RESEARCH ARTICLE

Genetic and Molecular Characterization of H9N2 Avian Influenza Viruses Isolated from Live Poultry Markets in Hubei Province, Central China, 2013–2017

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Abstract

H9N2 subtype avian influenza virus (AIV) is an influenza A virus that is widely spread throughout Asia, where it jeopardizes the poultry industry and provides genetic material for emerging human pathogens. To better understand the epidemicity and genetics of H9 subtype AIVs, we conducted active surveillance in live poultry markets (LPMs) in Hubei Province from 2013 to 2017. A total of 4798 samples were collected from apparent healthy poultry and environment. Real-time RT-PCR revealed that the positivity rate of influenza A was 26.6% (1275/4798), of which the H9 subtype accounted for 50.3% (641/1275) of the positive samples. Of the 132 H9N2 viral strains isolated, 48 representative strains were subjected to evolutionary analysis and genotyping. Phylogenetic analysis revealed that all H9N2 viral genes had 91.1%–100% nucleotide homology, clustered with genotype 57, and had high homology with human H9N2 viruses isolated from 2013 to 2017 in China. Using a nucleotide divergence cutoff of 95%, we identified ten distinct H9N2 genotypes that continued to change over time. Molecular analysis demonstrated that six H9N2 isolates had additional potential glycosylation sites at position 218 in the hemagglutinin protein, and all isolates had I155T and Q226L mutations. Moreover, 44 strains had A558V mutations in the PB2 protein and four had E627V mutations, along with H9N2 human infection strains A/Beijing/1/2016 and A/Beijing/1/2017. These results emphasize that the H9N2 influenza virus in Hubei continues to mutate and undergo mammalian adaptation changes, indicating the necessity of strengthening the surveillance of the AIV H9N2 subtype in LPMs.

Keywords Avian influenza · H9N2 · Genetic variation · Phylogenetic analysis · Live poultry markets (LPMs)

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Introduction

The influenza A virus has eight distinct gene segments that can be divided into 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes, of which H1–H16 are found in birds and H17 and H18 are found in bats (Webster et al. 1992; Tong et al. 2013). H9N2, H5Nx, and H7N9 subtype influenza A viruses cause the most harm to the poultry industry; however, as H9N2 subtype avian influenza viruses (AIVs) have low pathogenicity in poultry, their control has not been prioritized (Li et al. 2014). The first H9N2 influenza virus was isolated in the United States in 1966 (Homme and Easterday 1970) and appeared in China's Guangdong Province in 1994, where it has been widely prevalent since 1998 (Chen et al. 1994; Liu et al. 2003). The H9N2 influenza virus not only causes substantial damage to the poultry industry by direct infection

or stimulating infection, but also has the potential to infect mammals, and occasionally humans, and cause a pandemic (Peiris *et al.* 1999; Fusaro *et al.* 2011).

Influenza viruses can produce new viruses via mutation, recombination, and reassortment. H9N2 influenza viruses can provide some or all of their internal genes to produce new lethal reassortants that could infect humans, such as H5N1, H7N9, H10N8, and H5N6 (Guan *et al.* 1999; Chen *et al.* 2014; Yang *et al.* 2015; Feng *et al.* 2016; Pu *et al.* 2017). Based on genetic and phylogenetic analyses, the *HA* gene of H9N2 influenza viruses can be divided into Eurasian and American avian lineages. The Eurasian avian lineage mainly consists of three distinct lineages: A/chicken/Beijing/1/94-like (BJ/94-like); A/quail/Hong Kong/G1/97-like (G1-like); and A/duck/Hong Kong/Y439/97 (Y439-like) (Sun *et al.* 2010). A recent study identified 117 different genotypes from 730 H9N2 influenza viruses in Chinese birds, mammals, and human hosts from 1994 to 2013 (Li *et al.* 2017). Continuous research on the genetic evolution of H9N2 avian influenza virus is of great significance.

Live poultry markets (LPMs) provide a convenient location for the reorganization of influenza viruses from different species of poultry (Liu *et al.* 2014). As human-infecting AIVs have a history of exposure in LPMs, influenza surveillance in LPMs plays a key role in public health security. Although the H9N2 virus has been studied in LPMs in other regions of China and short-term local studies have been carried out in Hubei Province (Zhou *et al.* 2012; Huang *et al.* 2015; Wu *et al.* 2015; Chen *et al.* 2016), the presence and genetic structure of H9N2 viruses in LPMs in Hubei Province is still poorly understood. This is the first study to perform a comprehensive genetic evolution analysis of H9N2 influenza viruses in LPMs in Hubei Province, central China. Our results revealed the prevalence and genetic characteristics of H9N2 influenza viruses. In addition, our findings can help assess the potential pandemic risks of H9N2 influenza viruses.

Materials and Methods

Sample Collection

From October 2013 to December 2017, we carried out active surveillance in LPMs in 11 districts of Hubei Province, including: Wuhan, Huanggang, Huangshi, Ezhou, Jingzhou, Yichang, Xiangyang, Jingmen, Xianning, Xiaogan, and Shiyan (Fig. 1). A total of 4798 samples were collected from apparent healthy poultry, including 4342 swab samples (3036 chickens, 1261 ducks, 35 pigeons, 10 geese) and 456 environmental samples (Table 1). Throat and cloaca swabs from the same bird were placed in one

preservation tube according to previously described sampling methods (Deng *et al.* 2013). Poultry samples were collected in duplicate: one to detect AIV RNA and the other to inoculate chicken embryos. Each sample was placed in phosphate-buffered saline (PBS, pH 7.4) containing 10% glycerol, ampicillin (2000 U/mL), gentamycin (2000 μg/mL), and streptomycin (2000 μg/mL). The samples were immediately cryopreserved in the laboratory and stored at −70 °C.

