Heliyon

Received:
31 July 2018
Revised:
30 January 2019
Accepted:
14 February 2019

Cite as: Huang Zheng, Ou Xinhua, Zhang Rusheng, Yao Dong, Li Lingzhi, Liu Ruchun, Li Yelan, Chen Jingfang, Sun Biancheng. Evolved avian influenza virus (H7N9) isolated from human cases in a middle Yangtze River city in China, from February to April 2017.
Heliyon 5 (2019) e01253.
doi: 10.1016/j.heliyon.2019.e01253

Evolved avian influenza virus (H7N9) isolated from human cases in a middle Yangtze River city in China, from February to April 2017

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Abstract

Seven cases of avian influenza A H7N9 virus infection were reported from February to April 2017 in Changsha City. Viral genome was acquired by RT-PCR, aligned with other H7N9 viruses using Clustal W, and phylogenetic trees were constructed using the neighbor-joining method. Our results showed the representativeness of H7N9 virus infections in Middle Yangtze River City. The hemagglutinin segment contained Thr160Ala, Gly186Val and Gln226Leu substitutions, which are associated with increased binding affinity in humans. Phylogenetic analysis indicated that H7N9 viruses had an avian origin, and belonged to the Yangtze River Delta lineage. The proportion of PB2 Ala588Val substitutions in viruses revealed a significantly increasing in recent years, from 0.8 % (1 of 128 cases) to 84.9 % (275 of 324 cases). The data indicate that H7N9 viruses may be more capable of infecting mammals, even though they are still considered low pathogenic avian influenza virus. Hence, the prevalence and genetic evolution of this virus should be closely monitored to prevent more severe human pandemics.

https://doi.org/10.1016/j.heliyon.2019.e01253

Keywords: Infectious disease, Public health, Evolution, Genetics, Microbiology, Virology

1. Introduction

Avian influenza A viruses belong to the RNA virus family *Orthomyxoviridae*. The viruses have eight gene segments encoding hemagglutinin (HA), neuraminidase (NA), matrix protein (MP), nonstructural protein (NS), polymerase (PA), nucleocapsid protein (NP), polymerase basic protein 1 (PB1) and polymerase basic protein 2 (PB2). On the basis of two surface proteins, 18 HA subtypes and 11 NA subtypes had been found [1, 2]. Previous study indicated that certain subtypes of avian influenza viruses, including H5N1, H7N9 and H9N2, have co-circulated in live poultry markets (LPMs) in China for several decades [3, 4, 5, 6]. The infected chickens and ducks may provide an environment for reassortment between avian influenza viruses, as well as acting as a possible source of occasional transmission from avians to humans. The sporadic or occasional transmission of virus from poultry to humans in the LPM setting has been recognized [7, 8].

H7N9 viruses that can infect humans evolved into two categories, namely the low pathogenic avian influenza A (LPAI) viruses and highly pathogenic avian influenza A (HPAI) viruses [9, 10]. Most human LPAI infections can be cured [11, 12]. HPAI viruses infection usually cause high mortality in poultry. Otherwise, HPAI viruses naturally evolve from LPAI viruses when the HA gene acquires molecular changes that alter the tissue tropism of the virus in avians and poultry [13]. In January 2017, H7N9 HPAI human infection case was reported in China [10]. Phylogenetic tree analysis revealed that the HA gene of this virus belongs to the Yangtze River Delta (YRD) lineage and shows an insertion of three basic amino acid residues RKR at the cleavage site, which corresponds with an increased pathogenicity in humans [10, 14, 15]. Both viral types have been a persistent public health threat in mainland China. Moreover, previous research indicated that recently viruses of the YRD lineage reacted less well with post-infection ferret antiserum raised against the available A/Anhui/1/2013 and A/Shanghai/2/2013-derived candidate influenza vaccine viruses [16, 17]. These observations indicate that the viral gene segments are continuously evolving.

