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RESEARCH ARTICLE

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Emergence of a young case infected with avian influenza A (H5N6) in Anhui Province, East China during the COVID-19 pandemic

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Abstract

In the context of the coronavirus disease 2019 pandemic, we investigated the epidemiological and clinical characteristics of a young patient infected by avian influenza A (H5N6) virus in Anhui Province, East China, and analyzed genomic features of the pathogen in 2020. Through the cross-sectional investigation of external environment monitoring (December 29–31, 2020), 1909 samples were collected from Fuyang City. It was found that the positive rate of H5N6 was higher than other areas obviously in Tianma poultry market, where the case appeared. In addition, dual coinfections were detected with a 0.057% polymerase chain reaction positive rate the surveillance years. The virus was the clade 2.3.4.4, which was most likely formed by genetic reassortment between H5N6 and H9N2 viruses. This study found that the evolution rates of the hemagglutinin and neuraminidase genes of the virus were higher than those of common seasonal influenza viruses. The virus was still highly pathogenic to poultry and had a preference for avian receptor binding.

KEYWORDS

COVID-19, epidemiology, genetic, H5N6

Jun-Ling Yu, Sai Hou, Ya-Ting Feng and Ge Bu contributed equally to this study and should be considered as co-first authors.

1 | INTRODUCTION

Various avian influenza virus (AIV) subtypes naturally have caused zoonotic infections, but the subtypes H5N1 and H7N9 have caused a prominent impact. Based on specific molecular features and pathogenicity in birds, AIV could be classified as highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). Although HPAI viruses have an important impact on agriculture and the economy, infection with both LPAI and HPAI virus in humans typically caused a wide range of mild to fatal cases. H5N1 HPAI virus was first identified in 1996 in Guangdong province of China and subsequently spread to several continents around the world.[1] The global dissemination of H5 subtype viruses has facilitated the emergence of hemagglutinin (HA) gene variants, including 10 distinct clades (0–9), while some of these clades further evolved into subclades.[2]

Recently emerged H5Nx belongs to the clade 2.3.4.4, reassorts with different neuraminidase (NA) genes, including N2, N3, N6, and N8. In spite of the highly pathogenic to gallinaceous poultry, these H5 viruses were not uniformly pathogenic to domestic or wild ducks of different species.[3] The genetic dynamics and global transmission of H5N6 HPAI viruses pose grave concerns for public health. As of July 2020, the HPAI H5N6 viruses have caused a total of 24 laboratory-confirmed human infections in China since 2014, including seven deaths, for a CFR of ~30%.[4] The human cases of H5N6 infection were generally in older age groups, while only a few cases of infection were reported in young children.[5] Since a 65-year-old female case of H5N6 infection was reported in Anhui Province in 2016, there have been no new cases in that area.[6]

At present, the outbreak of novel coronavirus pneumonia (coronavirus disease 2019 [COVID-19]) has caused a worldwide pandemic. As of March 2021, a total of about 106 million human infections with COVID-19 had been reported globally, including about 2.6 million deaths (COVID-19 Data Repository by the Center for Systems Science and Engineering at Johns Hopkins University, available online: https://github.com/CSSEGISandData/COVID-19). To our knowledge, this is the youngest child infected with H5N6 subtype avian influenza in Anhui Province. Herein, we analyzed the epidemiology of the case and the characteristics of the pathogen genome, to find out the possible evolution of the virus.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and epidemiological data collection

A throat swab was obtained from patients at the onset of fever at Fuyang Peoples' Hospital in Anhui Province. The sample was collected by clinicians at the Fuyang Centre for Disease Control and Prevention (CDC; National Influenza Surveillance Network Laboratory). Meanwhile, the sample was transferred to the Anhui Province Influenza Reference Center according to the biological safety requirements for cold chain transportation. The samples were tested as influenza A (for M gene) and H5 positive with real-time reverse transcription–polymerase chain reaction (RT-PCR).

A standardized surveillance reporting form was used to collect epidemiological and clinical data, including demographical characteristics, diagnosis process, exposure history of live poultry, recent visits to live poultry markets, clinical laboratory testing results, and medication treatment. Environmental samples were collected in live poultry markets epidemiologically linked to the case. The survey of expanded sampling covered the entire Fuyang City, which finished in three days. "Close contacts" were defined as individuals who had provided care to, had been living with, or had potentially been directly exposed to respiratory secretions or bodily fluids of the patient. All close contacts were followed up for 14 consecutive days and throat samples were collected from them.

2.2 | RNA extraction and RT-PCR for subtyping

The viral RNAs were extracted using the nucleic acid purification kit (Cat. No: T014; Tianlong Technology Co., Ltd.) according to the manufacturer's instructions and were subsequently subjected to the detection by specific real-time RT-PCR with specific primer and probes. Positive samples were stored at −80°C for H5N6 virus isolation and sequencing.

