Novel Reassortant Avian Influenza A(H5N6) Virus, China, 2021

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Although reports of human infection with influenza A(H5N6) increased in 2021, reports of similar H5N6 virus infection in poultry are few. We detected 10 avian influenza A(H5N6) clade 2.3.4.4b viruses in poultry from 4 provinces in China. The viruses showed strong immune-escape capacity and complex genetic reassortment, suggesting further transmission risk.

Severe human infection with influenza A(H5N6) virus was identified in China in 2014. During 2014–2020, a total of 26 cases of human infection were laboratory confirmed (*1*,*2*). Sporadic cases did not attract widespread attention until 2021 (*3*,*4*). During February–October 2021, China reported 24 laboratory-confirmed cases of human infection with H5N6 virus and 5 deaths (Figure 1, panel A); the number of human infections within only 8 months was close to the total for the previous 7 years.

The policy of compulsory poultry immunizations in China was adopted to prevent and control infection with highly pathogenic avian influenza (HPAI) subtype H5Nx (*5*). Although vaccination can reduce the likelihood of severe clinical disease and reduce shedding of virus in poultry, it cannot prevent sporadic infections with H5N6 virus in waterfowl. Because it is difficult to achieve a qualified 100% rate of H5N6 virus antibodies in waterfowl (*6*), these birds have become a weak link in prevention and control of the virus. In the context of selection pressure for vaccines and the absence of immunity in waterfowl, antigenic drift causes the H5N6 virus to continuously evolve (*7*), making currently available H5N6 vaccines ineffective.

On November 27, 2020, an outbreak of influenza A(H5N8) virus infections among wild swans was reported in China, resulting in the death of 2 swans (*8*,*9*). Since then, H5N8 clade 2.3.4.4b virus has spread throughout China, resulting in co-endemicity of H5N6 clade 2.3.4.4h/b and H5N8 clade 2.3.4.4b viruses. This 2020 outbreak was not the first outbreak of H5N8 virus in China; the earliest introduction of the virus into China was reported in Liaoning on September 12, 2014 (*10*,*11*). Because of China's immunization policies for poultry, H5N8 virus was quickly eliminated, only to reemerge in 2020.

The reappearance and spread of H5N8 virus is a serious threat to the poultry industry. The ecologic environment of the virus has been altered, given the increasing number of influenza A(H5N6) cases in humans. The current prevalence and mode of virus reassortment is of great concern. We discovered a novel H5N6 virus that has spread throughout the poultry industry, and similar viruses caused a sharp rise in human infections.

The Study

In June 2021, we isolated an H5N6 virus from a sick duck on a live poultry farm in Chengdu, Sichuan, China. In July 2021, we isolated another H5N6 virus from a dead chicken in the backyard of a human patient with confirmed infection in Chongqing. In August 2021, we detected an H5N6 virus on a chicken farm in Maoming City and another on a goose farm in Huizhou City (both cities in Guangdong, China). In September 2021, we detected an H5N6 virus on a chicken farm in Qinzhou, Guangxi. Last, in October 2021, we detected 5 H5N6 viruses at live poultry

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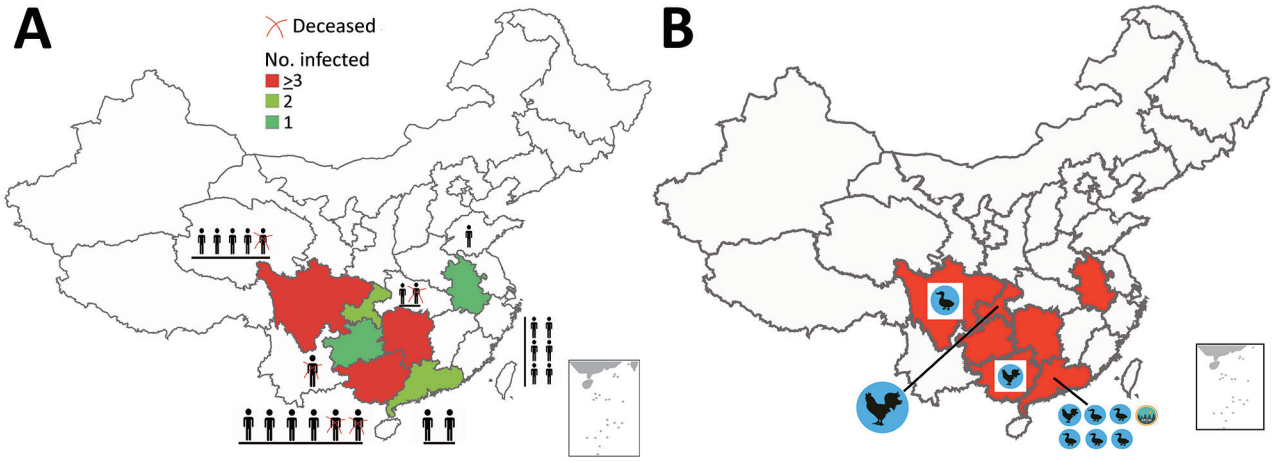