Detection of AIV RNA

RNA was extracted from the collected samples using a Thermo KingFisher Flex Purification System (ThermoFisher, Vantaa, Finland) with a Mag Pure Viral Nucleic Acid KF Kit (Mag, Guangzhou, China). Influenza A, H5, H7, and H9 subtypes were detected using TransScriptII Probe One-Step qRT-PCR SuperMix (Transgene, Beijing, China). Primers and probes were selected based on the Chinese National Influenza Center (CNIC) national influenza monitoring technical guide and the World Health Organization (WHO) information for the molecular diagnosis of influenza virus (CNIC 2011; WHO 2013, 2014). Samples with a cycle threshold (C_t) value of < 35 were considered positive.

Virus Isolation and Sequencing

To isolate the viruses, H9-subtype positive samples were inoculated into 9-day-old specific pathogen-free chicken embryos, and allantoic fluid was harvested as described previously (Deng *et al.* 2013) and tested for HA activity. HA-positive samples were characterized by real-time RT-PCR (CNIC 2011; WHO 2013, 2014). Briefly, viral RNA was extracted from allantoic fluid using a High Pure Viral RNA kit (Roche, Mannheim, Germany), and the surface genes and internal genes coding sequences were amplified by RT-PCR using PrimeScript^{TM} One Step RT-PCR Kit Ver. 2 (TaKaRa, Dalian, China) with specific primers (Hoffmann *et al.* 2001). RT-PCR was performed in a 50 μL reaction mix containing 5 μL of RNA, 25 μL of PrimeScript One step Enzyme Mix, 14 μL of RNase-Free dH$_2$O, 2 μL of 10 pmol forward primer, and 2 μL of 10 pmol reverse primer. Amplification parameters were as follows: initial step of 30 min at 50 °C, then 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C, followed by 10 min extension at 72 °C. The amplified products were sent to Shanghai Sangon Biotech for sequencing. AIV isolation and identification were conducted in Physical Containment Level 2 Plus laboratory.

Fig. 1 Map of sampling sites in Hubei Province, China. The eleven districts where samples were collected are shown in yellow.

Table 1 Detection of influenza A viruses by real-time RT-PCR in samples from live poultry markets in Hubei Province.

	Samples (*n*)	Influenza A (%)	H5 (%)	H7 (%)	H9 (%)	H5 + H9 (%)
Chicken	3036	763 (25.1)	54 (1.9)	10 (0.3)	575 (18.9)	63 (2.1)
Duck	1261	399 (31.6)	79 (6.2)	0	43 (3.4)	61 (4.8)
Pigeon	35	2 (5.7)	0	0	2 (5.7)	0
Goose	10	0	0	0	0	0
Environment	456	111 (24.3)	16 (3.5)	7 (1.5)	21 (4.6)	34 (7.4)
Total	4798	1275 (26.6)	149 (3.1)	17 (0.4)	641 (13.4)	158 (3.3)

Genetic and Phylogenetic Analyses

Nucleotide sequences were edited using the SeqMan module in DNAstar (7.0), and phylogenetic analyses were performed using MAGE 6.0 with the neighbor-joining algorithm and bootstrap analysis with 1000 replicates. Multiple sequence alignments were compiled using Clustal W (Tamura *et al.* 2013). Phylogenetic analyses were based on the following protein coding nucleotide sequences: PB2, 49-2289; PB1, 25-2233; polymerase acidic (PA), 25–2151; HA, 43-1572; nucleoprotein (NP), 46-1527; NA, 20–1408; matrix (M), 26-1001; and nonstructural (NS), 29-859. Potential HA glycosylation sites were predicted using

NetNGlyc1.0 (https://www.cbs.dtu.dk/services/NetNGlyc/). The "group" of each segment of H9N2 influenza viruses in the study was categorized using 95% sequence identity cutoffs refers to previously described (Deng *et al.* 2013). Genotyping was determined by the combination of group assignments of each of eight segments (Li *et al.* 2014). Reference strain sequences were obtained from the Influenza Virus Database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi). Nucleotide sequences were deposited in GenBank under accession numbers MN647131 to MN647514 (Supplementary Table S1).

Results

Prevalence of Influenza A Virus in Hubei Province

From October 2013 to December 2017, we collected 4798 swabs and environmental samples from LPMs throughout Hubei Province (Central China) and screened the samples for influenza A H5, H7, and H9 subtypes using real-time RT-PCR. A total of 1275 samples were positive for influenza A (26.6%), of which 641 were positive for the H9 subtype (13.4%), 149 were positive for the H5 subtype (3.1%), 158 were positive for the H5 + H9 subtype (3.3%), and 17 were positive for the H7 subtype (Table 1).

Phylogenetic Analysis and Genotyping of H9N2 Subtype

After molecular identification and virus isolation, 132 H9 subtype viruses were isolated and their *HA* and *NA* genes were sequenced. H9N2 subtype influenza viruses were the main subtype, and there were mixed infections. Highly homologous strains were removed based on sampling time, location, and poultry breed, leaving 48 H9N2 influenza viruses that underwent full-segment genetic analysis. The reference sequences included classic H9N2 strains, three genotype 57 strains, all full-length human H9N2 influenza virus strains from 1999 to 2017, and other reference strains.