2.3 | Deep sequencing and data analysis

The whole genomes were amplified and captured using MicroFuture ULSEN Kit (MicroFuture) and the amplification products were quantified by the Qubit 4.0. Genome-wide library construction was conducted by Ion XpressTM Plus Fragment Library method (4471269; Thermo Fisher Scientific Inc). Library preparation was conducted by Ion One TouchTM 2 system (4474779; Thermo Fisher Scientific Inc). The sequencing analysis was performed on the Ion S5TM next-generation sequencing system (A27212; Thermo Fisher Scientific Inc) using the 530 chip. The raw data were trimmed and de novo assembled under CLC Genomics Workbench 7.0.4, which was conducted by the bioinformatics team of BioGerm Medical Technology Co., Ltd.

A timetree was deduced by applying the RelTime for Dated Tips method[7,8] to the user-supplied phylogenetic tree whose branch lengths were calculated using the maximum likelihood (ML) method and the Hasegawa–Kishino–Yano substitution model. The timetree was computed using sampling tip dates for 23 taxa that were used as calibration constraints. The estimated log-likelihood value of the tree was −5142.10. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories [+G, parameter = 0.5040]). This analysis involved 23 nucleotide sequences. There were a total of 1381 positions in the final data set. Evolutionary analyses were conducted in MEGA X.[9]

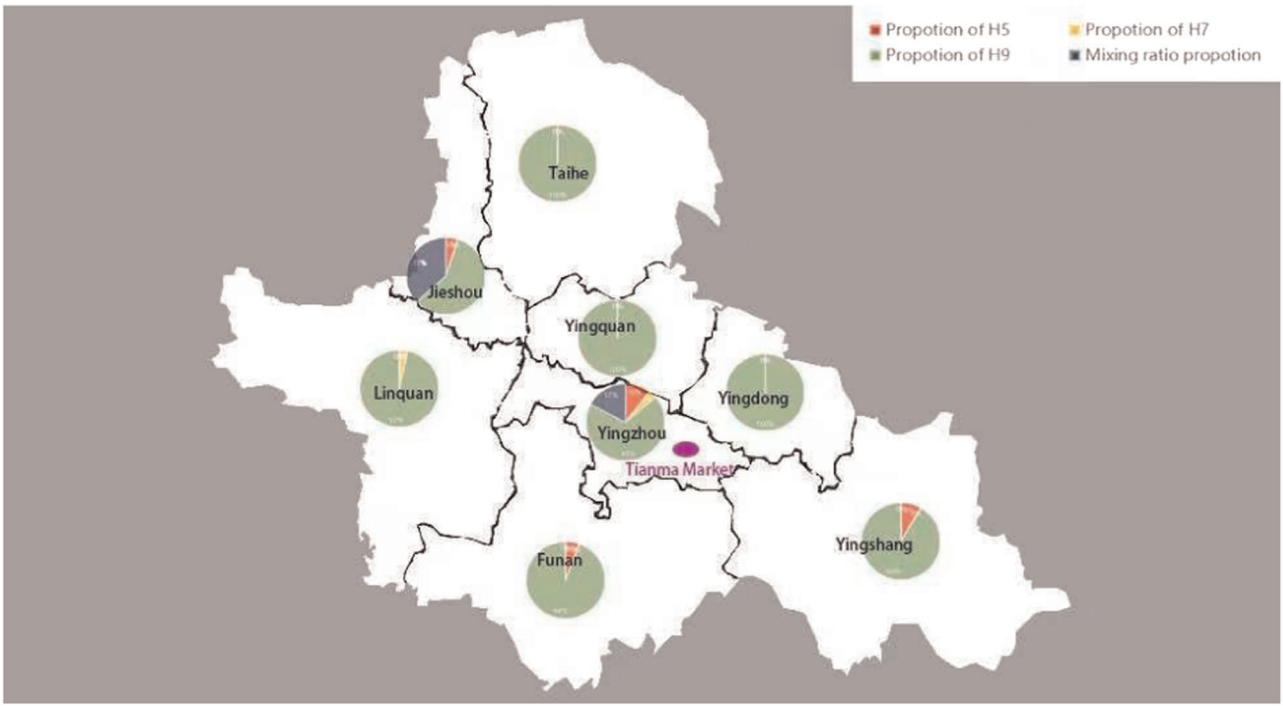

FIGURE 1 Geography of a positive rate of avian influenza

3 | RESULTS

3.1 | Case report

The patient was a 2-year-old female child from the urban area of Fuyang City, Anhui Province, East China. The patient developed a fever at about 1:00 a.m. on December 22, 2020, with a body temperature of 39.0°C and slight nasal obstruction, no cough, expectoration, or other symptoms. The patient took 4 ml ibuprofen suspension at home by herself while the fever persisted. Medical examination revealed that the patient had a clear mind, good spirit, pharyngeal congestion, body temperature of 39.5°C, and no other abnormalities. Throat swabs were collected for routine influenza surveillance and oseltamivir was prescribed.

