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Clinical, epidemiological and virological characteristics of the first detected human case of avian influenza A(H5N6) virus

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Abstract

A human infection with novel avian influenza A H5N6 virus emerged in Changsha city, China in February, 2014. This is the first detected human case among all human cases identified from 2014 to early 2016. We obtained and summarized clinical, epidemiological, and virological data from this patient. Complete genome of the virus was determined and compared to other avian influenza viruses via the construction of phylogenetic trees using the neighbor-joining approach. A girl aged five and half years developed fever and mild respiratory symptoms on Feb. 16, 2014 and visited hospital on Feb. 17. Throat swab specimens were obtained from the patient and a novel reassortant avian influenza A H5N6 virus was detected. All eight viral gene segments were of avian origin. The hemagglutinin (HA) and neuraminidase (NA) gene segments were closely related to A/duck/Sichuan/NCXN11/2014(H5N1) and A/chicken/Jiangxi/12782/2014(H10N6) viruses, respectively. The six internal genes were homologous to avian influenza A (H5N2) viruses isolated in duck from Jiangxi in China. This H5N6 virus has not gained genetic mutations necessary for human infection and was suggested to be sensitive to neuraminidase inhibitors, but resistant to adamantanes. Epidemiological investigation of the exposure history of the patient found that a live poultry market could be the source place of infection and the incubation period was 2–5 days. This novel reassortant Avian influenza A(H5N6) virus could be low pathogenic in humans. The prevalence and genetic evolution of this virus should be closely monitored.

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Keywords

Avian influenza; H5N6; The first detected human case; Reassortant; Sequence

1. Introduction

Avian influenza virus is a member of the *Orthomyxoviridae* family, *Influenzavirus* A genus. On the basis of the external glycoproteins hem-agglutinin (HA) and neuraminidase (NA), currently 18 HA (H1–H18) and 11 NA (N1–N11) subtypes are known. Subtypes H17N10 and H18N11 were described recently in bats (Tong et al., 2012, 2013). The avian influenza subtypes capable of infecting humans are H5N1, H5N2, H6N1, H9N2, H7N7, H7N2, H7N3, H10N7, H7N9 and H10N8 (Arzey et al., 2012; Chen et al., 2014; Cheng et al., 2011; Gao et al., 2013; Hirst et al., 2004; Koopmans et al., 2004; Ogata et al., 2008; Ostrowsky et al., 2012; To et al., 2012; Wei et al., 2013).

On Feb. 22, 2014, the National Laboratory for Influenza Surveillance at Changsha Municipal Center for Disease Control and Prevention (CDC) detected an avian influenza virus in a throat swab sample collected by a sentinel hospital of the China influenza surveillance system. The sample was tested positive for H5 but negative for N1 by the sentinel hospital. The test result was confirmed by Hunan Provincial CDC and the Chinese National Influenza Center. On Mar. 20, 2015, the virus was further confirmed to be avian-origin influenza A H5N6 by full genome sequencing conducted at Changsha Municipal CDC. The onset date of this case was earlier than all known H5N6-infected cases reported by World Health Organization (WHO) or by the National Health and Family Planning Commission (NHFPC) of the People's Republic of China, suggesting that this case from Changsha was likely the first detected human infection with the novel reassortant avian influenza A virus. Here, we report the result of a clinical investigation on this patient and the characteristics of this virus.

2. Materials and methods

2.1. Clinical and epidemiological data collection

A standardized case reporting form was used to gather the following epidemiological and clinical data: demographic characteristics; underlying medical conditions; recent exposures to pigs, poultry, or other animals; recent visits to live animal markets; clinical signs and symptoms; laboratory testing methods and results; antiviral treatment; and clinical outcomes. According to the regulations and guidelines of the NHFPC of China, data collection on this patient was part of the routine surveillance and outbreak investigation, and was therefore exempt from the oversight by institutional review board (IRB).

Close contacts, defined as individuals who had provided care to, had been living with, or had potentially been directly exposed to respiratory secretions or body fluids of the patient in 14 days before the illness onset of the patient, were identified. The IRB of Changsha CDC approved the assessment of these close contacts. Written consent was obtained from each close contact.

