Original Article

Genetic Characterization of a Novel Reassortant H5N6 Avian Influenza Virus Identified from a 10-Year-Old Girl

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SUMMARY: A 10-year-old girl was confirmed, by laboratory tests, to be infected with the H5N6 subtype of the highly pathogenic avian influenza virus in Suzhou city in November, 2018 (JSSZ01). The hemagglutinin (HA) gene of this H5N6 virus belonged to the 2.3.4.4 H5 clade, and the HA linkage peptide of the JSSZ01 strain was RERRRKR ↓ GLF with multiple basic amino acids. Q226L and G228S mutations were not observed, but S128P, S137A, and T160A substitutions were identified in the receptor binding sites. The resistance mutation D198N in the neuraminidase (NA) protein was also identified in this strain. Additionally, an 11 amino acid (AA positions 59–69) deletion was identified in the NA stalk region. Most genes of JSSZ01 exhibited highest identity with previously characterized H5N6 viruses, but its PA segment sequence was highly similar to previously identified avian H3 viruses (Accession Number: EPI596567 or EPI590058), indicating that JSSZ01 may be created by gene reassortment.

INTRODUCTION

H5 subtype highly pathogenic avian influenza (HPAI) virus is spread widely and known to cause high mortality in both poultry and human beings in the world. The first human infection with H5N1 subtype HPAI virus was identified in Hong Kong in 1997 (1) with re-emergence in Asia in 2003 (2,3). To date, H5N1 subtype avian influenza virus has caused at least 860 human infections including 454 deaths in 16 countries worldwide (4). Since the first reported human infection with avian-origin virus H5N6 in Sichuan province in 2014 (5), a total of 22 laboratory-confirmed H5N6 cases, including 15 deaths, have been reported in nine provinces in China as of November 1, 2018. The general fatality rate of human infection with H5 subtype avian influenza virus is greater than that of H7N9 subtype avian influenza (6,7). The main source of H5N6 infection is contact with live poultry or exposure to contaminated environments (7), although a few mutations for human adaption in H5N6 viruses have been documented (8).

Suzhou is an important city with 10.6 million permanent residents and located in the Yangtze River Delta region of China. All severe acute respiratory infection cases with unexplained severe pneumonia have been tested for influenza virus since the fatal human infection with H7N9 avian virus outbreak in 2013 (9). A laboratory-confirmed H5N6 case was identified in

Suzhou city on November 7, 2018. The patient was a 10-year-old girl, and is the first case of human infection with H5N6 HPAI virus in Jiangsu province, China. Here, we describe the genetic and epidemiological features of the latest H5N6 virus.

MATERIALS AND METHODS

Data collection: Close contacts include individuals who did not take effective protection against exposure to influenza A (H5N6) or the contaminated environment from patient illness onset to the time of confirmed infection. After the girl was confirmed to be a H5N6 patient, all of her 15 close contacts, including medical staff, family members and classmates, were placed under medical observation for at least seven days and monitored for possible infection. Investigators of the Suzhou Center for Disease Control and Prevention (Suzhou CDC) used standardized questionnaires to collect epidemiological and clinical data from the patient and her close contacts. Primary information, such as demographic data, live poultry contact history, close contacts, main symptoms, and laboratory findings, were collected by checking the medical records and interviewing related personnel.

Sample collection and detection of virus: Throat-swab samples were collected from the patient and close contacts. A total of 60 environmental samples suspected of being contaminated with H5N6 avian influenza virus were collected from live poultry market and mobile stalls that sold live birds in areas that the patient had passed by before symptoms occurred. All samples were detected within 24 h in the viral laboratory of Suzhou CDC. Viral RNA was extracted from every sample using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Basel, Switzerland). The different subtype of influenza virus (A/B, H1 to H16, and N1 to N9) was determined by real-time fluorescence quantitative test kit (Jiangsu Biopertectus, Co., Ltd. Jiangsu, China) according to the operational instructions. Positive samples were store

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at -80°C for H5N6 virus isolation and sequencing. Virus isolation was performed in a BSL-3 laboratory in the Chinese National Influenza Center. The throat-swab specimen obtained from the A/H5N6 patient was propagated in 9-day-old specific pathogen-free embryonated chicken eggs for 48 h at 37°C .