To better understand the genetic evolution of the H9N2 AIVs isolated from LPMs, we performed phylogenetic analysis on all eight genes of the 48 H9N2 isolates. Genetic evolution analysis revealed that all gene segments of the 48 H9N2 viruses belonged to the Eurasian lineage and genotype 57. The surface genes of all H9N2 viruses analyzed in the study were highly homologous with the human strains after 2013, while the *HA* gene shared 93.4%–99.9% identity at the nucleotide level and formed three phylogenetic groups (Groups 1–3). Group 1 contained six isolates and human origin strain A/Lengshuitan/11197/2013 (H9N2). Group 2 contained seven isolates. Group 3 contained 35 isolates and five H9N2 human strains (Fig. 2A). The *NA* genes shared 91.1%–100% identity at the nucleotide level and formed five phylogenetic groups (Groups 1–5). Groups 1, 2, and 3 each contained one isolate and group 3 contained the human strain A/Lengshuitan/11197/2013 (H9N2), while groups 4 and 5 contained the other five H9N2 human strains (Fig. 2B).

The internal genes displayed respective nucleotide homologies of 94.4%–100% for the *PB2* gene, 94.8%–99.9% for the *PB1* gene, 93.2%–100% for the *PA* gene, 93.6%–100% for the *NP* gene, 95.3%–100% for the *M* gene, and 94.3%–100% for the *NS* gene. Based on their nucleotide identity, the *PA* and *NP* genes were divided into

two phylogenetic groups, while the *PB2*, *PB1*, *M*, and *NS* genes were categorized into one phylogenetic group. Group 2 of the *PA* gene contained only one A/chicken/Hubei/S389/2013 isolate (H9N2). The internal genes of all H9N2 viruses were clustered into genotype 57, whose representative strain is A/chicken/Zhejiang/HJ/2007 (H9N2). These results indicated that the internal genes of the H9N2 viruses were highly homologous with the human H9N2 strains from 2013 to 2017 and AIVs such as H7N9, H10N8, H5N2, H5N6, and H10N6 (Supplementary Figure S1 and S2).

Based on the 95% genetic diversity of each segment, the 48 H9N2 viruses in this study were divided into ten genotypes (Fig. 3A). Genotypes 1 (2013), 2 (2013), 6 (2016), and 7 (2016) each contained one isolate and were thus deemed to be transient genotypes. Genotype 4 only appeared in 2016. Genotype 5 (2014–2016) was the main genotype, accounting for 43.8% of the isolated strains. Genotypes 8 and 9 existed in 2015–2017, whereas genotype 10 was detected in 2015 and 2017, with a significant change in its *NP* gene (Fig. 3B). These findings indicated that the genotypes of H9N2 influenza viruses continued to change over time.

Molecular Characterization

All H9N2 isolates possessed a "PSRSSR/GLF" motif at the HA cleavage site (Fig. 4), which is a typical characteristic of low pathogenicity in chickens (Guo et al. 2000; Ge et al. 2009). The HA amino acid receptor binding sites were S138A, I155T, H183N, A190V/T, Q226L, 227M, and 228G (H3 numbering, as used throughout the manuscript). I155T and Q226L mutations were detected in all strains, which favor the affinity of influenza viruses for human-type receptors (Matrosovich et al. 2001; Wan et al. 2008). In all viruses, HA has eight known potential glycosylation sites (Asn-X-Ser/Thr, where X =/=P) at positions 29, 82, 141, 298, 305, 313, 492, and 551 (Wu *et al.* 2015; Zhu *et al.* 2018). Six of the H9N2 viruses in this study had an additional potential glycosylation site at position 218, which was detected in the human H9N2 influenza viruses A/Hong Kong/1074/1999 and A/Hong Kong/3239/2008 (Supplementary Table S2A).

In NA, three amino acids (sites 63–65) were deleted at the stalk region in all virus strains in this study, as described previously (Huang *et al.* 2015; Wu *et al.* 2015; Ge *et al.* 2018; Wang *et al.* 2018). However, no H274Y or R292K substitutions were observed, indicating that isolated viruses would be sensitive to NA inhibitors such as oseltamivir (Hurt *et al.* 2009) (Supplementary Table S2B).