Since the first H7N9 virus was identified in 2013 in China, avian influenza viruses have continuously caused human infections [6, 18, 19]. Subsequently, H7N9 infections have been confirmed in mainland of China [20, 21, 22, 23]. Up to 30 April 2017, a total of 1393 laboratory-confirmed cases of human infections with H7N9 viruses in China have been reported to the World Health Organization (WHO), representing the fifth wave (since September 2016) of the epidemic [24]. In Changsha City, the first case of H7N9 virus infection in humans was reported in 2013 [25]. As of 13 April 2017, seven cases have been laboratory-confirmed in the fifth

https://doi.org/10.1016/j.heliyon.2019.e01253

wave of the H7N9 virus epidemic in the city. In this study, we analyzed the genetic characteristics of the H7N9 virus to understand its evolution among the influenza A (H7N9) viruses in this wave of infections.

2. Materials and methods

2.1. Patients and samples collection

Respiratory samples from suspected H7N9 patients were collected and detected by Changsha center Disease Control and Prevention (CSCDC), and tested for avain influenza virus subtypes, such as H5, H7 and H9, using Real-time PCR kits (Jiangsu Bioperfectus Technologies, China). All epidemiological and clinical information of laboratory-confirmed human infection cases with H7N9 virus is collected by CSCDC staff or clinical doctors from hospital.

Ethical approval was given by the Ethics Committee of the Changsha Center for Disease Control and Prevention with the following reference number: CSCDC-2018- 003.

2.2. RNA extraction, RT-PCR and sequencing

Viral RNA of H7N9 positive samples were extracted using QIAamp RNA mini kit (Qiagen). All segments of H7N9 viruses were amplified by RT-PCR using Super-Script™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kits. RT-PCR program was performed on GeneAmpR PCR System 9700(Thermo). Primers were obtained from Invitrogen life company, and desiged by colleague [25] in our laboratory. RT-PCR products were sequenced. And the sequences were obtained from Invitrogen life company.

2.3. Phylogenetic analysis

The whole genome sequences were edited and assembled using SeqMan (Lasergene) software. Subsequently, phylogenetic tree was constructed by Molecular Evolutionary Genetic Analysis (MEGA) version 6.0 using a neighbour-joining method with 1,000 bootstrap replicates. Sequences in this research were download from Genbank and GISAID database. The filter condition of human infection cases is collection date from January 2013 to December 2017 in China and that infected with LPAI virus.

3. Results

3.1. Epidemiological features

As of 30 April 2017, seven local human cases of H7N9 virus had been confirmed in Changsha City in the fifth wave of infections. The epidemiological and clinical

https://doi.org/10.1016/j.heliyon.2019.e01253

information of these cases are presented in Table 1. The median age of the patients was 52 years. None of the patients had an occupation that involved frequent direct exposure to poultry. All patients suffered from fever, headache, pneumonia, and other common clinical symptoms, including arthralgia, shortness of breath, cough, and sore throat. Five patients experienced severe pneumonia and acute respiratory distress syndrome (ARDS) and most were admitted to the intensive care unit. Case 6 had no history of LPM exposed because the patient was hospitalized with leukemia during the time of the infections. Case 3 and 5 had a history of direct exposure to poultry before onset of disease, especially case 5 have been exposure to dead poultry. The remainders were lived nearby LPM, and had no direct contact with birds or poultry but the family members had. The mortality in our human infection case is 14.29% (1 of 7).

3.2. Amino acid mutations

Sequences of the isolates were submitted to the GenBank database and the accession numbers were MF370243 to MF370262, MG572470 to MG572504, and MG645390 to MG645393.

To identify the key substitutions, the amino acid sequences of the H7N9 viruses in this study were compared to those of the sequences in GISAID database (Table 2). The Thr160Ala, Gly186Val, and Gln226Leu mutations in the HA segment, which are associated with the relative binding affinity between hemagglutinin and the sialic acid receptor on the host cell surface, and which prefer the α-2,6 sialic acid receptor, have been found in H7N9 strains in our research [26]. H7N9 viruses are incapable of transmitting between humans because of the absence of substitutions in the HA segment, including Asn158Asp, Asn224Lys, Gly228Ser, and Thr318Ile [27]. The HA cleavage site of the H7N9 viruses in the current study had an PKGRGLF motif, similar to the human isolates obtained in 2013, which are known to cause low pathogenicity in humans [9].