Sequencing and analysis: A novel reassortant H5N6 avian influenza virus identified from a 10-year-old girl in Suzhou city in 2018 is called JSSZ01. The full genome of the H5N6 virus isolated from the patient was sequenced using the Sanger method (Accession number: MK300699-MK300706). BioEdit v7.0 software was used for sequence assembly and editing. The highest similarity of every gene segment of JSSZ01 virus strain was analyzed using the Basic Local Alignment Search Tool. The hemagglutinin (HA) and neuraminidase (NA) phylogenetic tree was constructed with the MEGA v7.0 software, using the neighbor-joining method with 1000 bootstrap replicates. N-glycosylation sites in the HA and NA proteins were predicted using the NetNGlyc 1.0 Server. Reference sequences were downloaded from the GISAID EpiFluTM DATABASE <<https://platform.gisaid.org/epi3/frontend#4c9b48>>.

Ethical statement: To effectively deal with potential outbreaks and protect public health, data collection of the H5N6 case and her close contacts in the event was permitted by the Health Commission of Suzhou city. Data collection and sample tests were conducted under the guidance of the procedure document approved by the China Center for Disease Control and Prevention (China CDC).

RESULTS

Case information: The patient was a 10-year-old girl, who lived in Gusu district of Suzhou city. She did not have direct contact with live poultry but may have been exposed to a virus-contaminated environment, such as live poultry stalls, within seven days before illness onset.

She first consulted physicians in hospital A due to fever ($T_{\text{max}}=39.1^{\circ}\text{C}$) and cough in October 29, 2018. The white blood cell counts and C-reactive protein concentration was $6.19 \times 10^9/\text{L}$ and 6.19 mg/L, respectively. The proportion of lymphocyte and neutrophils was 24.3% and 62.6%, respectively. Three days later, the patient developed severe unexplained pneumonia and was admitted to the ICU in hospital B on November 6, 2018. Viral RNA, which was extracted from a throat swab sample of the patient, was detected using real-time RT-PCR assay by Suzhou CDC laboratory. Once H5N6-positive result was reported the following day, the girl was treated with oseltamivir in isolation. The patient was not suffering from any other underlying diseases. Moreover, the patient had not been vaccinated against influenza and immunoglobulin in the last year. The H5N6 virus test on the 17th day after illness onset was still positive in the respiratory sample. The medical staff tried their best to save her, but unfortunately, she eventually died in early December 2018 (Fig. 1). The H5N6 virus strain was successfully isolated from the throat swab sample of the patient by the Chinese National Influenza Center. The hemagglutination titer of H5N6 virus in allantoic fluid was 256.

Close contacts and source of infection: Within seven days of medical observation, no one else among the close contacts displayed symptoms of influenza-like illness, including fever, cough, headache, and fatigue. All pharyngeal swab samples of these close contacts were negative for H5N6 viral RNA test using the real-time RT-PCR method. For HA and NA genotyping detection, two chicken feces samples from the 60 environmental samples were confirmed to be positive for H5N6.

Phylogenetic analysis: Sequence alignment showed that HA and NA segments of H5N6 viruses obtained from the patient and environmental sample shared greater than 99% identity at the nucleotide level. The HA and NA gene of the JSSZ01 strain were both

Fig. 1. Time-line of the key indicators of the first H5N6 patient in Jiangsu province.