In PB2, the A588V mutation, which is a new marker of mammalian AIV adaptation, was detected in 44 of the 48 H9N2 virus strains (Xiao *et al.* 2016). L89V, G309D,
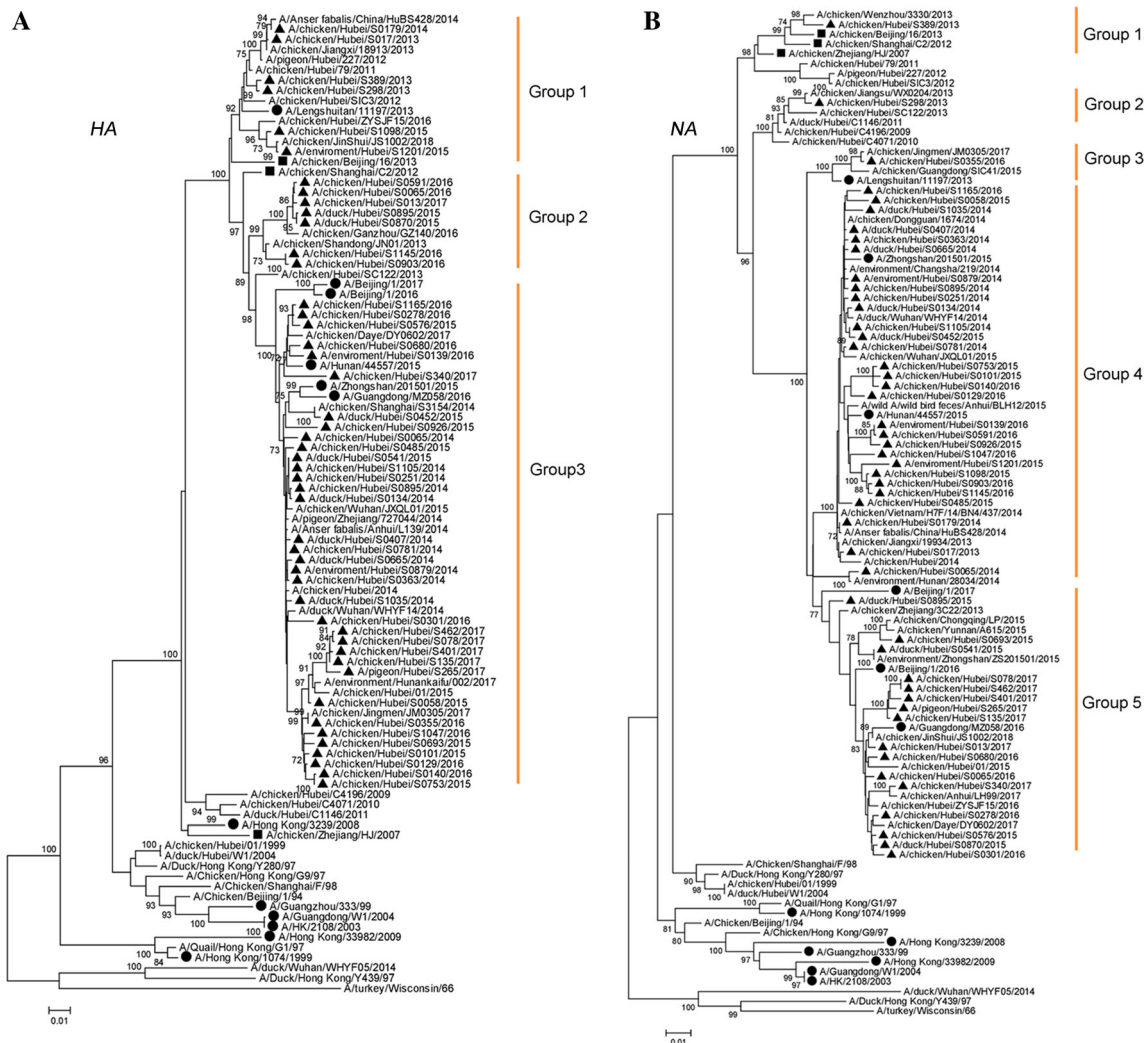
Fig. 2 Phylogenetic analysis of the surface genes of H9N2 avian influenza viruses collected from live poultry markets in Hubei Province between 2013 to 2017. **A** *HA* gene. **B** *NA* gene. Solid triangles indicate strains isolated in this study. Solid circles indicate H9N2 strains that caused human infection. Solid squares indicate genotype 57 strains. For each node, bootstrap values ≥ 70% are shown. The tree was created using the neighbor-joining method and bootstrapped with 1000 replicates using MEGA6.0.

R477G, and I495V mutations were also detected in PB2, which have been shown to increase AIV virulence in mice (Li *et al.* 2009). No E627K or D701N mutations were detected in PB2; however, four viruses possessed an E627V mutant, which was identified in human H9N2 influenza virus A/Beijing/1/2016 and A/Beijing/1/2017.

In PB1, L13P and 368V mutation were found. A515T was found in PA. The mutations N30D and T215A in the M1 and the P42S and V149A substitutions in NS1 were detected in all strains, which associated with increased virulence in mice (Fan et al. 2009; Li et al. 2006; Jiao et al. 2008). In addition, S31N was identified in M2 in all isolates, suggesting that these viruses were resistant to antiviral drugs (Belshe et al. 1988). (Supplementary Table S2B). The results indicated that the H9N2 avian

influenza virus in this study had undergone mutations adapted to mammals (Fig. 4).

Discussion

The H9N2 virus poses a threat to public health as it can act as a genetic donor and directly infect humans as an emerging human influenza virus (Gu *et al.* 2017). In this study, we found that the rate of avian influenza A virus positivity in LPMs was 26.57% and H9N2 was the main subtype, consistent with a previous report from Wuhan in Hubei Province over the same period (Chen *et al.* 2016). In addition, the positivity rate in chickens was higher than that in waterfowl duck, and mixed infections of different influenza viruses were observed, as reported previously
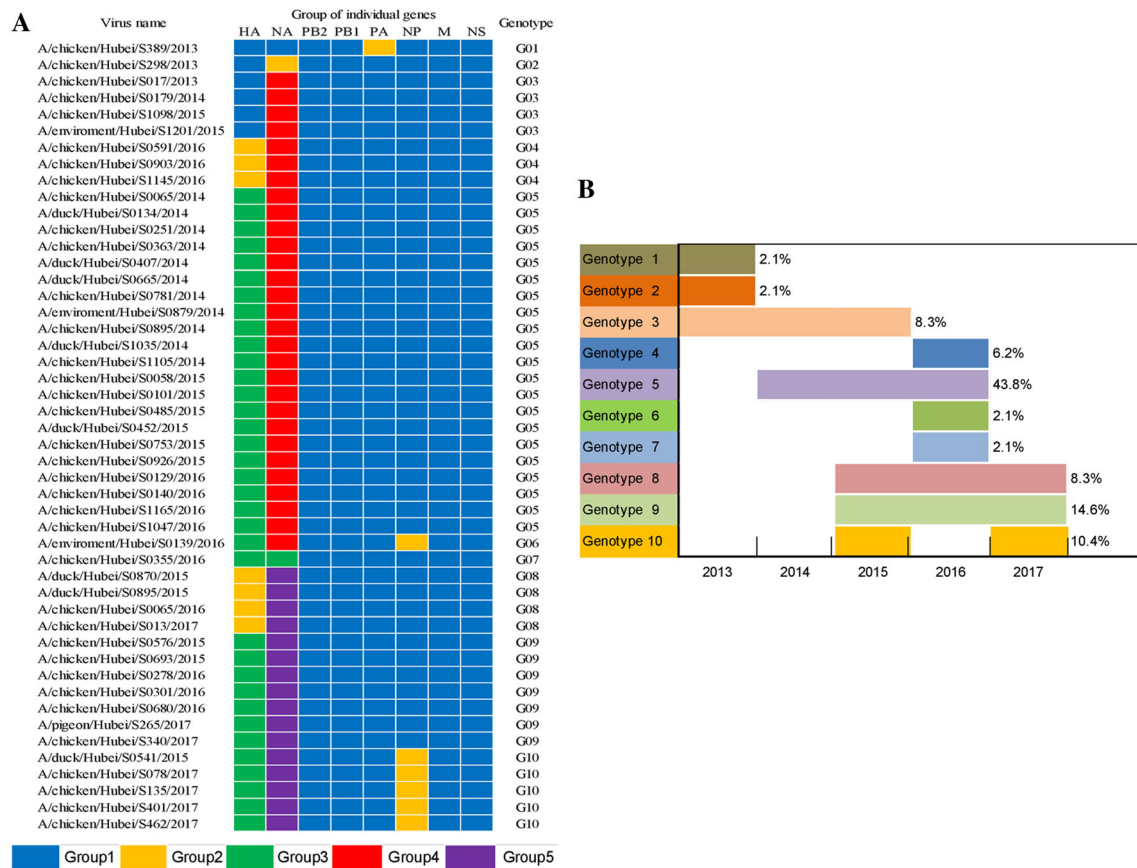
Fig. 3 The genotypes and timeline of 48 H9N2 viruses collected in Hubei Province from 2013 to 2017. **A** The genotypes of 48 H9N2 viruses. **B** Timeline.