2.2. Viral analysis

2.2.1. RNA extraction and real-time RT-PCR—Throat swab specimens were obtained from the patient on day 2 since the illness onset (illness onset counted as day 1). Specimen collection, storage and transportation were performed according to WHO guidelines (WHO Global Influenza Surveillance Network, 2011). Real-time RT-PCR or conventional RT-PCR, or both, were used for influenza typing and subtyping by the Changsha CDC, Hunan provincial CDC, and the Chinese National Influenza Center (CNIC). The sample was confirmed to contain M and HA genes of influenza A virus with subtype H5, but the NA gene could not be subtyped. Throat swab specimens were also obtained from the patient on days 7, 17, 24, and 27 since illness onset and showed similar results.

The virus was inactivated by heating at 65 °C for 30 min prior to RNA extraction. RNA was extracted from the throat-swab sample using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Primers and probes specific to seasonal influenza viruses (H1, H3, or B), avian influenza viruses (H5, H7, H9) and neuraminidase (N1, N2, N9) were used to sequence the virus. Sequences for comparison were provided by the CINC and have been published elsewhere (World Health Organization, 2014).

On day 8 since illness onset of the patient (Feb 23, 2014), five environmental samples were collected and stored in viral transport medium, including one sample of poultry drinking-water and one sample of water used for cleaning slaughtered poultry from the epidemiologically linked live poultry trading site (Fig. S1), and two swabs of poultry cages and one sample of sewage water from the epidemiologically linked restaurant. These samples were tested by real-time RT-PCR or conventional RT-PCR or both for influenza typing and subtyping by the Changsha CDC, using primers and probes specific to H5, H7, H9, N1, N2, and N9 provided by the CNIC.

2.2.2. Genome sequencing and phylogenetic analysis—A total of 8 universal primer sets were used to amplify the full genome of all influenza A viruses for sequencing (Hoffmann et al., 2001), with the use of SuperScript® III One-Step RT-PCR System with Platinum® Taq RT-PCR (Life Technologies Corporation, USA). PCR products were sequenced by Life Technologies biotechnology (Shanghai, China) CO. Full genome sequences of the virus from the patient were deposited in the Influenza Virus Database of the National Center for Biotechnology Information (accession number KR063684-91).

The sequence homology of each gene of the virus was analyzed with the online Basic Local Alignment Search Tool (BLAST). The ClustalW Multiple alignments were constructed using the BioEdit Sequence Alignment Editor (USA, Borland). The phylogenetic trees for the HA and NA genes were constructed using the neighbor-joining method provided by MEGA 5.2 software, coupled with the Tamura-Nei model for nucleotide substitution. One thousand bootstrap data sets were generated to evaluate the reliability of the phylogenetic tree. The molecular characteristics of each gene of the virus were summarized.

3. Results

This patient was a five-year-old kindergarten student living with her father. Looking for employment, the father migrated with his daughter from Chenzhou to Changsha, Hunan province, on Feb. 5, 2014. From Feb. 5 to Feb. 10, the child stayed at home as her father worked outside. During the four days following Feb. 10, the girl went out with her cousin and was exposed to a live poultry trading site for about 5 min a day.

Fig. S1 shows the path of the patient's activity outside her house during the four days. Every morning, she and her cousin went out for breakfast nearby, bypassing a restaurant with live chicken and wild birds in cages. After breakfast, they shopped for snacks in a supermarket across

the street. Then they went to shop for vegetables at a vegetables trading site next to a live poultry trading site with live chicken and ducks for sale. About 5 min later, they returned home. The girl stayed home on Feb. 15, 2014. On Feb. 16, 2014, she developed influenza-like illness (ILI) including sore throat and fever (38.0 °C). Her father bought her some herb medicine (Table 1), but her situation got no better. On Feb. 17, 2014, her father brought her to the outpatient care of Changsha Central Hospital which is a sentinel hospital for influenza surveillance. Physical examination found a body temperature of 38.9 °C and a red pharynx. Due to the presentation of ILI symptoms, a throat swab sample was collected to be tested for influenza virus. In addition, a blood sample was drawn for routine tests and found that the number of white blood cells was 23.12×10^9/L, twice the upper bound of the normal range, the proportion of neutrophils was higher than the normal range, and the proportion of lymphocytes was lower than the normal range. These test results suggested a probable bacterial infection. A diagnosis of “suppurative tonsillitis” was made by the outpatient physician, and a combination of antibiotics, febrifuge and herb medicine was prescribed for treatment (Table 1). The patient was not hospitalized and returned home in the evening.