Fig. 2. The neighbor-joining phylogenetic trees of H5 and N6 genes were constructed by MEGA v7.0 software with 1,000 bootstrap. The black dots represented Suzhou H5N6 viruses.

closest to previous Jiangxi or Guangdong H5N6 viruses (A/chicken/Ganzhou/GZ27/2015-like) (Fig. 2), sharing over 98% in nucleotide identity. According to phylogenetic analysis results, the HA sequences of H5 subtype viruses were divided into several groups. The HA sequence of the Suzhou isolates were clearly clustered in the same branch and may originate from 2.3.4.4 H5Nx viruses. The N6 gene of H5N6 viruses likely originated from H6N6 viruses and is divided into two main groups (A and B); the JSSZ01 H5N6 virus clearly belonged to group A (Fig. 2). The PB1, PB2, MP, NP, and NS internal segments were also highly similar to previous H5N6 strains, but the PA gene exhibited closest homology with H3 viruses such as A/H3N8 (EPI596567) and A/H3N2 (EPI590058) isolated from Muscovy duck (Fig. 3). These results indicated that the JSSZ01 virus may have been generated by genetic reassortment between previous H5N6 viruses and previous avian H3 viruses (Fig. 4).

Amino acid sequence: The JSSZ01 and environment/038 H5N6 viruses obtained from this event possessed the same amino acids in key sites that were

related to viral replication, receptor-binding and drug resistance. The critical amino acid (226Q and 228G) in HA receptor binding sites (RBS) retained avian-like receptor binding preference. However, the JSSZ01 H5N6 virus possessed 128P, 137A and 160A, indicating that this H5N6 virus had acquired partial ability to adapt to human hosts. As shown in Table 1, the amino acid sequence of the HA cleavage sites of Suzhou JSSZ01 strain is PERRRKR ↓ GLF. This is not quite the same as that of A/Sichuan/26221/2014(H5N6) and A/Hong Kong/156/97(H5N1), though they all possess multiple basic amino acids, which is the characterization of HPAI virus strain. E119G, I122V, H274Y, R292K, and N294S substitutions in the NA protein were not observed, but D198N mutation was identified in the Suzhou strain, suggesting that JSSZ01 had obtained some form of resistance to oseltamivir. Additionally, the NA protein of the JSSZ01 strain possessed an 11 AA (at AA positions 59–69) deletion in the stalk region. Moreover, the PB1-F2 protein of JSSZ01 contained only 52 amino acids, which was shorter than the A/Sichuan/26221/2014(H5N6) strain with 57 amino acids

Fig. 3. The neighbor-joining phylogenetic tree of PA gene was constructed by MEGA v7.0 software with 1,000 bootstrap.

Fig. 4. (Color online) JSSZ01 H5N6 virus may be generated by genetic reassortment between previous H5N6 viruses and previous avian H3 viruses.

(Table 1). Neither E627K nor K526R substitutions were observed in the PB2 protein. According to the prediction by NetNGlyc, there are seven potential N-glycosylation sites (positions 27,39,180,208,301,498, and 557) in the HA protein and five potential N-glycosylation sites (positions 51,54,59,135, and 190) in the NA protein (Fig. 5).

DISCUSSION

Human infection with H5N6 virus manifests as flu-like symptoms such as fever and cough in the early stage, but rapidly progresses to severe pneumonia (10,11). The fatality rate of human infection with H5N6 avian influenza virus is higher than that of low pathogenic avian influenza virus (H7N9 and H9N2) (12) and seasonal influenza virus (subtype H1N1, H3N2, and B) (13–15). Treatment with oseltamivir within 48 hours of illness onset can effectively reduce the severity and mortality caused by avian influenza virus infection (16,17). However, the first antiviral treatment with oseltamivir in the Suzhou case was applied one week after illness onset. Missing the optimal antiviral time may be one cause of the patient's death. It is well-known that contact with live poultry or exposure to a contaminated environment is the main source of human infection with the H5 subtype HPAI virus. Unfortunately, the H5 subtype avian influenza virus has

been circulating in live poultry in China (18–20), and along with chicken consumption, this will increase the risk of human infections. Moreover, several clusters of human infection with H5 subtype virus epidemics have been confirmed (21–23). Therefore, we should be on high alert to the potential threat to human health.