(Wu *et al.* 2015). As such, LPMs likely played a key role in the emergence of new influenza viruses, and disinfection should be used to stop the spread of the disease.

H9N2 influenza virus has low pathogenicity in birds and humans; however, its gene segments can reassort with other influenza viruses with the potential to produce new virus epidemics that can cause greater harm. The H5N6, H7N9, and H10N8 subtypes that infect humans possess all the H9N2 influenza virus internal genes (Gao *et al.* 2013; Qi *et al.* 2014; Shen *et al.* 2016), while the *PB2* and *PB1* genes of early human H5N1 virus infections are highly homologous to those in H9N2 (Guan *et al.* 1999). In this study, we found that the internal genes of the H9N2 isolates were highly homologous with many other influenza viruses, posing the risk of a new influenza virus epidemic. Genotype 57 appeared in China in 2007 and dominated from 2010 to 2013 (Pu *et al.* 2015; Li *et al.* 2017), providing a genetic source for more than ten influenza reassortants, including H7N9 and H10N8, and thus posing a potential influenza pandemic concern (Pu *et al.* 2015). The H9N2 viral genes in this study belonged to genotype 57 and were divided into ten genotypes based on 95% nucleotide homology, indicating a high degree of genetic diversity.

Studies carried out over the same period in Hunan and Jiangxi Provinces found that the isolated H9N2 viruses also belonged to genotype 57 (Huang *et al.* 2015; Han *et al.* 2018). Recently, new H9N2 genotypes were identified from environmental samples in China (Zou *et al.* 2019). In this study, the surface genes of the H9N2 isolates displayed great genetic diversity; however, there were only a few changes in the internal genes and continuous changes in genotypes. Together, these results indicate that the H9N2 AIV in Hubei continues to evolve and should be given high priority.

HA is an AIV receptor binding and membrane fusion glycoprotein that is the main inducer of neutralizing antibodies against viral infection (Skehel and Wiley 2000). HA receptor binding preferences play an important role in the replication and spread of influenza viruses. For instance, the characteristics of H9N2 viruses in southern China from 2009 to 2013 indicated that natural chicken-derived H9N2 isolates gradually gained preference for human α-2,6 sialic acid receptors, with several variants displaying airborne transmission in ferrets (Li *et al.* 2014). Previous studies have shown that the HA I155T mutation is important for the binding of H9N2 to mammalian receptors (Li *et al.*
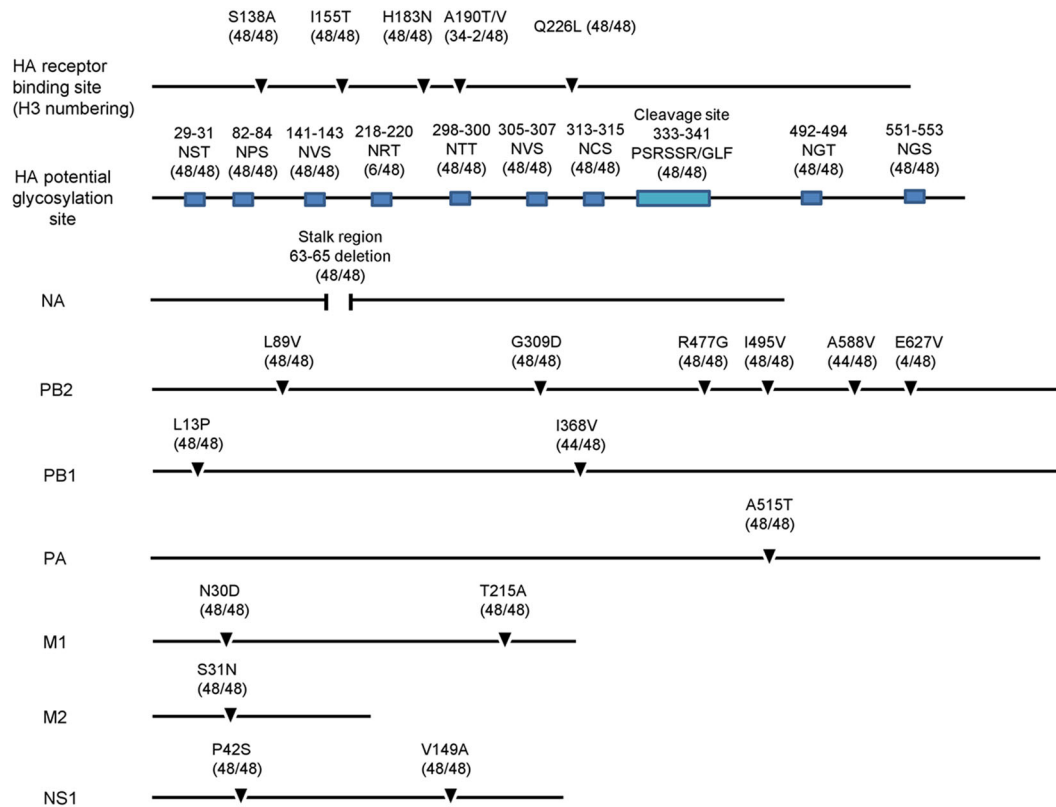
Fig. 4 Summary of molecular features of H9N2 isolates. Key amino acids and their change frequencies are indicated.