The fever and sore throat lasted for 3 days, and the patient felt chilly after that. Throat swab samples were collected on day 7 and day 11 respectively (Fig. S2), which were tested positive for H5 but negative for N1 by Changsha Municipal CDC using Real-time PCR. The results were confirmed by Hunan Provincial CDC and China CDC. In an attempt to reduce the viral activity, antivirals (Oseltamivir) were prescribed on day 11 and taken for seven days (75 mg twice a day). Tests on the blood sample drawn on day 2 showed increased levels of white blood cells, neutrophilic granulocytes, monocytes, proportion of neutrophils, and proportion of monocytes. The number of lymphocytes was normal but proportion of lymphocytes was lower than the normal range (Table S1).

Real-time RT-PCR and full genome sequencing found the throat swabs obtained on day 7 and day 11 to be positive for avian influenza A H5N6 virus [The virus designated as A/Changsha/1/2014(H5N6)] and negative for influenza viruses H1, H3, B, H5N1, H7N9, and H9N2. Phylogenetic analyses revealed that the HA gene of A/Changsha/1/2014(H5N6) was closely related to A/duck/Sichuan/NCXN11/2014(H5N1) virus (98% identity), and the neuraminidase (NA) gene was closely related to A/chicken/Jiangxi/12782/2014(H10N6) virus (99% identity) (Table 2, Fig. 1). The RNA polymerase basic subunit (PB) 1 protein,

PB2 protein and nucleocapsid protein (NP) genes were closely related to A/duck/Jiangxi/JXA132023/2013(H5N2) virus (99% identity). The sequences of the remaining viral genes were closely related (99% identity) to A/duck/Jiangxi/JXA131996/2013(H5N2) virus, which was isolated in poultry in Jiangxi province, China (Table 2). Phylogenetic analysis demonstrated that the new isolate in this study, A/Changsha/1/2014(H5N6), is a novel triple reassortant H5N6 virus, similar to A/Sichuan/26221/2014 and A/Guangzhou/39715/2014, the two isolates from human H5N6 cases reported in Sichuan and Guangdong provinces in 2014, but different from A/Yunnan/0127/2015, the isolate from a human H5N6 case in Yunnan province in 2015 which is a complex reassortant virus. The A/Changsha/1/2014 (H5N6) virus might have acquired its HA gene from A/duck/Sichuan/NCXN11/2014(H5N1)-like viruses, NA gene from A/chicken/Jiangxi/12782/2014(H10N6)-like viruses and the six internal genes from viruses similar to the avian influenza A(H5N2) viruses in ducks of Jiangxi province, China (Table 2, Fig. 2). Each gene was of an avian origin. The A/Chang-sha/1/2014(H5N6) isolate shared moderate to high levels of nucleotide identity in the eight gene segments with the other three isolates from human H5N6 cases in China: PB2:84.2 ~ 98.9%, PB1: 88.8 ~ 99.1%, PA: 88.0 ~ 99.0%, HA: 95.4 ~ 99.1%, NP: 92.8 ~ 99.3%, NA: 89.6 ~ 99.8%, M: 90.0 ~ 99.7%, and NS: 84.2 ~ 98.7%. In particular, A/Changsha/1/2014(H5N6) was closely related (98.5 ~ 99.7% identity) to A/Guang-zhou/39,715/2014 (Table 3), which was isolated from a 59-year-old male who developed symptoms on Dec. 4, 2014 in Guangzhou, Guang-dong province in southern China (Yang et al., 2015). Guangdong province is located to the south of Hunan province. It is also clear that A/Changsha/1/2014(H5N6) and the other three isolates from human H5N6 cases belong to the Eurasian lineage of avian influenza viruses, and their HA genes belong to clade 2.3.4.4 H5 viruses reported recently (Fig. 1) (Smith et al., 2015). A/Changsha/1/2014 (H5N6) was clustered with A/Guangzhou/39715/2014(H5N6) and the avian strains from Laos and Vietnam in the phylogenetic trees for both HA (Fig. 1A) and NA (Fig. 1B), suggesting these viruses likely had a common source.