At around 14:00 p.m., the patient's mother took her to the community health service station in Fuyang again, where she was tested for white blood cells 8.2×10^9/L, neutrophil 6.72×10^9/L, lymphocyte 0.74×10^9/L, and CRP 5.3 mg/L. The patient returned home at around 16:00 p.m. and took ibuprofen suspension, cefixime, anti-inflammatory granules, and oseltamivir at home. At about 20:00 p.m., the fever subsided and did not recur. On December 28, a retrospective epidemiological investigation was conducted by Fuyang CDC, by then the child had recovered.

On December 28, by means of real-time RT-PCR and sequencing, the throat swabs were confirmed to be positive for influenza A (H5N6) virus but negative for seasonal influenza viruses (H1, H3, or B) or AIVs (H5N1, H9N2, H7N9, H7N4, H6N1, or H10N8). The virus obtained from the patient was designated as A/Anhui/38/2020 (H5N6) (AH38) and the full genome sequences of AH38 were deposited in the Global Initiative on Sharing Avian Influenza Data database (Accession No. EPI_ISL_1273104, EPI1850652-EPI1850659).

3.2 | Epidemiological investigation

On December 20, the patient's grandmother bought live poultry in Tianma Farmers Market of Fuyang City, slaughtered it on site and took it home. The live poultry stalls of the Tianma Farmers Market were located about 100 m away from the patient's home. The environment around the live poultry stalls was poor. A total of five close contacts of the patient were identified, including four family members (parents, grandparents) and one neighbor, whose test results were negative. These close contacts were placed under medical observation for 14 days.

Environmental samples were collected in epidemiologically linked live poultry markets by Fuyang CDC. The survey covered the entire Fuyang City and finished in 3 days. A total of 1909 samples were collected from live poultry markets, poultry farms, free-range areas, slaughterhouses, and processing plants in Fuyang City, including throat swabs of the exposed people and live poultry; meanwhile, the environmental specimens from live poultry markets (including cages, cutting boards, knives, sewage, etc.) were also collected. A total of 311 samples were positive with a positive rate of 16.29%, 52 samples were positive for H5 with a positive rate of 2.72%, 5 samples were positive for H7 with a positive rate of 0.26%, and 287 samples were positive for H9 with a positive rate of 15.03%.

Among 360 samples from Yingzhou District, 116 samples were avian influenza-positive, with a positive rate of 32.22%. Among them, 12 samples were H5 positive, accounting for 10.34%, 4 samples were H7 positive, accounting for 3.45%, 80 samples were H9 positive, accounting for 68.97%, and 20 samples were mixed type, accounting for 17.24%. It is obvious that the infection rate of avian influenza in the Yingzhou district is significantly higher than that in other areas, with the Tianma market being the highest (Figure 1).

3.3 | Phylogenetic analysis

To search homologous sequences in the nucleotide database of the National Center for Biotechnology Information for HA and NA, we concurrently ranked them by similarity from top to bottom. A sequence was selected every 20 sequences to construct the phylogenetic tree. The HA gene of the H5N6 virus in this study was probably derived from H5N1 and homologous with H5N2 and H5N8. The AH38 virus showed a high similarity to the virus A/chicken/Vietnam/HU9-842/2018(H5N6). The AH38 virus was clustered within a clade that was hosted by chickens, but some of them were mink, suggesting that chickens probably acted as the intermediate hosts leading to the infection in humans. The A/Anhui/33163/2016(H5N6) (AH33163) virus has the same host range as the AH38, so both of the two strains might have undergone a transmission pattern from the chicken to humans (Figure 2).

The NA gene of the AH38 H5N6 virus was closely related to H6N6 and its ancestors probably came from H3N6 and H4N6 AIVs. For the NA genes, both the AH38 and the A/goose/China/GS42-6.seq/2016 (H5N6) (EPI_ISL_372817) viruses were in the same clade, suggesting that their hosts may be from the waterfowl. Similarly, AH33163 was clustered in the same clade with A/chicken/Anhui/MZ33/2016(EPI_ISL_297930), suggesting that its host might be from chickens (Figure 3).

Subsequently, to better understand the evolution and emergence of the AH38, we predicted the time evolution of the HA and NA gene of the H5N6 virus. For HA gene, A/chicken/Vietnam/HU9-842/2018(H5N6) (EPI_ISL_389657)-like H5N6 virus from Vietnam was the probable predecessor of AH38, and the estimated divergence time was around 2017. Nevertheless, the estimated divergence time of the AH33163 might be 2014, its probable predecessor was A/wild/bird/Jiangxi/P560/2015(H5N6) (KX960164.1). Compared to the root of the ML tree of the HA gene, rooted using A/duck/Vietnam/LBM360c1-4-1/2013 (H5N6) (EPI_ISL_173146), the earliest divergence time was between 2010 and 2011. For NA gene, AH38 was clustered together with A/goose/China/GS42-6. Seq/2016 (H5N6) (EPI_ISL_372817), they had an estimated common ancestor that circulated between 2015 and 2016. However, the AH33163 was clustered with A/wild bird/Jiangxi/P5/2015(H5N6) (KX960177.1), the common ancestors of the NA gene segment were estimated to have circulated between 2013 and 2014. The earliest divergence time of the NA gene segment was between 2009 and 2010. In addition, we estimated that evolutionary rates of the HA

