, the H7N9 viruses isolated from Suzhou during the fifth epidemic shared the same ancestor as previous strains, but clustered in an independent branch. Additionally, the H7N9 viruses isolated from different years clustered in different subgroups. These results indicated that the H7N9 virus is evolving continuously. The nucleotide homologies of different gene fragments are shown in Table 2.

Amino acid sequence analysis

Unlike the HPAIV mutants isolated from areas in Guangdong and Taiwan, there was no RKRT amino acid insertion between amino acids 337th and 338th of the HA protein in the Suzhou isolates. The amino acid sequence of the HA cleavage site of all the Suzhou isolates was PEIPKGR↓GLF (333–342). There were only two discontinuous basic amino acids (K and R) in the cleavage site, which accorded with the characteristics of a low pathogenic avian influenza virus, indicating that the H7N9 viruses isolated from Suzhou are likely to be low pathogenicity to poultry currently.

Compared with A/Shanghai/2/2013(H7N9), no significant changes were observed in key sites (amino acids 186, 226, and 228 in the H3 numbering) of the HA receptor binding sites, suggesting that the ability of the H7N9 virus to bind the receptors SA-α2,3-Gal and SA-α2,6-Gal had not changed. In the NA protein, the E120G or R153K substitutions were not observed in any of the Suzhou isolates, but the H276Y and R294K substitutions, which are associated with reduced sensitivity to Oseltamivir, which is used to treat H7N9 cases, were observed in the A/Suzhou/88/2016(H7N9) and A/Suzhou/54/2016(H7N9) viruses, respectively. Other strains lacked mutations at these