2014). In this study, all H9N2 strains had I155T mutations, indicating that all these viruses had changes that enable mammalian infection. The HA Q226L mutation, which has been shown to contribute to the binding between human receptor and the H9N2 virus (Vines *et al.* 1998), was detected in all H9N2 isolates in our study. Thus, our findings indicate that H9N2 has the potential for cross-species transmission.

The E627K and D701N mutations in influenza virus PB2 proteins are critical for enhancing mammalian pathogenicity (Chen *et al.* 2013; Nieto *et al.* 2017). Previous studies have shown that H7N9 and H10N8 viruses readily acquire E627K or D701N mutations in PB2 during human replication (Qi *et al.* 2014; Nieto *et al.* 2017; Qi *et al.* 2018), and PB2 E627K mutations have recently been discovered in wild birds for the first time (Ge *et al.* 2018). Although no E627K or D701N mutations were identified in PB2 in this study, four of the isolates had E627V mutations similar to those in the H9N2 influenza virus that infected humans in 2016 and 2017. A recent study found that PB2 E627V mutations could increase H9N2 virus replication and enhance its pathogenicity (Arai *et al.* 2019). Similarly, we found that most isolates in this study had recently acquired the mammalian adaptation mutation A588V in PB2 protein, which can enhance polymerase activity, virus

replication, and virulence of influenza H7N9, H10N8, and H9N2 viruses since 2013.

In conclusion, we conducted an epidemiological survey of AIVs in LPMs in Hubei Province and performed genetic analysis on representative H9N2 isolated strains. We revealed that the H9N2 subtype virus is the dominant influenza virus in LPMs and mixed infections are prevalent, posing a greater risk of genetic recombination to generate new influenza viruses. Phylogenetic analysis indicated that all H9N2 influenza viruses in this study were divided into multiple genotypes that change over time. Their internal genes were highly homologous with H7N9, H10N8, H5N6, and other influenza viruses that infect humans, thus may act as a gene donor to promote the emergence of new influenza viruses. Multiple specific amino acid mutations were observed in HA and PB2 proteins, among which the E627V mutation in PB2 was also identified in the H9N2 influenza strains that infected humans in 2016 and 2017, indicating that H9N2 has the potential for cross-species transmission. Together, our findings demonstrate that the H9N2 subtype influenza virus in central China continues to mutate and displays the potential for human infection. Subsequent studies on the biological characteristics of the isolates are required to further assess the risk of infection. In addition, the

surveillance of H9N2 avian influenza virus should be strengthened to elucidate the genetic characteristics.

Author Contributions DRB and NS designed and coordinated the study. ZH, XJ, JX, JH, and HX collected samples. ZH, FP, and ZX carried out most of the experiments. ZH, TL, YS, and ZL analyzed the data. ZH, WZ, and ZL wrote and finalized the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement The study was approved by the Hubei Center for Animal Disease Control and Prevention. All institutional and national guidelines for the care and use of animals were followed.

References

Arai Y, Kawashita N, Ibrahim MS, Elgendy EM, Daidoji T, Ono T, Takagi T, Nakaya T, Matsumoto K, Watanabe Y (2019) PB2 mutations arising during H9N2 influenza evolution in the middle east confer enhanced replication and growth in mammals. PLoS Pathog 15:e1007919

Belshe RB, Smith MH, Hall CB, Betts R, Hay AJ (1988) Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J Virol 62:1508–1512

Chen B, Zhang Z, Chen W (1994) The study of avian influenza: I. The isolation and preliminary serological identification of avian influenza virus in chicken. Chin J Vet Med 20:3–5

Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, Yao H, Wo J, Fang Q, Cui D, Li Y, Yao X, Zhang Y, Wu H, Zheng S, Diao H, Xia S, Zhang Y, Chan KH, Tsoi HW, Teng JL, Song W, Wang P, Lau SY, Zheng M, Chan JF, To KK, Chen H, Li L, Yuen KY (2013) Human infections with the emerging avian influenza a H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. Lancet 381:1916–1925

Chen H, Yuan H, Gao R, Zhang J, Wang D, Xiong Y, Fan G, Yang F, Li X, Zhou J, Zou S, Yang L, Chen T, Dong L, Bo H, Zhao X, Zhang Y, Lan Y, Bai T, Dong J, Li Q, Wang S, Zhang Y, Li H, Gong T, Shi Y, Ni X, Li J, Zhou J, Fan J, Wu J, Zhou X, Hu M, Wan J, Yang W, Li D, Wu G, Feng Z, Gao GF, Wang Y, Jin Q, Liu M, Shu Y (2014) Clinical and epidemiological characteristics of a fatal case of avian influenza a H10N8 virus infection: a descriptive study. Lancet 383:714–721