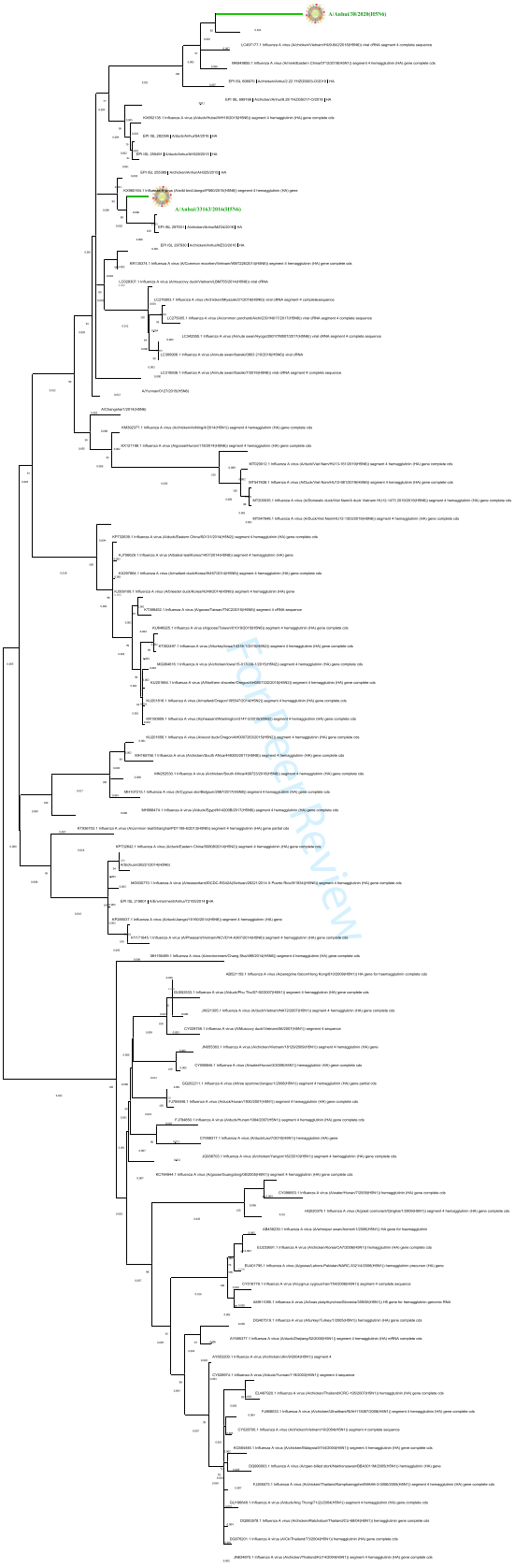

FIGURE 2 Phylogenetic analysis of hemagglutinin (HA) genes of H5N6 viruses isolated from the patient in Anhui Province, which are highlighted in green