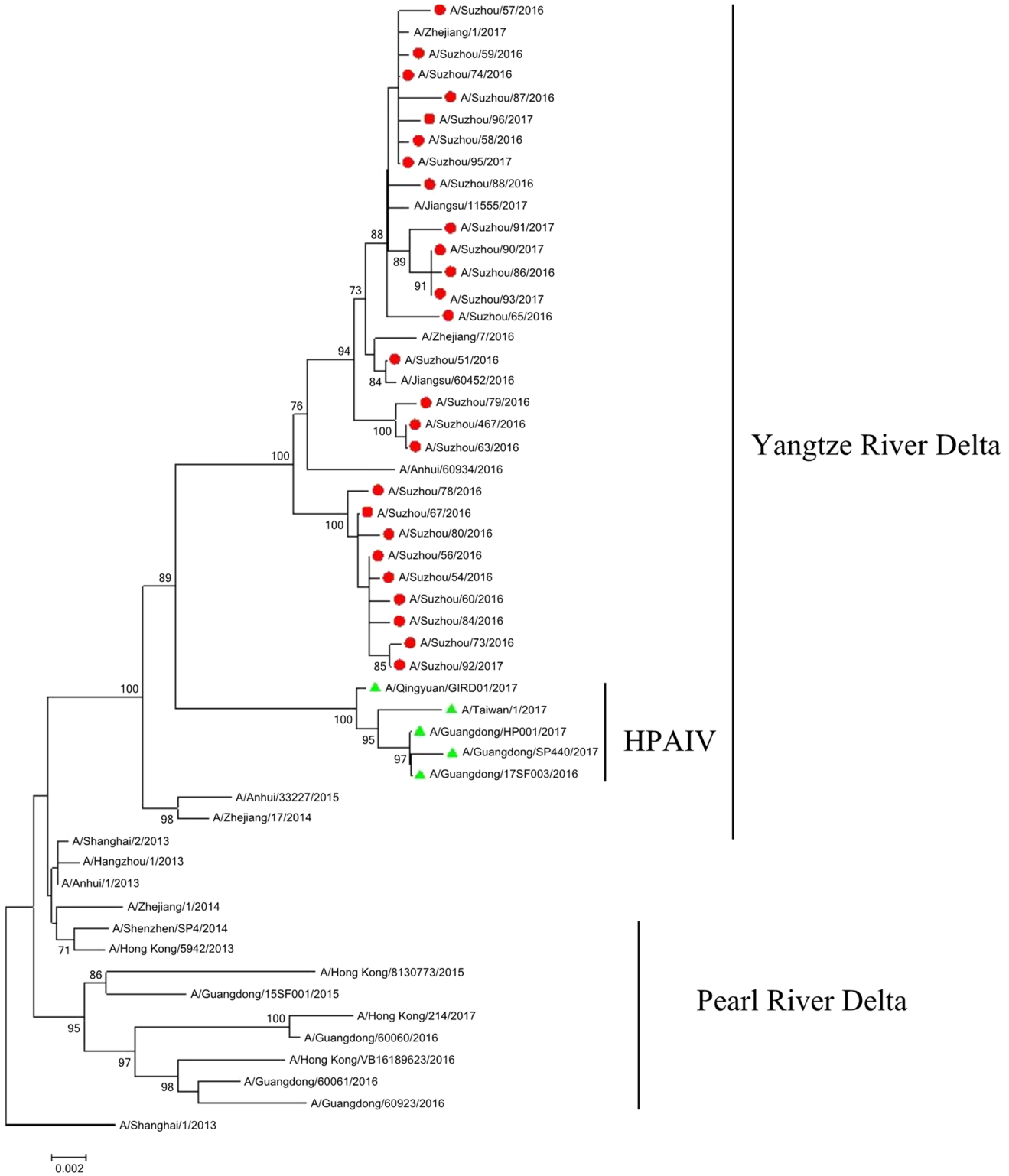


Fig. 2 Phylogenetic tree of the HA sequences of the H7N9 viruses isolated from Suzhou during the fifth epidemic. Red circles represent H7N9 strains isolated from Suzhou. Green triangles indicate highly pathogenic avian influenza virus (HPAIV) mutant strains (Color figure online)

Table 2 The nucleotide homology of the H7N9 viruses isolated from Suzhou 2016/17

Segments	Internal homology (%)	Compared with A/Shanghai/2/2013 (%)
HA	98.7–100	97.6–98.1
NA	98.8–100	97.7–98.1
PB1	94.8–100	96.0–98.9
PB2	97.8–100	96.5–97.1
NP	94.2–99.9	95.4–98.5
MP	98.7–100	97.1–97.6
NS1	98.7–100	98.4–99.4
PA	95.4–100	96.7–98.5

two positions, suggesting that most of the H7N9 viruses isolated from Suzhou were still sensitive to Oseltamivir.

Moreover, the E627K substitution in the PB2 protein was observed in most strains, except A/Suzhou/56/2016, A/Suzhou/60/2016, A/Suzhou/467/2016, A/Suzhou/63/2016, A/Suzhou/67/2017, A/Suzhou/73/2017, A/Suzhou/86/2017, A/Suzhou/87/2017, and A/Suzhou/96/2017. In these nine strains, the amino acid at position 627 of PB2 was glutamic acid (E). As shown in Table 3, some other mutations were also identified.