To identify key mutation sites, the amino acid sequence of A/Chang-sha/1/2014 (H5N6) was compared to those of the H5N6, H5N1, H7N9, H10N8 and H9N2 viruses reported in the influenza virus database. There are six basic amino acids (R, K) at the protein cleavage site (amino acids 338–346) of the A/Changsha/1/2014 (H5N6) HA protein, similar to a human H5N6 isolate (A/Guangzhou/39715/2014),a duck H5N6 isolate (A/duck/Dongguan/3069/2013) and a human H5N1 isolate (A/Hubei/1/2010) but different from those in human H7N9, H10N8 and H9N2 isolates which are known to be low pathogenic in poultry (Table 4), indicating that A/Changsha/1/2014 (H5N6) might be highly pathogenic in poultry (Senne et al., 1996). Substitutions Q226L and G228S at the 210-loop in the HA protein that may facilitate human infection (Srinivasan et al., 2013) were not found in A/Chang-sha/1/2014 (H5N6), A/Guangzhou/39,715/2014 (H5N6), A/duck/Dongguan/3069/2013(H5N6), or A/Hubei/1/2010(H5N1) viruses (Table 4). The resistance-conferring mutation, R294K in NA, did not occur in A/Changsha/1/2014(H5N6), indicating possible sensitivity of this virus to neuraminidase inhibitors (McKimm-Breschkin et al., 1998). However, the substitution S31N was noted in the M2 gene, indicating possible drug resistance of this virus to adamantanes (Table 4) (Hay et al., 1985; Pinto et al., 1992). A deletion of A/Changsha/1/2014(H5N6) virus was observed in the stalk region of NA residue 69 to 73 (Table 4), which was also

found in A/Guangzhou/39715/2014 (H5N6), A/duck/Dongguan/3069/2013(H5N6), A/Hubei/1/2010(H5N1) and A/Changsha/2/2013(H7N9) but not in A/Jiangxi/IPB13/2013(H10N8) and A/Lengshuitan/11197/2013(H9N2) (Table 4). Other mutations in the A/Changsha/1/2014(H5N6) were also observed, such as N30D and T215A in M1, L89V in PB2, and P42S in the NS1 (Table 4). E627K, another mutation in PB2 facilitating adaption to mammalian hosts (Hatta et al., 2001), appeared in two human H5N6 isolates (A/Guangzhou/39715/2014, A/Yunnan/0127/2015) and a human H10N8 isolate A/Jiangxi/IPB13/2013, but was absent in A/Changsha/1/2014 (H5N6), a duck H5N6 isolate (A/duck/Dongguan/3069/2013) and other virus in Table 4.

H5 and N2 genes were detected in the poultry drinking-water sample from the live poultry trading site which the patient had visited, and the result was confirmed by sequencing. This H5 gene belongs to the Eurasian lineage, but is not closely related to the one isolated from the patient (Fig. 1A).

4. Discussion

A novel avian influenza A H5N6 virus [A/Changsha/1/2014(H5N6)] was isolated in China from a patient with fever and mild respiratory symptoms who recovered after a few days, implying the presence of mild cases or even asymptomatic carriers of avian influenza H5N6 in human. Three human infections with avian influenza A H5N6 virus in China have been previously reported — one in Sichuan province on May 7, 2014, one in Guangdong province on Dec 23, 2014, and one in Yunnan province on February 9, 2015, all presenting severe pneumonia (Pan et al., 2016; Yang et al., 2015; World Health Organization, 2015). However, the human case infected with influenza A/Changsha/1/2014(H5N6) virus reported in this study is the earliest among all known cases.

Our phylogenetic analysis showed that the A/Changsha/1/2014(H5N6) and A/Guangzhou/39715/2014 isolates are both novel triple reassortant H5N6 virus, and they likely shared a common source. A/Sichuan/26221/2014(H5N6) is also a triple reassortant virus but with somewhat different sources of genes as compared to A/Changsha/1/2014(H5N6): HA gene belonging to Clade 2.3.4.4 H5, internal genes belonging to Clade 2.3.2.1 H5, and NA gene closely related to H6N6 avian virus (Pan et al., 2016). A/Yunnan/0127/2015(H5N6) (GenBank: KT245143–50) is a complex reassortant that might have acquired its HA gene from A/chicken/Tonghai/802/2014(H5N1)-like viruses, NA gene from A/chicken/Jiangxi/12782/2014(H10N6)-like viruses, and the six internal genes from viruses similar to the H9N2, H7N9 and H10N8 viruses of poultry origin, China. These results indicated the diversity in the sources of the gene segments of the reassortant H5N6 virus in China during 2014–2015.