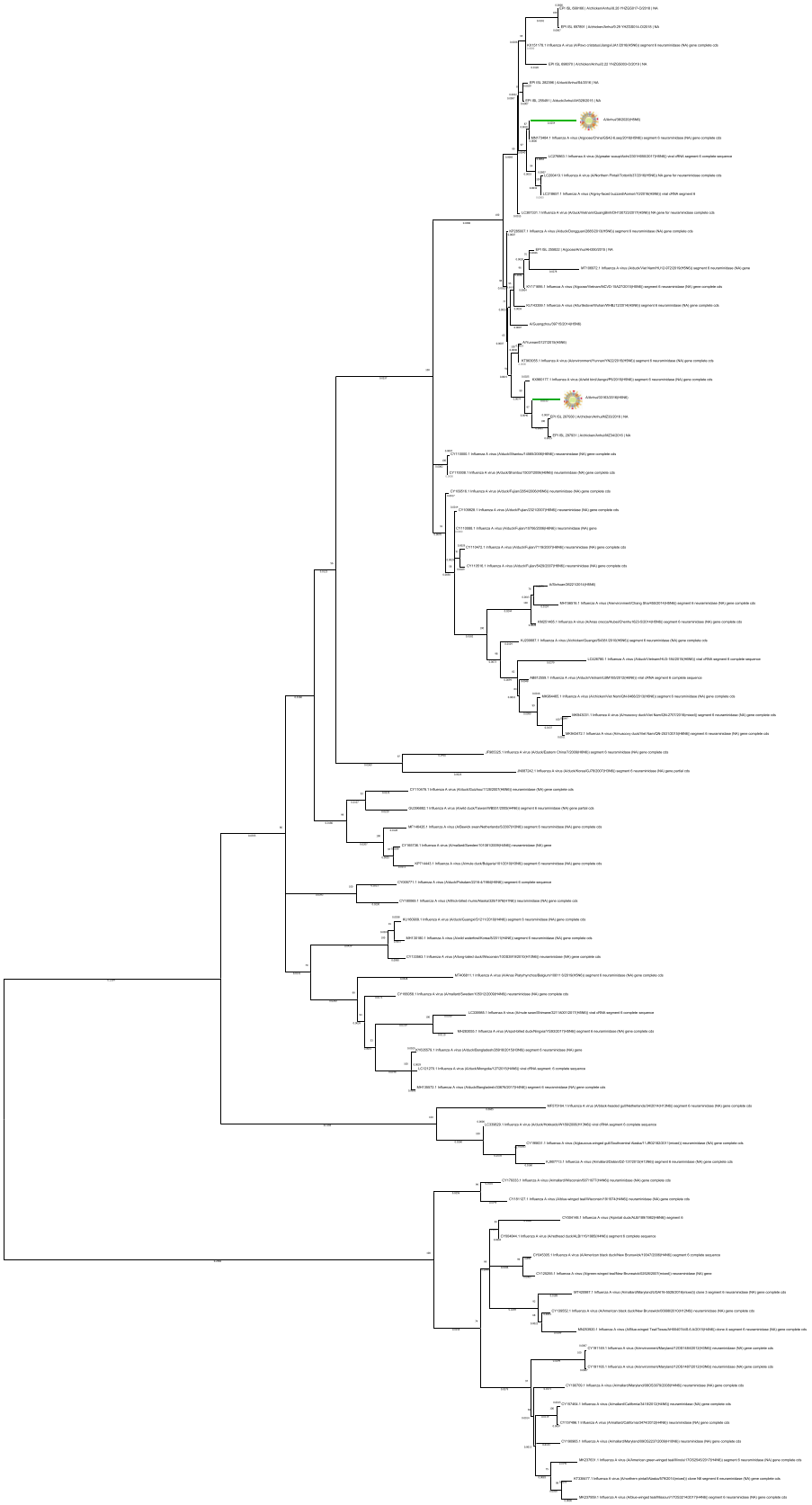

FIGURE 3 Phylogenetic analysis of neuraminidase (NA) genes of H5N6 viruses isolated from the patient in Anhui Province, which are highlighted in green