Chen LJ, Lin XD, Guo WP, Tian JH, Wang W, Ying XH, Wang MR, Yu B, Yang ZQ, Shi M, Holmes EC, Zhang YZ (2016) Diversity and evolution of avian influenza viruses in live poultry markets, free-range poultry and wild wetland birds in China. J Gen Virol 97:844–854

Chinese National Influenza Center (CNIC) (2011) national influenza monitoring technical guide. Available: http://www.chinaivdc.cn/cnic/zyzx/jcfa/201605/t20160520_129702.htm. Accessed 9 Aug 2011

Deng G, Tan D, Shi J, Cui P, Jiang Y, Liu L, Tian G, Kawaoka Y, Li C, Chen H (2013) Complex reassortment of multiple subtypes of avian influenza viruses in domestic ducks at the Dongting Lake Region of China. J Virol 87:9452–9462

Fan S, Deng G, Song J, Tian G, Suo Y, Jiang Y, Guan Y, Bu Z, Kawaoka Y, Chen H (2009) Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 avian influenza viruses in mice. Virology 384:28–32

Feng Y, Guan W, Yuan B, Wang Y, Li Z, Song Y, Li S, Yang Z, Zhong N, Zhang Y, Xia X (2016) Emergence of triple-subtype reassortants of fatal human H5N6 avian influenza virus in Yunnan, China. J Infect 72:753–756

Fusaro A, Monne I, Salviato A, Valastro V, Schivo A, Amarin NM, Gonzalez C, Ismail MM, Al-Ankari AR, Al-Blowi MH, Khan OA, Maken Ali AS, Hedayati A, Garcia Garcia J, Ziay GM, Shoushtari A, Al Qahtani KN, Capua I, Holmes EC, Cattoli G (2011) Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol 85:8413–8421

Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y (2013) Human infection with a novel Avian-origin Influenza A (H7N9) virus. N Engl J Med 368:1888–1897

Ge FF, Zhou JP, Liu J, Wang J, Zhang WY, Sheng LP, Xu F, Ju HB, Sun QY, Liu PH (2009) Genetic evolution of H9 subtype influenza viruses from live poultry markets in Shanghai, China. J Clin Microbiol 47:3294–3300

Ge Y, Yao Q, Chai H, Hua Y, Deng G, Chen H (2018) A 627 K variant in the PB2 protein of H9 subtype influenza virus in wild birds. Influenza Other Respir Viruses 12:728–741

Gu M, Xu L, Wang X, Liu X (2017) Current situation of H9N2 subtype avian influenza in China. Vet Res 48:49

Guan Y, Shortridge KF, Krauss S, Webster RG (1999) Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci USA 96:9363–9367

Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, Norwood M, Shortridge KF, Webster RG, Guan Y (2000) Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 267:279–288

Han L, He W, Yan H, Li X, Wang C, Shi Q, Zhou T, Dong G (2018) The evolution and molecular characteristics of H9N2 avian influenza viruses in Jiangxi of China. J Med Virol 91(4):711–716

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146:2275–2289

Homme PJ, Easterday BC (1970) Avian influenza virus infections. I. Characteristics of influenza A/Turkey/Wisconsin/1996 virus. Avian Dis 14:66–74

Huang Y, Zhang H, Li X, Hu S, Cai L, Sun Q, Li W, Deng Z, Xiang X, Zhang H, Li F, Gao L (2015) Detection and genetic characteristics of H9N2 avian influenza viruses from live poultry markets in Hunan Province, China. PLoS One 10:e0142584

Hurt AC, Holien JK, Parker MW, Barr IG (2009) Oseltamivir resistance and the H274Y neuraminidase mutation in seasonal, pandemic and highly pathogenic influenza viruses. Drugs 69:2523–2531

Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, Liu W, Bu Z, Kawaoka Y, Chen H (2008) A single-amino-acid substitution in the NS1

protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol 82(3):1146–1154

Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H (2006) The nS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 80(22):11115–11123

Li J, Ishaq M, Prudence M, Xi X, Hu T, Liu Q, Guo D (2009) Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 avian influenza virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2. Virus Res 144:123–129

Li X, Shi J, Guo J, Deng G, Zhang Q, Wang J, He X, Wang K, Chen J, Li Y, Fan J, Kong H, Gu C, Guan Y, Suzuki Y, Kawaoka Y, Liu L, Jiang Y, Tian G, Li Y, Bu Z, Chen H (2014) Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 avian influenza viruses. PLoS Pathog 10:e1004508

Li C, Wang S, Bing G, Carter RA, Wang Z, Wang J, Wang C, Wang L, Wu G, Webster RG, Wang Y, Sun H, Sun Y, Liu J, Pu J (2017) Genetic evolution of influenza H9N2 viruses isolated from various hosts in China from 1994 to 2013. Emerg Microbes Infect 6:e106

Liu H, Liu X, Cheng J, Peng D, Jia L, Huang Y (2003) Phylogenetic analysis of the hemagglutinin genes of twenty-six avian influenza viruses of subtype H9N2 isolated from chickens in China during 1996–2001. Avian Dis 47:116–127

Liu D, Shi W, Gao GF (2014) Poultry carrying H9N2 act as incubators for novel human avian influenza viruses. Lancet 383:869

Matrosovich MN, Krauss S, Webster RG (2001) H9N2 influenza a viruses from poultry in Asia have human virus-like receptor specificity. Virology 281:156–162