All eight viral gene segments of A/Changsha/1/2014 (H5N6) were of avian origin. Previous and our studies confirmed that the H5N6 virus can be transmitted from poultry to humans. Live poultry markets have been suggested to be an important source of human infection with avian influenza A H5N1, H7N9 and H10N8 viruses (Shi et al., 2013; Wan et al., 2011; Zhang et al., 2014). The field investigation in our study to establish the source of the infection did not reach a clear conclusion. The patient had visited a live poultry market

multiple times a few days before illness onset and could have acquired the infection during one of those visits, indicating an incubation period of 2–5 days, similar to other influenza viruses. We did not find avian influenza A H5N6 in the environmental samples collected form the live bird market that the patient had visited.

With amino acids at the protein cleavage site similar to other H5 viruses, the HA protein of human H5N6 isolate A/Changsha/1/2014(H5N6) virus might be highly pathogenic in poultry (Senne et al., 1996). Based on the amino acid sequence of A/Changsha/1/2014(H5N6), it is most probably sensitive to neuraminidase inhibitors, but resistant to adamantanes. In addition, A/Changsha/1/2014(H5N6) encoded a deletion in the stalk region of NA, and other mutations were observed, such as N30D and T215A in M1, L89V in PB2, and P42S in the NS1. These substitutions were associated with increased virulence in mice (Fan et al., 2009; Jiao et al., 2008). In this study, the patient infected with A/Changsha/1/2014(H5N6) virus did not present severe symptoms such as pneumonia. The young age of the patient might have played a role in the mildness of the symptoms, as the other three known H5N6-infected cases were all adults (Pan et al., 2016; Yang et al., 2015; World Health Organization, 2015). Another possible contributor to the mildness is the lack of mutation E627K in PB2 protein. This mutation was found to promote viral adaption to mammalian cells (Hatta et al., 2001) and was seen in two human H5N6 isolates (A/Guangzhou/39715/2014, A/Yunnan/0127/2015) and a human H10N8 isolate A/Jiangxi/IPB13/2013, all of which were associated with severe respiratory symptoms and fatality in human cases (Chen et al., 2014; Pan et al., 2016).

Some probable person-to-person transmission of avian influenza A(H5N1) (Wang et al., 2008) and A(H7N9) (Qi et al., 2013) viruses has been reported. In this study, medical observation and laboratory testing of close contacts of the patient showed no evidence of infection. No secondary infections were reported for the other three cases either. Mutations Q226L and G228S (in the H3 numbering system) of the HA protein of avian influenza H2, H3 and H7 subtypes were shown to be associated with increased affinity of HA to the glycan-receptors in the lower respiratory tracts of humans (Liu et al., 2013; Srinivasan et al., 2013; van Riel et al., 2006; Yang et al., 2010; Zhang et al., 2013), although these mutations not necessarily facilitate the binding of H5 (Zhang et al., 2013). We did not find these mutations in A/Changsha/1/2014 (H5N6) HA protein. These epidemiological and genetic observations suggest that avian influenza A(H5N6) virus has not yet gained transmissibility from person to person (Liu et al., 2013; Srinivasan et al., 2013; van Riel et al., 2006; Yang et al., 2010; Zhang et al., 2013).

Two surveillance methods are employed by the China influenza surveillance system — viral culture and nucleic acid screening. Viral culture used to be the only method for detecting influenza viruses. However, from January to March, 2014, the nucleic acid screening was added for screening all the samples collected from the routine surveillance, in response to the epidemic of human infections with avian influenza A H7N9 virus in China. The H5N6 case we reported here was first detected through the China influenza surveillance system using the nucleic acid screening, suggesting that this method is sensitive and efficient in discovering novel influenza viruses.

Our study is limited in the following aspects. First, we were not able to isolate the virus from the patient's throat swab specimens for further research. Second, this or similar H5N6 viruses were not found in the live poultry market to which the patient was exposed, and therefore the source of this human infection cannot be pinpointed.