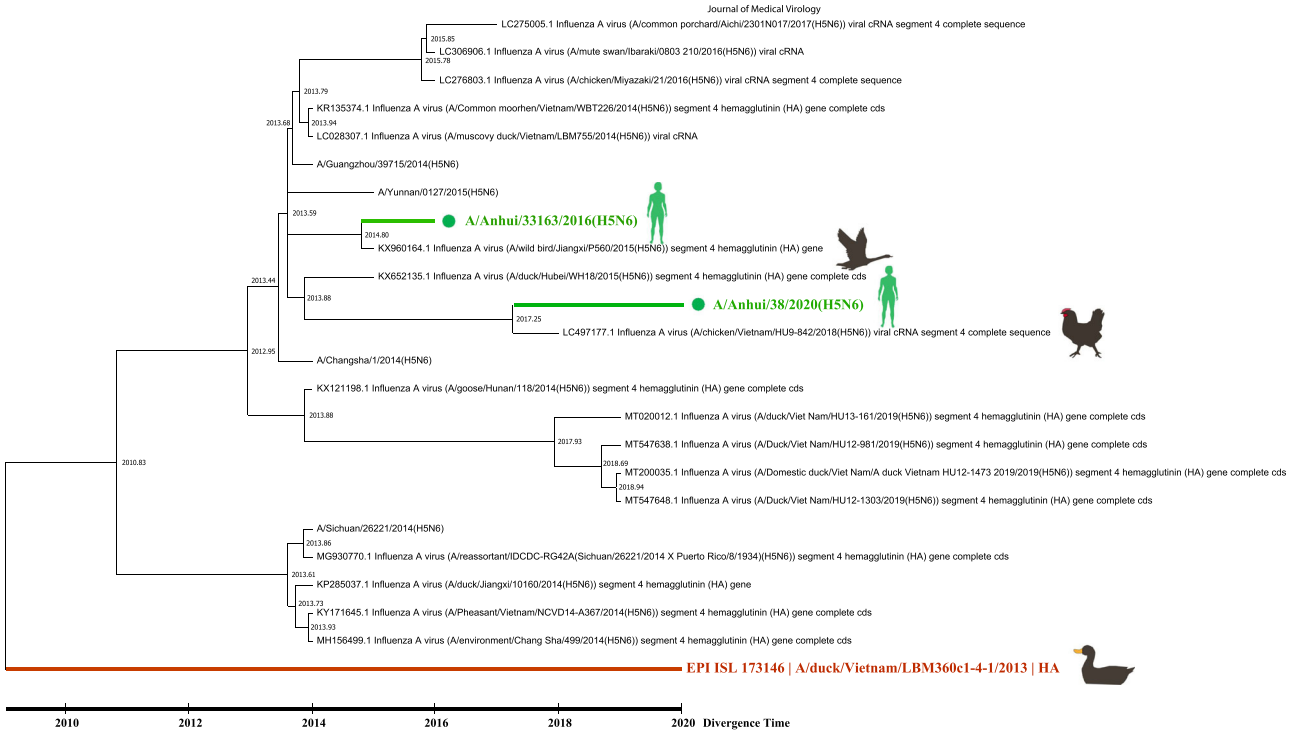

FIGURE 4 Phylogeny of the hemagglutinin (HA) genes was inferred using the maximum likelihood method with 1000 bootstrap replicates. The virus A/duck/Vietnam/LBM360c1-4-1/2013 was defined as the root of the tree, which was used to calculate the divergence time. The root of the tree is shown in red and the H5N6 viruses isolated from the patient in Anhui Province are highlighted in green

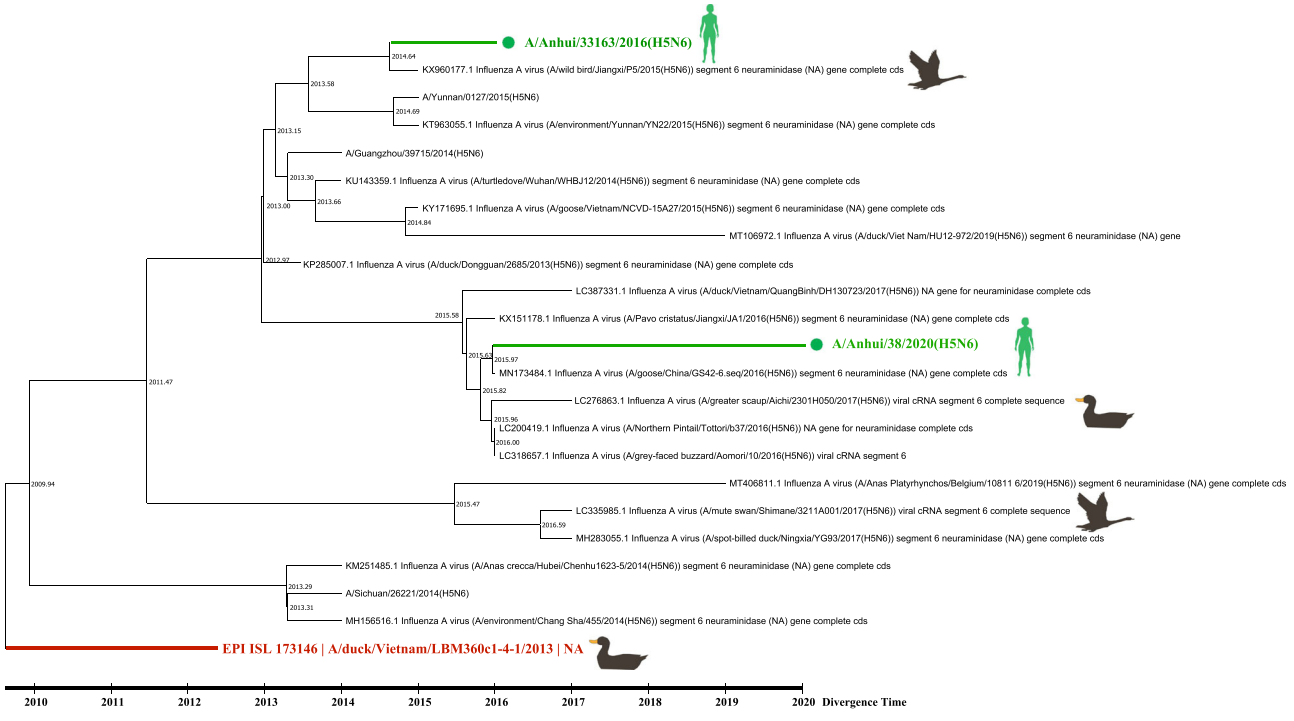

FIGURE 5 Phylogeny of the neuraminidase (NA) genes was inferred using the maximum likelihood method with 1000 bootstrap replicates. The virus A/duck/Vietnam/LBM360c1-4-1/2013 was defined as the root of the tree, which was used to calculate the divergence time. The root of the tree is shown in red and the H5N6 viruses isolated from the patient in Anhui Province is highlighted in green

TABLE 1 Similarity analysis of H5N6 virus sequence from the youngest case in Anhui Province

Gene	Length (bp)	Strain with the highest similarity	GenBank ID	Similarity(%)
PB2	2369	A/chicken/Qing yuan/zd201601/2016(H9N2)	MK249994.1	98.804
PB1	2392	A/chicken/Vietnam/HU9-842/2018(H5N6)	LC497175.1	97.341
PA	2275	A/chicken/Vietnam/HU9-847/2018(H5N6)	LC497192.1	97.476
HA	1808	A/chicken/Vietnam/HU9-842/2018(H5N6)	LC497177.1	97.214
NP	1575	A/Muscovy duck/Japan/AQ-HE30-77C2/2018(H5N6)	LC550072.1	98.714
NA	1452	A/Muscovy duck/Japan/AQ-HE30-77C1/2018(H5N6)	LC550068.1	97.602
MP	1035	A/chicken/Shao guan/zd201603/2017(H9N2)	MK250370.1	99.221
NS	886	A/duck/Hubei/ZYSYF1/2015(H5N6)	KY415927.1	98.743