N-glycosylation site analysis

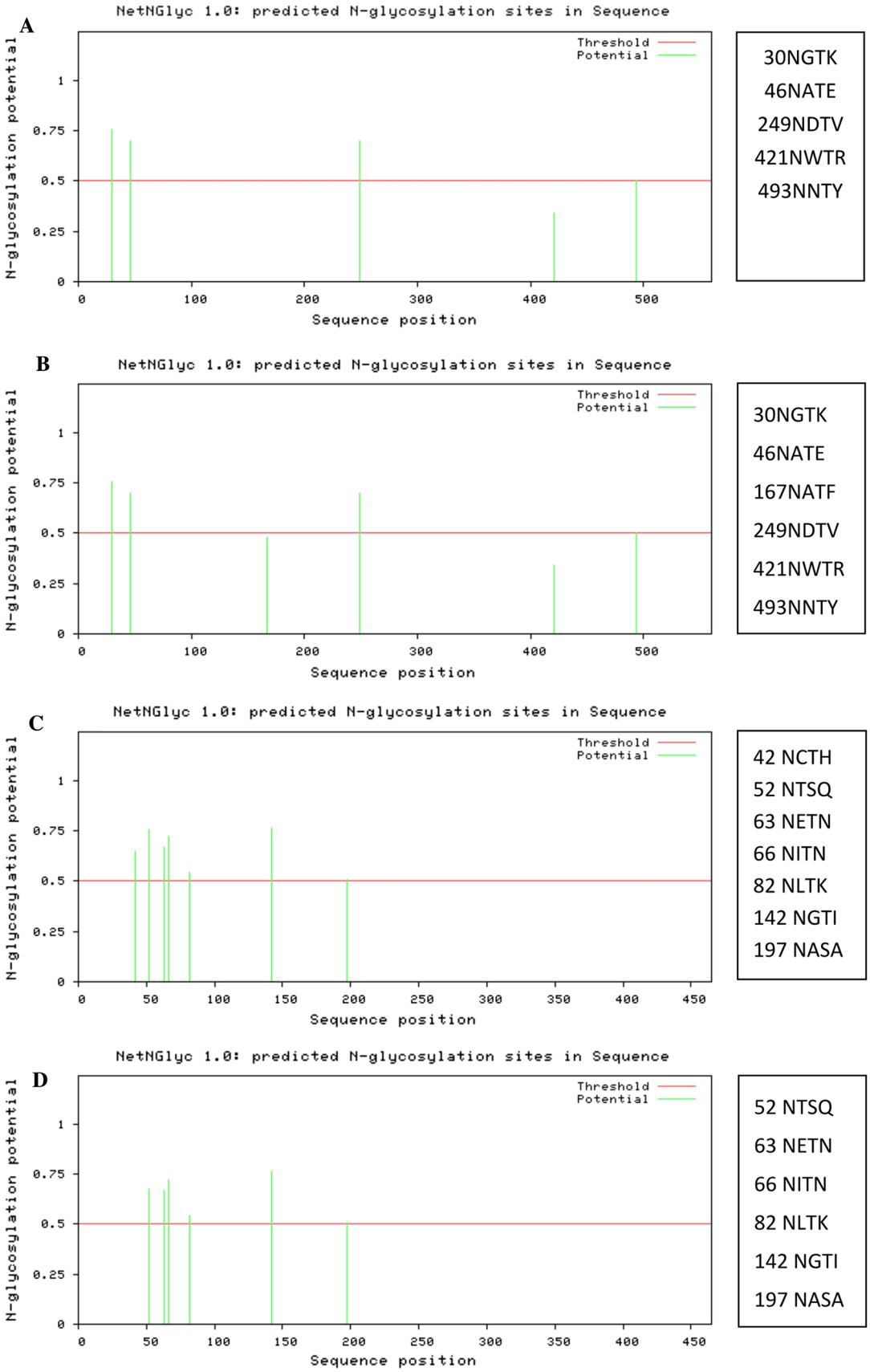
The number and location of *N*-glycosylation sites of currently circulating strains in Suzhou are shown in Fig. 3. According to the prediction of NetNGlyc, there are five potential *N*-glycosylation sites (position 30, 46, 249, 421, and 493; Fig. 3a) in the HA protein, except A/Suzhou/65/2016 (which has an addition site at position 167; Fig. 3b), while there are seven potential *N*-glycosylation sites (Fig. 3c) in the NA protein of most strains. Most of the H7N9 viruses isolated from Suzhou during the current wave retain the same *N*-glycosylation sites compared with previous strains; however, a potential *N*-glycosylation site (position 42; Fig. 3d) of the NA protein was removed in some strains.