Despite the lack of efficient poultry-to-human and human-to-human transmission of influenza A(H5N6) viruses at current stage, the recent outbreaks of this virus in poultry in Asia (Wong et al., 2015; Wu et al., 2015) and the four human cases in a one-year period (Pan et al., 2016; Yang et al., 2015; World Health Organization, 2015) warrant close monitoring of the viral evolution and timely detection of new human cases. The possibility of reassortment of H5N6 with other avian influenza strains, e.g., H7N9 and H10N8, into a new highly pathogenic strain of pandemic potential should not be ruled out. In addition to strengthening surveillance, we recommend resources be allocated to initiate development of vaccines targeting avian influenza A(H5N6) for both poultry and human.

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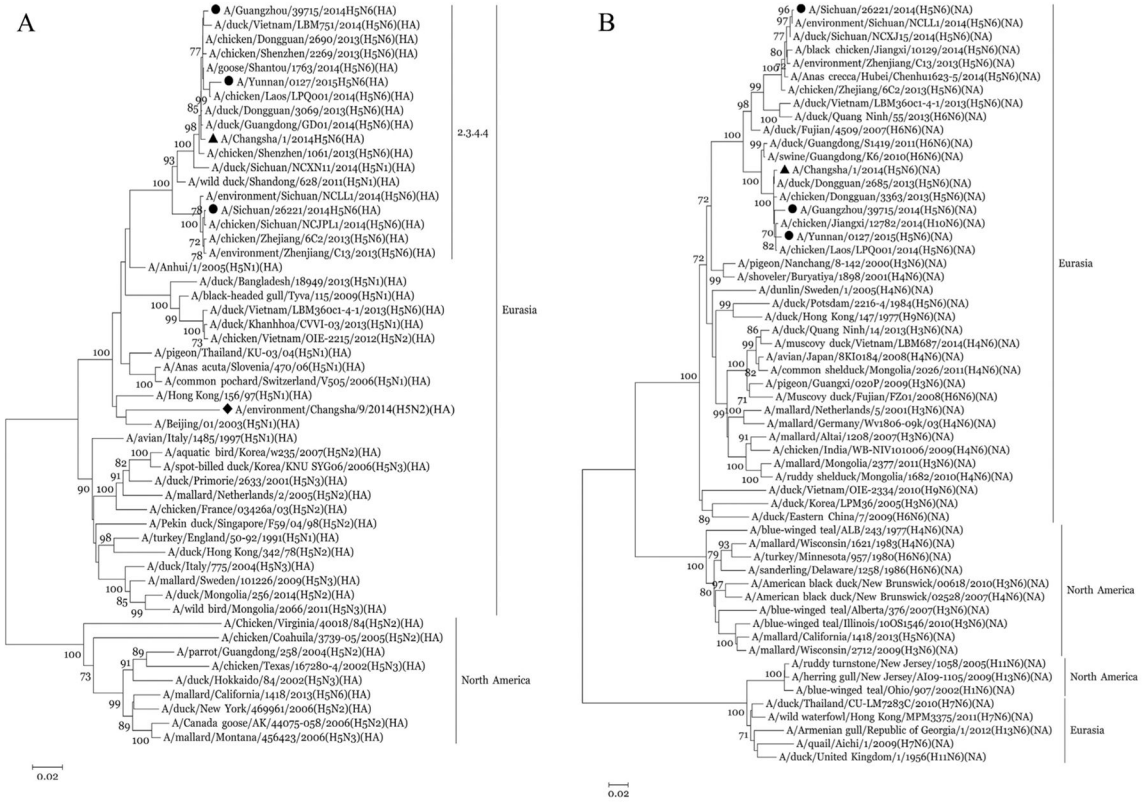

Fig. 1.

Phylogenetic analysis of the HA (panel A) and NA(panel B) genes of the novel influenza A(H5N6) virus isolated from a patient in Changsha, China in 2014. The sequence of the H5N6 virus in our study is marked by ▲, the sequence of the H5N2 virus in our study is marked by ◆, and the sequences of H5N6 viruses isolated from the other three H5N6 cases in China are marked by ●.