TABLE 2 Molecular features of the hemagglutinin gene in H5N6 viruses isolated from birds and human

	Receptor binding site								Glycosylation site						
Strain name	133	137	160	187	193	196	226–228	Cleavage site	21	33	158	169	289	483	541
AH38	S	A	A	S	D	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
AH33163	P	A	S	N	N	K	QSG	PLRERRRKKR↓GLF	NST	NVT	NDS	NNT	NSS	NGT	NGS
SC26221	L	A	A	N	N	K	QRG	PLREKRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
GZ39715	L	A	T	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	NDT	NNT	NSS	NGT	NGS
CS1	S	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
YN0127	S	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
Astrakhan3212	L	A	A	N	N	K	QRG	PLREKRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
AH1/2005	S	S	T	D	K	Q	QSG	PLRERRKR↓GLF	NST	NVT	NNT	NNT	NSS	NGT	NGS
AH1/2006	S	S	T	D	K	Q	QSG	PLRERRKR↓GLF	NST	NVT	NNT	NNT	NSS	NGT	NGS
AH1/2007	S	S	T	D	K	Q	QSG	PLRERRKR↓GLF	NST	NVT	NNT	NNT	NSS	NGT	NGS
Chicken/DG2690	L	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
Duck/GD01	L	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
Duck/LBM758	L	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
Chicken/LPQ001	L	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
Duck/LP002	L	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS
Feline/GD1	L	A	A	N	N	K	QRG	PLRERRKR↓GLF	NST	NVT	/	NNT	NSS	NGT	NGS

and NA gene of the AH38 virus were 6.2×10^{-3} to 8.7×10^{-3} substitutions/site/year for HA and 4.5×10^{-3} to 6.8×10^{-3} substitutions/site/year for NA, respectively, which were higher than that of seasonal influenza virus[10–12] (Figures 4 and 5).

3.4 | Genetic molecular characteristics

We sequenced eight genomic fragments of the AH38 virus and found that the virus possessed some mutations, which were related to viral replication, receptor-binding, and mammalian virulence-related markers. The HA and PB1 gene of AH38 virus showed high homology with that of A/chicken/Vietnam/HU9-842/2018(H5N6) virus, with 97.214% and 97.341 similarities, respectively. Additionally, the following genes showed high homology (>97% nucleotide identity) with the reference strains: The PB2 gene of AH38 with A/chicken/Qing yuan/zd201601/2016(H9N2), the PA with A/chicken/Vietnam/HU9-847/2018(H5N6), the NP with A/Muscovy duck/Japan/AQ-HE30-77C2/2018(H5N6), the NA with A/Muscovy duck/Japan/AQ-HE30-77C1/2018(H5N6), the M gene with A/chicken/Shao guan/zd201603/2017(H9N2), the NS gene with A/duck/Hubei/ZYSYF1/2015(H5N6) (Table 1).

The HA cleavage site of the AH38 virus possesses a multiple basic amino acids motif (PLRERRKR↓GLF), indicating its potentially high pathogenicity in chickens. The 226–228 site of the HA protein was a QRG motif, while the QSG site of the H5N6 (AH33163) virus

TABLE 3 Molecular features of the other genes of H5N6 viruses isolated from birds and human

Proteins	Phenotypic effect	Sites substitution or molecular feature	Origin from avian species	AH38	AH33163	SC26221	GZ39715	CS1	YN0127	References
NA	Antiviral oseltamivir resistance[a]	E119V	E	E	D	E	E	E	E	17
		I222V	I	I	I	I	I	I	I	18
		H274Y	H	H	H	H	H	H	H	19
		R292K	R	R	R	R	R	R	R	17
		N294S	N	N	N	N	N	N	N	20
PB2	Enhanced growth capacity in human and mammalian cells	K389R	R	R	K	R	R	R	K	21
		V598T/I	T	T	V	T	T	T	V	21
	Enhanced polymerase activity and increased virulence	E627K	E	E	K	E	K	E	K	22
PB1	Transmissible among ferrets	I368V	I	I	V	I	I	I	V	23
PA	Increased virus replicative ability in mammalian systems	N409S	S	S	N	S	S	S	N	24
M1	Increased virulence in mice	N30D	D	D	D	D	D	D	D	25
		T215A	A	A	A	A	A	A	A	
M2	Antiviral amantadine resistance	S31N	S	S	N	S	S	N	N	26
NS1	Virulence increase of H5N1 viruses in chickens and mice	P42S	S	S	S	S	S	S	S	27
		80–84 Deletion	Yes	Yes	No	Yes	Yes	Yes	No	28
		D92E	E	E	D	E	E	E	D	