Discussion

Because of the severity and high fatality rate of human H7N9 infections, compared with seasonal influenza virus infections, the epidemic of H7N9 avian influenza has aroused widespread social concern and become a focus of public health prevention and control. Suzhou is suffering from a fifth wave of H7N9 virus infections, with a significantly

Table 3 Molecular analysis of the H7N9 viruses isolated from Suzhou, during the fifth epidemic

Segments	Sites	Suzhou isolates	A/Shanghai/2/2013
HA	Cleavage site	PEIPKGR↓GLF	PEIPKGR↓GLF
	T160A	A	A
	G186V	V	V
	Q226L	L	L
	G228S	G	G
NA	E120G	E	E
	R153K	R	R
	H276Y	A/Suzhou/88/2016:Y others: H	H
	R294K	A/Suzhou/54/2016:K others: R	R
PB2	L89V	V	V
	K526R	A/Suzhou/88/2016:R others: K	K
	Q591K	Q	Q
	E627K	65.4% (17/26)was K and 34.6%(9/26)was E	K
	D701N	A/Suzhou/87/2016:N others: D	D
PB1	H99Y	H	H
	I368V	A/Suzhou/65/2016:I others: V	V
M1	N30D	D	D
	T215A	A	A
NS1	P42S	S	S
	V192I	V	V
NP	R246K	A/Suzhou/74/2016:K others: R	R
	V280M	A/Suzhou/73/2016:M others: V	V
PA	I308V	I	I
	T618K	T	T


A
NetNGlyc 1.0: predicted N-glycosylation sites in Sequence
Threshold
Potential
N-glycosylation potential
Sequence position
30NGTK
46NATE
249NDTV
421NWTR
493NNTY
B
NetNGlyc 1.0: predicted N-glycosylation sites in Sequence
Threshold
Potential
N-glycosylation potential
Sequence position
30NGTK
46NATE
167NATF
249NDTV
421NWTR
493NNTY
C
NetNGlyc 1.0: predicted N-glycosylation sites in Sequence
Threshold
Potential
N-glycosylation potential
Sequence position
42 NCTH
52 NTSQ
63 NETN
66 NITN
82 NLTK
142 NGTI
197 NASA
D
NetNGlyc 1.0: predicted N-glycosylation sites in Sequence
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52 NTSQ
63 NETN
66 NITN
82 NLTK
142 NGTI
197 NASA

◂**Fig. 3** The *N*-glycosylation sites of HA and NA protein of H7N9 viruses isolated from Suzhou, China. 3**a**, **b**: potential *N*-glycosylation sites in the HA protein; 3**c** , 3**d**: potential *N*-glycosylation sites in the NA protein

increased number of confirmed cases compared with the four previous waves. In Suzhou, the detection peak appeared in the month before the Spring Festival. This may be associated with lower temperatures and increased poultry consumption, which increased the opportunity for people to contact live poultry or visit live poultry markets. Contacting live poultry or exposure to virus-contaminated environments are the critical risk factors for H7N9 virus infection. This is similar to previous studies [3, 6], and the main epidemiological characteristics of the H7N9 cases have not changed.

Clustered cases of H7N9 virus infections were reported in previous studies [7–9]. Another family cluster was identified during the fifth epidemic wave in Suzhou, and the two confirmed cases (a father and daughter) did not have a common history of visiting live poultry markets. The secondary case (the daughter) may have become infected in the absence of protection while taking care of her father in the hospital. Additionally, the nucleotide identities of all eight gene fragments of the H7N9 viruses isolated from the two cases were greater than 99.9%. Among all the close contacts of the initial case, no one, except his daughter, was confirmed to have an H7N9 infection. The evidence suggests that the H7N9 virus has a limited human-to-human transmission ability, and cannot be spread effectively from person-to-person.

The homologies of the HA gene and NA gene sequences of the H7N9 viruses isolated from Suzhou during the fifth wave were 98.7–100% and 98.8–100%, respectively. Compared with the sequence of candidate vaccine strain A/Shanghai/2/2013(H7N9), the nucleotide homologies of all eight gene segments of the Suzhou isolates were greater than 95%. The genetic analysis of HA revealed that the H7N9 viral strains isolated from Suzhou belonged to the Yangtze River Delta branch and were distant from the Pearl River Delta branch. The HA gene sequences of viruses isolated in different years were distributed in different sub-branches, suggesting that the virus is evolving continuously. Recently, some viruses isolated from Guangdong Province were shown to have a four-amino-acid insertion in the HA protein, which is the cleavage site of an HPAIV [5]. This shows that the H7N9 virus, which is thought to be less virulent to poultry, could be converted to an HPAIV. However, in all the Suzhou isolates, the amino acid sequence of the HA cleavage site did not change. This means that the Suzhou isolates probably exhibit low pathogenicity to poultry.