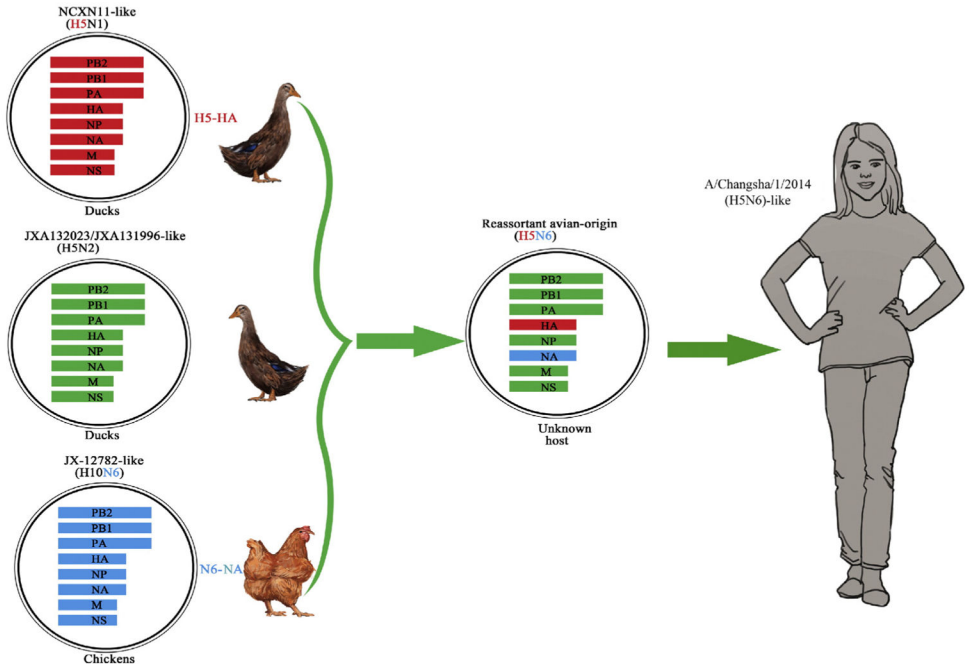

Fig. 2.

Possible source of the genes of the avian influenza A(H5N6) virus isolated from a patient in Changsha, China in 2014.

Table 1

Clinical characteristics, treatment, and outcome of the patient infected with the avian influenza A(H5N6) virus in Changsha, China in 2014.

Signs and treatment	Starting day	Stopping day	Details
Fever	1	2	38.0–38.9 °C
Sore throat	1	3	
Chills	3	3	
Other complications			None
Bacterial co-infection			Probable (white blood cell count was twice the upper bound of normal range)
Antibiotic treatment	2	4	Amoxicillin and Clavulanate Potassium (two 25 mg/kg doses given orally)
Febrifuge treatment	2	4	Ibuprofen suspension (four 5 mg/kg doses given orally)
Traditional Chinese medicine	1	11	Kangbingdu oral liquid, Pudilan anti-inflammatory oral liquid, Andrographolide
Antiviral treatment	12	18	Tamiflu (75 mg twice a day)

Table 2

Nucleotide identity of A/Changsha/1/2014 (H5N6) virus genes. The date of this BLAST search was Apr 10, 2015.

Viral gene[a]	Closest influenza virus relative	Nucleotide identity (%)
PB2	A/duck/Jiangxi/JXA132023/2013(H5N2)	99.6
PB1	A/duck/Jiangxi/JXA132023/2013(H5N2)	99.3
PA	A/duck/Jiangxi/JXA131996/2013(H5N2)	99.5
HA	A/duck/Sichuan/NCXN11/2014(H5N1)	98.0
NP	A/duck/Jiangxi/JXA132023/2013(H5N2)	99.7
NA	A/chicken/Jiangxi/12782/2014(H10N6)	99.2
M	A/duck/Jiangxi/JXA131996/2013(H5N2)	99.7
NS	A/duck/Jiangxi/JXA131996/2013(H5N2)	99.0

[a]PB2: RNA polymerase basic subunit 2; PB1: RNA polymerase basic subunit 1; PA: RNA polymerase acidic subunit; HA: haemagglutinin; NP: nucleoprotein; NA: neuraminidase; M: matrix gene; NS: non-structural gene.

Table 3

Nucleotide identity levels between the human isolate A/Changsha/1/2014 (H5N6) and the other three human isolates of influenza A(H5N6) viruses in China during 2014–2015.