[a]Refers to N2 numbering.

isolated in 2016 was a QSG, suggesting that these viruses were fowl-like receptors (α2,3-SA). The T160A mutations, which resulted in the absence of the glycosylation at site 158, were detected in the HA protein of AH38, indicating the increased human-like (α2,6-SA) receptor recognition of the virus[13] and making viral pathogenicity milder and favoring virus spread.[14] Q226L and G228S mutations in the HA gene are important amino acid mutations that lead to changes in the binding preference of the H5N1 AIV. No mutations were found in both H5N6 HPAV strains. However, mutations in S137A, T160A, and S227R have been found in our strain, and studies have shown that these mutations make H5N6 AIV easier to adapt to mammalian hosts.[15,16] In addition, the AH38 H5N6 virus had the characteristic NA stalk deletion, although no amino acid substitution associated with resistance to NA inhibitors was observed. The K389R, V598T mutations in PB2 protein and the N409S mutation in PA protein, which had been reported to enhance growth capacity in human and mammalian systems, were observed in the AH38 virus. The N30D and T215A mutations in the M1 protein of the AH38 virus were related to increased virulence in mice. Moreover, the P42S, D92E, and deletion were at positions of 80–84, which revealed the virulence increase of H5N1 viruses in chicken and mice (Tables 2 and 3).

4 | DISCUSSION

Throughout human history, the world has been increasingly prone to emerging respiratory infections. From 2010 to date, over 400 cases and more than 150 human deaths by an HPAI have been reported in Egypt, Ghana, Hong Kong, Indonesia, Nigeria, Bangladesh, Cambodia, Vietnam, and Canada (https://www.who.int/influenza/human_animal_interface/en/, https://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Zoonoses). There is evidence that the spread of different subtypes of avian influenza in domestic poultry is geographically continuous and may be related to the intensity of the live poultry trade in China. Long-distance transmission may be related to bird migration.[17,18,29] At the same time, the increased mobility of people in the context of globalization also makes it easier for respiratory infections to spread widely. As of October 2020, a total of 24 laboratory-confirmed cases of human infection with influenza A

(H5N6) virus, including seven deaths, for a CFR of 30% (https://iris.wpro.who.int/handle/10665.1/14460), pose grave concerns for public health.

The patient of this report developed fever and nasal congestion after being infected with avian influenza and recovered after medical treatment with ibuprofen, oseltamivir, and other drugs. Epidemiological investigations revealed that it may be related to his grandmother's purchase of live poultry at the Tianma market. Samples taken in Fuyang City showed that the positive rate of avian influenza in the Tianma market was significantly higher than in other regions of Fuyang City. And the positive rate of the Yingzhou district where the Tianma market is located was also higher than that of other districts in Fuyang City. In addition, the H5 positive rate in Jieshou was also high, but no human cases had been reported. It is probably for this very reason that the virus retained an avian virus-like character, which is why occasional human infections may be inevitable, but the risk of a pandemic is low. The results of HA and NA gene sequencing showed that the host of the H5N6 strain in Anhui Province might be chicken, which might be the mode of transmission from chicken to human. The time evolution of the H5N6 sequence showed that the average evolution rate of HA and NA was higher than that of influenza. This may be related to the mutation of some gene sites. At the same time, the virus possessed some mutations, which were related to viral replication, receptor-binding, and mammalian virulence-related markers. In particular, the absence of the glycosylation at site 158 was more resistant to heat and bound host cells. Meanwhile, this made viral pathogenicity milder and favored virus spread.[14] For this reason, the H5N6 case presented mild clinical symptoms, but the ability of heat resistance and virus spread may facilitate the stability of the virus in nature.

The current study has several limitations, as the patient was too young to be examined very carefully and the absence of some retrospective epidemiological investigations was unavoidable. Due to the COVID-19 pandemic, the availability of BSL-3 laboratory was very limited, thus obtaining a quantitative level of viral load or further characterization of the virus was difficult. Secondly, there is not enough direct evidence that the patient infected with avian influenza was due to the Tianma market live poultry market because the deep sequencing data of environmental samples were not available. In the following work, it was necessary to carry out serological surveillance among the occupational poultry-exposed workers in the area.

At the moment, the world is focused on controlling and mitigating the current COVID-19 epidemic. At the same time, the emergence of avian influenza is another major threat that deserves more attention and control. If there is an outbreak of both diseases in one place at the same time, the differential diagnosis of clinical patients, the prevention of infection, the management of patients, as well as the capacity of medical care, will pose a great challenge and pressure. Therefore, on the premise of preventing and controlling the novel coronavirus pneumonia, we should strengthen the management and reform of the live poultry market, reduce people's contact with poultry as far as possible, improve dietary hygiene, and protect them by vaccination of avian influenza vaccine. All in all, as the COVID-19 epidemic situation is still severe now, we should be very alert to the emergence of cases of avian influenza and strengthen the prevention and control of this disease.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

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