Nieto A, Pozo F, Vidal-Garcia M, Omenaca M, Casas I, Falcon A (2017) Identification of rare PB2-D701NMutation from a patient with severe influenza: contribution of the PB2-D701NMutation to the pathogenicity of human influenza. Front Microbiol 8:575

Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF (1999) Human infection with influenza H9N2. Lancet 354:916–917

Pu J, Wang S, Yin Y, Zhang G, Carter RA, Wang J, Xu G, Sun H, Wang M, Wen C, Wei Y, Wang D, Zhu B, Lemmon G, Jiao Y, Duan S, Wang Q, Du Q, Sun M, Bao J, Sun Y, Zhao J, Zhang H, Wu G, Liu J, Webster RG (2015) Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. Proc Natl Acad Sci USA 112:548–553

Pu J, Sun H, Qu Y, Wang C, Gao W, Zhu J, Sun Y, Bi Y, Huang Y, Chang KC, Cui J, Liu J (2017) M gene reassortment in H9N2 influenza virus promotes early infection and replication: contribution to rising virus prevalence in chickens in China. J Virol. https://doi.org/10.1128/JVI.02055-16

Qi W, Zhou X, Shi W, Huang L, Xia W, Liu D, Li H, Chen S, Lei F, Cao L, Wu J, He F, Song W, Li Q, Li H, Liao M, Liu M (2014) Genesis of the novel human-infecting influenza A(H10N8) virus and potential genetic diversity of the virus in poultry, China. Euro Surveill 19:20841

Qi W, Jia W, Liu D, Li J, Bi Y, Xie S, Li B, Hu T, Du Y, Xing L, Zhang J, Zhang F, Wei X, Eden JS, Li H, Tian H, Li W, Su G, Lao G, Xu C, Xu B, Liu W, Zhang G, Ren T, Holmes EC, Cui J, Shi W, Gao GF, Liao M (2018) Emergence and adaptation of a novel highly pathogenic H7N9 influenza virus in birds and humans from a 2013 human-infecting low-pathogenic ancestor. J Virol. https://doi.org/10.1128/JVI.00921-17

Shen YY, Ke CW, Li Q, Yuan RY, Xiang D, Jia WX, Yu YD, Liu L, Huang C, Qi WB, Sikkema R, Wu J, Koopmans M, Liao M (2016) Novel reassortant avian influenza A(H5N6) viruses in humans, Guangdong, China, 2015. Emerg Infect Dis 22:1507–1509

Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69:531–569

Sun Y, Pu J, Jiang Z, Guan T, Xia Y, Xu Q, Liu L, Ma B, Tian F, Brown EG, Liu J (2010) Genotypic evolution and antigenic drift of H9N2 influenza viruses in China from 1994 to 2008. Vet Microbiol 146:215–225

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO (2013) New World Bats Harbor Diverse Iinfluenza AViruses. PLoS Pathog 9:e1003657

Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, Kawaoka Y (1998) The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J Virol 72:7626–7631

Wan H, Sorrell EM, Song H, Hossain MJ, Ramirez-Nieto G, Monne I, Stevens J, Cattoli G, Capua I, Chen LM, Donis RO, Busch J, Paulson JC, Brockwell C, Webby R, Blanco J, Al-Natour MQ, Perez DR (2008) Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS ONE 3:e2923

Wang D, Wang J, Bi Y, Fan D, Liu H, Luo N, Yang Z, Wang S, Chen W, Wang J, Xu S, Chen J, Zhang Y, Yin Y (2018) Characterization of avian influenza H9N2 viruses isolated from ostriches (*Struthio camelus*). Sci Rep 8:2273

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56:152–179

World Health Organization (WHO) (2013) Real-time RT-PCR Protocol for the detection of Avian Influenza A (H7N9) virus. Available: https://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf?ua=1. Accessed Apr 2013

World Health Organization (WHO) (2014) WHO information for molecular diagnosis of influenza virus—update. Available: https://www.who.int/influenza/gisrs_laboratory/molecular_diagnosis/en/. Accessed Mar 2014

Wu H, Peng X, Peng X, Cheng L, Lu X, Jin C, Xie T, Yao H, Wu N (2015) Genetic and molecular characterization of H9N2 and H5 avian influenza viruses from live poultry markets in Zhejiang Province, Eastern China. Sci Rep 5:17508

Xiao C, Ma W, Sun N, Huang L, Li Y, Zeng Z, Wen Y, Zhang Z, Li H, Li Q, Yu Y, Zheng Y, Liu S, Hu P, Zhang X, Ning Z, Qi W, Liao M (2016) PB2-588 V promotes the mammalian adaptation of H10N8, H7N9 and H9N2 avian influenza viruses. Sci Rep 6:19474

Yang ZF, Mok CK, Peiris JS, Zhong NS (2015) Human infection with a Novel Avian Influenza A (H5N6) virus. N Engl J Med 373:487–489

Zhou JP, Ge FF, Liu J, Ju HB, Yang DQ, Wang J, Zhang WY, Liu PH (2012) Epidemiological survey and genetic evolution of H9 subtype influenza viruses in Shanghai, China, from 2006 to 2010. Arch Virol 157:1193–1198

Zhu R, Yang X, Zhang J, Xu D, Fan J, Shi H, Wang S, Liu X (2018) Identification, sequence analysis, and infectivity of H9N2 avian influenza viruses isolated from geese. J Vet Sci 19:406–415

Zou S, Zhang Y, Li X, Bo H, Wei H, Dong L, Yang L, Dong J, Liu J, Shu Y, Wang D (2019) Molecular characterization and receptor binding specificity of H9N2 avian influenza viruses based on poultry-related environmental surveillance in China between 2013 and 2016. Virology 529:135–143