Importantly, the PB2 gene Ala588Val and both Ala588Val/Glu627Lys mutations in the H7N9 virus have been demonstrated to enhance virulence in mice and the transmission of influenza viruses in mammals [28, 29, 30]. We also found that the proportion of Ala588Val substitution in human infection cases was increasing from 2013 to 2017 years (Table 2). In changsha city, PB2 Ala588Val mutation site was presented in five of the isolates and K526R mutation site was presented in one of the isolates in this wave. In addition, two of the strains, A/changsha/34/2017 and A/changsha/72/2017, acquired both Ala588Val/Glu627Lys mutations. Another crucial mutant, a PB2 Asp701Asn substitution, was associated with increased viral polymerase activity, and was also present in A/changsha/26/2017 and A/changsha/58/2017 [31].

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Table 1. Clinical and epidemiological information of seven human infection cases of H7N9 viruses in 2017.

Group	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Strains	A/changsha/26/2017	A/changsha/34/2017	A/changsha/41/2017	A/changsha/44/2017	A/changsha/50/2017	A/changsha/58/2017	A/changsha/72/2017
Age	69	42	63	52	46	53	41
Gender	Female	Male	Male	Female	Female	Male	Male
Occupation	Unemployed	Builder	Retired	Commercial service	Farmer	Farmer	Teacher
Poultry market exposure	Indirect	Indirect	Before 10 days	Indirect	Before 7 days	Indirect	No
Symptom	ARDS	ARDS	ARDS	Mild	ARDS	ARDS	Severe pneumonia
ICU administration	Yes	Yes	Yes	No	Yes	Yes	Yes
Outcome	Cured	Cured	7 days to death	Cured	Cured	Cured	Cured
History of diseases	Type 2 diabetes Hypertension	No	No	Type 2 diabetes Hypertension	Hypertension	Type 2 diabetes Hypertension	Leukemia

https://doi.org/10.1016/j.heliyon.2019.e01253

Table 2. The importance of mutations in amino acids sequence in H7N9 viruses, from 2013 to 2017 in China.

Gene segment	Importance of the mutation [27]	H7N9 (2013)	H7N9 (2014)	H7N9 (2015)	H7N9 (2016)	H7N9 (2017)[a]
Haemagglutinin H7 Numbering						
Ala144Thr	Increased viral replication	128[b]/128[c]	156/156	106/106	121/121	324/324
Thr169Ala	Increased binding to α-2,6-linked sialic acid receptor	128/128	151/156	105/106	120/121	321/324
Gly195Val	Increased binding to α-2,6-linked sialic acid receptor	125/128	155/156	95/106	120/121	323/324
Gln235Leu	Increased binding to α-2,6-linked sialic acid receptor	120/128	154/156	97/106	121/121	324/324
Neuraminidase(NA)						
Deletions in stalk region	Increased virulence	126/128	156/156	106/106	121/121	324/324
Arg292Lys	Neuraminidase resistance	4/128	5/156	1/106	2/121	21/324
Matrix protein(M1)						
Asn30Asp, Thr215Ala	Increased virulence	128/128	156/156	106/106	121/121	324/324
Matrix protein(M2)						
Ser31Asn	Amantadine resistance	128/128	156/156	106/106	121/121	324/324
Polymerase(PB2)						
Leu89Val	Enhanced polymerase activity	128/128	156/156	106/106	121/121	324/324
Ile292Val	Enhanced polymerase activity	126/128	154/156	100/106	89/121	263/324
Ala588Val	Mammalian adaption	1/128	6/156	24/106	89/121	275/324
Glu627Lys	Improved viral replication at 33 °C	99/128	122/156	56/106	68/121	192/324
Asp701Asn	Mammalian adaption	7/128	9/156	6/106	8/121	15/324
Polymerase(PB1)						
His99Tyr	Enables droplet transmission in ferrets	ND[d]	ND	ND	ND	ND
Ile368Val	Enables droplet transmission in ferrets	118/128	156/156	106/106	111/121	295/324
Nonstructural(NS1)						
Pro42Ser	Increased virulence in mice	128/128	156/156	106/106	121/121	324/324

[a] Seven human infections in Changsha city are included.