Avian influenza viruses preferentially bind to SA-α-2,3-Gal, whereas seasonal human influenza A viruses bind to SA-a-2,6-Gal [10, 11]. To infect humans, it is necessary for avian influenza viruses to break through the inter-species barrier. Besides, HA protein to acquire the ability to bind the receptor SA-a-2,6-Gal is necessary for human-to-human transmission. Key amino acids of the receptor binding site of the HA protein of the influenza virus determine whether it preferentially binds the human- or avian-type receptors. The T160A, G186 V, and G226L (together with G228S) substitutions of the avian influenza virus increase the ability of the virus to bind the human-type receptor [12–15]. Compared with the A/Shanghai/2/2013(H7N9) virus, no substitutions were observed at these positions in any of the Suzhou isolates. This is conjecture; the lack of certain mutations might suggest that human-to-human transmission is unlikely.

Studies have shown that the R294K and H276Y substitutions reduce the sensitivity of the virus to NA inhibitors [16–19]. In the first H7N9 wave, the R294K substitution was identified in the A/Shanghai/1/2013(H7N9) virus [1, 20, 21], but the NA inhibitor-resistant substitution H276Y in the NA protein of the H7N9 viruses isolated from patients was not reported in previous studies. The amino acids of the key loci of the NA protein in most of the strains isolated in Suzhou did not change, but the resistant R294K substitution was observed in the A/Suzhou/54/2016(H7N9) virus, and the H276Y substitution occurred in the A/Suzhou/88/2016(H7N9) virus. It is necessary to closely monitor the probable emergence of these mutations and the spread of resistant strains. Most of the H7N9 viruses isolated from Suzhou city had no drug resistance mutations; this is a piece of evidence that most of the H7N9 viruses may remain sensitive to Oseltamivir.

The E627K substitution in the PB2 protein enhances the ability of avian influenza virus to replicate and cause serious disease in mammals [22, 23]. PB2-K526R contributes to enhanced replication for certain influenza virus sub-types, particularly in combination with the E627K substitution [24]. The E627K substitution, a potential virulence mutation of the H7N9 virus, occurred in most of the Suzhou isolates. The A/Suzhou/88/2016(H7N9) virus carried both the K526R and E627K substitutions, which is of great concern.

The influenza virus may benefit from the reduction of *N*-glycosylation sites, which may improve its ability to evade the immune system and bind its host cell receptor [25, 26]. For the H7N9 virus, the additional *N*-glycosylation site at position 167 of the HA protein may reduce the transmissibility of the virus, while the removal of *N*-glycosylation site at position 42 of the NA protein may increase the viral transmission efficiency [27]. The removal of *N*-glycosylation site at position 42 of the NA protein was observed in some of the Suzhou isolates; thus, enhanced surveillance is essential.

In conclusion, the epidemiological and genetic characteristics of the fifth wave of the H7N9 virus in Suzhou were preliminary discussed in this study. Human infections with the H7N9 virus are sporadic events, and there was no epidemiological relationship among most of the cases. There is

not enough evidence to suggest that the transmission pattern and virulence of the H7N9 viruses isolated from Suzhou have changed, but further studies are still required to provide a more accurate scientific basis for dealing with the potential pandemic of the H7N9 virus.

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Author Contributions Q.S. designed this study and Z.D. wrote the manuscript. Z.D., Y.X., and X.Y. performed the laboratory detections and analyses. C.L. and L.C. contributed to the case sample collection and epidemiological investigation. R.W. provided some helpful suggestions for improving the manuscript.

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Compliance with ethical standard

Conflict of interest The authors declare no competing interests exist.

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