Strain	Nucleotide identity, %							
	PB2	PB1	PA	HA	NP	NA	MP	NS
A/Sichuan/26221/2014(H5N6)	97.9	97.8	98.4	95.5	98.2	89.6	98.3	98.3
A/Guangzhou/39715/2014(H5N6)	98.9	99.1	99.0	99.1	99.3	98.5	99.7	98.7
A/Yunnan/0127/2014(H5N6)	84.2	88.8	88.0	95.4	92.8	98.8	90.0	84.2

Table 4

Molecular analysis of important amino acids in the HA, NA, PB2, PB1, PB1-F2, NS1, M1 and M2 proteins of 7 avian influenza virus strain.

Viral protein	Sites	Amino acid position	Virus 1[c]	Virus 2[d]	Virus 3[e]	Virus 4[f]	Virus 5[g]	Virus 6[h]	Virus 7[i]	Function
HA	Cleavage site	338–346	RERRRKRGL	RERRRKRGL	RERRRKRGL	RERRRKRGL	-EIPK GRGL	-ELIQGRGL	–SRSSRGL	Indicating pathogenic effects in poultry (Senne et al., 1996)
	Q226L	226/238[a]	Q	Q	Q	Q	**L**	Q	**L**	Q226L,G228S increases binding affinity for a-2,6-linked sialic acid receptor (Srinivasan et al., 2013)
	G228S	228/240[a]	G	G	G	G	G	G	G	
NA	R294K	294/290[b]	R	R	R	R	R	R	R	R294K reduces susceptibility to oseltamivir and zanamivir (McKimm-Breschkin et al., 1998)
		69–73	Deletion	Deletion	Deletion	Deletion	Deletion	No	No	Deletion of amino acids 69–73: increased virulence in mice (McKimm-Breschkin et al., 1998)
PB2	L89V	89	**V**	**V**	**V**	**V**	**V**	**V**	**V**	Enhanced polymerase activity and increased virulence in mice (Gao et al., 2013)
	E627K	627	E	**K**	E	E	E	**K**	E	Mammalian host adaptation (Hatta et al., 2001)
	D701N	701	D	D	D	D	D	D	D	Enhanced transmission in guinea pigs (Chen et al., 2014)
PB1	H99Y	99	H	H	H	H	H	H	H	H5 virus transmissible among ferrets (Gao et al., 2013)
	I368V	368	I	I	I	I	**V**	**V**	**V**	

Viral protein	Sites	Amino acid position	Virus 1[c]	Virus 2[d]	Virus 3[e]	Virus 4[f]	Virus 5[g]	Virus 6[h]	Virus 7[i]	Function
PB1-F2	Full length		Nonfunctional PB1-F2 protein due to mutation	Nonfunctional PB1-F2 protein due to mutation	Nonfunctional PB1-F2 protein due to mutation	90 aa	52 aa	52 aa	90 aa	Increased virulence in mice (Gao et al., 2013)
NS1	P42S	42	S	S	S	S	*S*	S	S	Increased virulence in mice (Jiao et al., 2008)
		218–230	No	No	No	No	Deletion	Deletion	Deletion	Lack of PDZ domain binding motif:decreased virulence in mice (Jackson et al., 2008)
M1	N30D	30	**D**	**D**	**D**	**D**	**D**	**D**	**D**	Increased virulence in mice (Fan et al., 2009)
	T215A	215	**A**	**A**	**A**	**A**	**A**	**A**	**A**	
M2	S31N	31	**N**	S	S	S	**N**	**N**	**N**	Reduced susceptibility to amantadine and rimantadine (Hay et al., 1985; Pinto et al., 1992)

Substitutions of particular concern are shown in bold.

[a] Amino acid sites of H3/H5N6.

[b] Amino acid sites of N9/N6.

[c] A/Changsha/1/2014(H5N6).

[d] A/Guangzhou/39715/2014(H5N6).

[e] A/duck/Dongguan/3069/2013(H5N6).

[f] A/Hubei/1/2010(H5N1).

[g] A/Changsha/2/2013(H7N9).

[h] A/Jiangxi/IPB13/2013(H10N8).

[i] A/Lengshuitan/11197/2013(H9N2).