[b] The number of relevant mutation sites in human infection cases.

[c] The number of human infecion cases had been reported in GISAID database in each year.

[d] ND: Not detected.

A number of H7N9 viruses had acquired Arg292Lys substitution associated with oseltamivir-resistance in NA gene until April 2017 [32]. But Arg292Lys substitution was not observed, and antivirals including oseltamivir were still administrated to all patients with H7N9 infections in our research. A deletion in the neuraminidase stalk

https://doi.org/10.1016/j.heliyon.2019.e01253

region, which was correlated to an increase in virulence, has also been revealed [33]. Amantadine resistance was evident and the resistance-conferring mutation was identified as Ser31Asn in the M2 gene (Table 3) [34,35]. Substitutions of other segments, including Asn30Asp and Thr215Ala in M1, Pro42Ser in NS1, and Ile368Val in PB1, which increase virulence in mouse models, were observed in key functional loci of the isolates from the fifth H7N9 wave in our preliminary analyses [36, 37].

3.3. Phylogenetic analysis

Phylogenetic analysis indicated that the HA segments of newly isolated H7N9 viruses from the environment and humans belonged to the YRD branch. These viruses were genetically distant from those of the infection cases reported in Changsha City in 2013, which belonged to the Pearl River Delta (PRD) lineage (Fig. 1A). HA segments from the environment and human isolates clustered near the isolates from the Guangdong province, which had been reported with three amino acids inserted in the HA segment (Fig. 1A). The NA gene of the strains were dispersedly distributed in one branch (Fig. 1B).

4. Discussion

H7N9 virus infections in humans have been reported continuously in mainland China since 2013. Up to 2017, China has experienced five waves of human infections with the H7N9 virus. Hunan province, which includes Changsha City, has reported seven infections in the fifth wave. In this study, we found that H7N9 viruses are transforming to be more capable of infecting mammals compared to previous cases reported in Changsha City, due to the substitution in the PB2 gene associated with mammalian adaptation. Moreover, the HAs of H7N9 viruses are avian in origin and feature conservative cleavage sites, which still classify the viruses as LPAI. Fortunately, human-to-human transmission has not been found in this wave.

Key functional sites that are mutated in gene segments are crucial for H7N9 virus invasion and proliferation. In our research, the changes occurred in the PB2 protein in all strains, which enhanced polymerase activity, and increased mammalian adaption in H7N9 virus infection [38, 39, 40]. Previous studies demonstrated that one or more of the Ile292Val, Ala588Val, Glu627Lys, and Asp701Asn substitutions are important for avian influenza viruses to break the host species barrier to infect mammals [41, 42]. PB2 E627K mutation can be detected in most of human infection cases, and this result in significantly higher polymerase activity and virulence in H5N1 or H7N9 virus infections [36, 38]. However, a minority of successfully infections are presented with deficiency of PB2 E627K mutant. The mutation in other sites, including Ile292Val, Ala588Val and D701N in PB2, could be found effective to compensate for the absence of E627K. H7N9 infection cases in this wave also

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Table 3. Comparison of critical amino acid residues in proteins of influenza A (H7N9) viruses from Changsha city, Hunan province, China.