The full genome analysis indicated that PB1, PB2, HA, MP, NA, NS, and NP segments of the Suzhou isolates shared strong homology to previous H5N6 viruses, but the PA segment was most similar to previously identified avian H3 viruses. This provides evidence that the Suzhou JSSZ01 H5N6 virus may have been generated by genetic reassortment between previous H5N6 viruses and previous avian H3 viruses. The HA phylogenetic tree indicated that the H5N6 virus obtained from this case belong to the 2.3.4.4 H5 clade. The key amino acid mutations in RBS of avian influenza viruses (G226L and G228S) can make them bind more effectively to human-like receptors (24–26). Fortunately, G226L and G228S substitutions were not observed in JSSZ01, indicating that the H5N6 virus isolated from this case retained the preferential binding to avian-like receptors. Additionally, all close contacts to the patient were not identified as having such infections during the medical observation period. These results supported that H5N6 viruses have not yet acquired the ability of sustained transmission in humans, and that poultry-

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Table 1. Comparison of the critical amino acids in representative H5 subtype viruses obtained from human host

Segment	Comment	Substitute	Suzhou JSSZ01 strain (H5N6)	A/Sichuan/26221/2014 (H5N6)	A/Hong Kong/156/97 (H5N1)
HA ¹⁾	Related to pathogenicity in poultry	cleavage sites	RERRRKR ↓ GLF	REKRRKR ↓ GLF	RERRRKKR ↓ GLF
		S128P	P	T	S
	Receptor binding sites (RBS)	S137A	A	A	S
		T160A	A	A	A
		Q226L	Q	Q	Q
		S227N	G	R	S
		G228S	G	G	G
NA ²⁾	Increased resistance to Antiviral drugs	E119V	E	E	E
		D198N	N	N	D
		I222V	I	I	I
		H274Y	H	H	H
		R292K	R	R	R
		N294S	N	N	N
	Enhanced virulence in mice	59-69AA deletion (N6 Numbering)	Yes	NO	NA
PB2	Enhanced virulence and replication in mice	L89V	V	V	V
		K526R	K	K	K
		E627K	E	E	E
		D701N	D	N	D
PB1	Enhanced transmission in ferrets	H99Y	H	H	H
		I368V	I	I	I
PB1-F2	Related to virulence	Full length	52AA	57AA	90AA
PA	enhanced virulence in mice	T97I	T	T	T
	Suppressed polymerase activity	I668V	I	I	I
M1	Enhanced virulence in mice	M30D	D	D	D
		T215A	A	A	A
M2	Amantadine resistance	S31N	S	S	S
NS1	Enhanced virulence in mice	P42S	S	S	S
		D92E	E	E	E
NP	Related to virus adaptability	S314N	S	S	S
		D375N	D	D	D

¹⁾: H3 numbering.

²⁾: N2 numbering.

Fig. 5. (Color online) Potential N-glycosylation sites in HA protein and NA protein of JSSZ01 strain.

to-human transmission was still the source of human infection with H5N6 virus. No mutation occurred in the resistance loci (I122V, H274Y, R292K, and N294S) in the NA gene of H5N6 virus isolated from the Suzhou patient; however, resistance loci D198N mutation, which can increase resistance to neuraminidase inhibitors such as oseltamivir (27), was identified. This mutation may have also contributed to the patient's death. Unlike A/Sichuan/26221/2014(H5N6), the JSSZ01 strain contained deletion at AA positions 59–69 in the NA stalk region which may enhance replication and virulence of the H5N6 virus.

In conclusion, novel reassortant avian influenza virus strains are constantly emerging (28,29), which will endanger human health and cause great economic losses. Several types of avian influenza virus (H5N1, H5N6, H7N9, H7N4, H6N1, H9N2, and H10N8) have been confirmed to cause severe human infections in China (12,30–32). In order to effectively respond to potential influenza pandemics, surveillance of viral mutations should be strengthened, especially mutations that may enhance drug resistance and transmission capacity.

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Conflict of interest None to declare.

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