Strains	HA					NA		M1		M2	NS1	PB1		PB2					
	144	169	195	235	Cleavage site	292	Stalk deletions	30	215	31	42	99	368	89	292	526	588	627	701
A/Changsha/26/2017	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	V	K	V	E	N
A/Changsha/34/2017	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	V	K	V	K	D
A/Changsha/41/2017	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	I	R	A	K	D
A/Changsha/44/2017	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	V	K	V	E	D
A/Changsha/50/2017	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	I	K	A	E	D
A/Changsha/58/2017	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	V	K	V	E	N
A/Changsha/72/2017	T	T	V	L	PKGRGLF	R	Yes	D	A	N	S	H	I	V	V	K	V	K	D
A/Changsha/1/2013	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	V	K	A	E	D
A/Changsha/2/2013	T	A	V	L	PKGRGLF	R	Yes	D	A	N	S	H	V	V	V	K	A	E	D

https://doi.org/10.1016/j.heliyon.2019.e01253

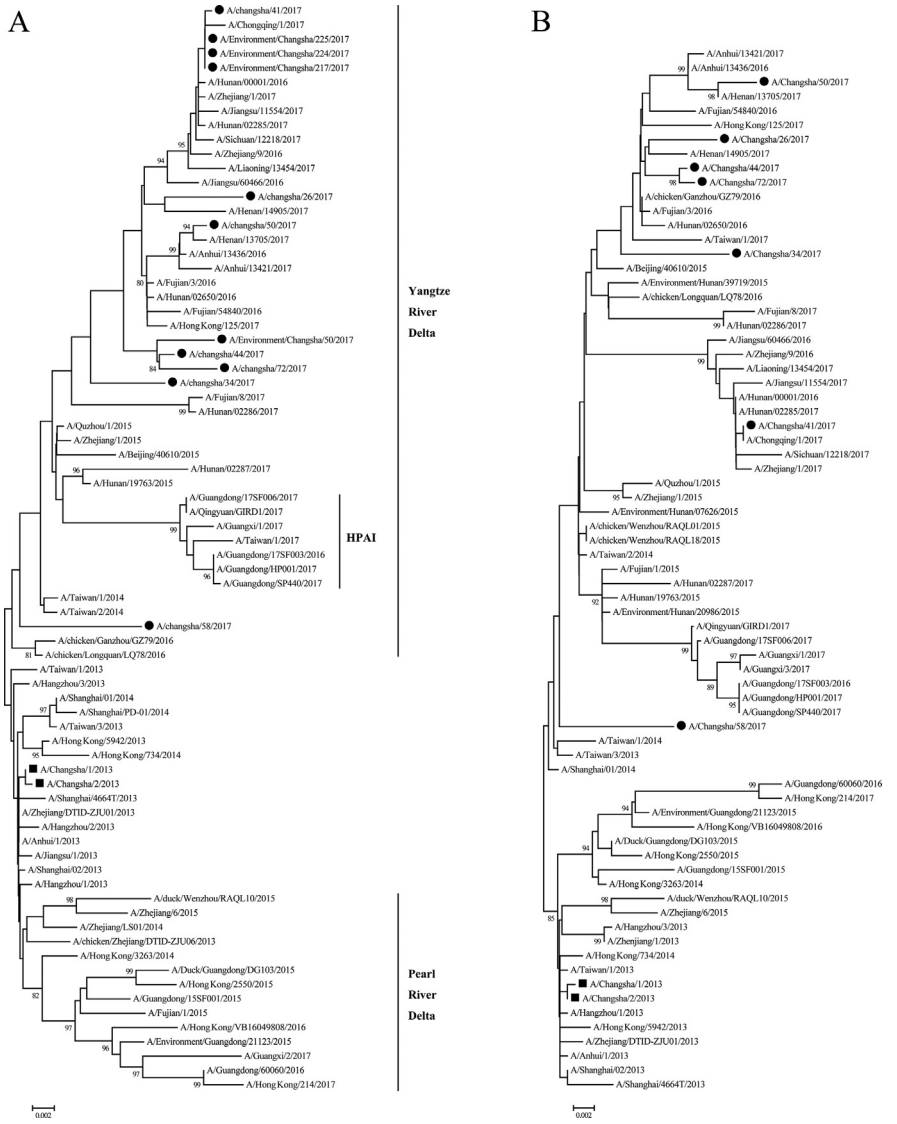

Fig. 1. Phylogenetic relationships of A (H7N9) HA(A) and NA(B) genes. Human and environment viruses in our research in 2017 are indicated by a black dot. The human viruses isolated from Changsha city in 2013 are indicated by a black square. The scale bar represents the number of substitutions per site.

presented with one or more substitutions, but not all occurred in the PB2 gene (Table 3). Our results showed that the ratio of Ala588Val mutant in PB2 gene was increasing continuously, which may causing more capable of infection in mammals. Moreover, PB2 Ala588Val showed more efficiently enhanced virulence than the PB2 Glu627Lys in mice, but similar growth dynamics in cell lines [28]. These observations suggest that LPAI viruses may have gained key substitutions on the PB2 segment after years of evolution. These evidences strongly indicate that the adaptation to mammals have become stronger.

https://doi.org/10.1016/j.heliyon.2019.e01253

Previous research confirmed that the infection source was epidemiologically linked with LPMs [8]. Cases of human infection with the H7N9 virus generally follow a high positive detection in LPMs, and the situation of this was also presented in Changsha city (data not show). Phylogenetic analysis indicated that the HA of H7N9 virus from humans is closely related to the isolates from LPMs. A/Environment/changsha/50/2017 was obtained from a nearby poultry market where the case 4 exposure occurred, and A/environment/changsha/217/2017, A/environment/changsha/224/2017, and A/environment/changsha/225/2017 were obtained from the poultry market that featured a high positive rate of virus. Owing to the increasing trend of H7N9 virus detection rate in poultry markets in Changsha City since December 2016, a government-imposed temporary comprehensive closure of LPMs was implemented on 01 April 2017. Since then, human infections were not reported in April and May. This indicates that the H7N9 viruses wave are avian origin, and visiting low hygiene level LPMs is still the main risk factor for H7N9 infection for the public [21]. The LPAI viruses, including H5N1, H5N6, H7N9 and H9N2 virus, have generally infected chickens and ducks, and have been asymptomatic. The infected poultry may provide an environment for reassortment between avain influenza viruses, as well as acting as a possible source of occasional transmission from polluted poultry in LPMs to humans. For another, previous research had reported that H9N2 virus can be isolated from the air nearby the LPMs, indicating that there might be a risk of avain influenza viruses transmission from polluted aerosols in LPMs to humans [43]. This situation in avians and poultry infections is worth heeding in H7N9 virus monitoring and prevention efforts.

In summary, despite the limited sample of subtype H7N9 avian influenza A virus isolates in Changsha City, the data indicate that H7N9 viruses are still evolving, and are transforming to be more capable of infecting mammals, even though they are still considered LPAI virus. An overwhelming majority of the reported patients were severely infected in the fifth wave of infection, showing transformed PB2 amino acid sites. Our findings suggest the need for careful surveillance of avian influenza virus infection in the middle Yangtze River in the coming years. Thus far, close monitoring of H7N9 avian influenza viruses in environments and poultry markets is essential to prevent a more serious H7N9 pandemic.

Declarations

Author contribution statement

Zheng Huang: Analyzed and interpreted the data; Wrote the paper.

Xinhua Ou, Rusheng Zhang, Biancheng Sun: Conceived and designed the experiments.

https://doi.org/10.1016/j.heliyon.2019.e01253

Dong Yao, Lingzhi Li: Performed the experiments.

Ruchun Liu, Yelan Li, Jingfang Chen: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the Natural Science Foundation of Hunan Province (CN) (No. 2017JJ3339).

Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study has been deposited at GenBank under the accession numbers MF370243 to MF370262, MG572470 to MG572504, and MG645390 to MG645393.

Acknowledgements

We acknowledge all the authors and clinicians in data and samples collection.

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https://doi.org/10.1016/j.heliyon.2019.e01253