Figure 1. Distribution of confirmed cases of avian influenza A(H5N6) virus in humans, China, 2021. A) Provinces of the outbreaks and number of infected persons. A red X indicates a deceased person, and colors represent the number of infected persons. B) Region of novel H5N6 virus isolation from birds (chickens, ducks) and the environment (green icon). The red areas on the map indicate the provinces where human cases occurred in 2021. Insets indicate islands of China, additional sites of poultry breeding and human habitation.

markets in Dongguan City of Guangdong Province (3 from ducks, 1 from a goose, and 1 from the environment) (Figure 1, panel B).

To examine the genetic relationships of the viruses, we sequenced the genomes of the 10 H5N6 viruses and constructed maximum-likelihood phylogenetic trees divided into H5N6 epidemic clades (*12*), according to the protocol established by the World Health Organization (Figure 2, panel A). The H5N6 virus fell within 8 hemagglutinin (HA)

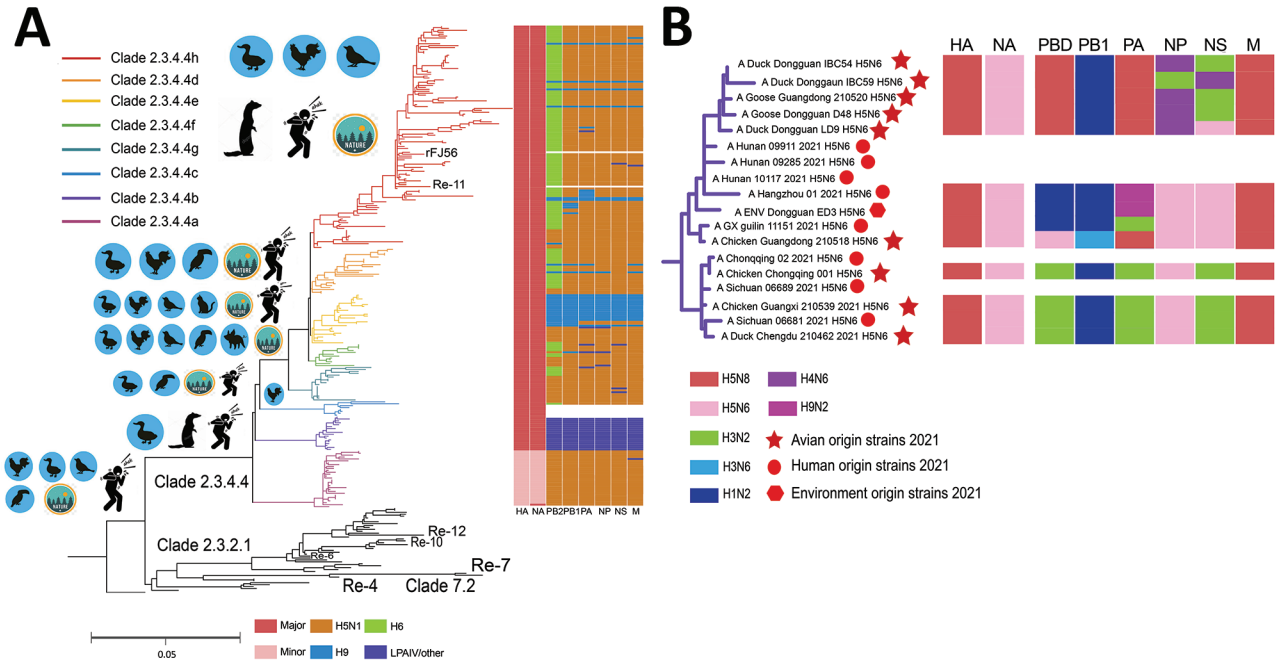

Figure 2. Visual depictions of avian influenza A(H5N6) viruses from China, 2021, and reference viruses. A) Maximum-likelihood phylogenetic tree showing comparisons with 332 H5 reference sequences downloaded from the GISAID database (http://www.gisaid.org). The Guizhou strain (A/Guizhou/1/2012) was set as the tree root, and all influenza A(H5N1) strains were set as the outgroup. Re-X/rFJ56 represents vaccine strains. To the left of each clade are images showing the corresponding primary hosts. On the right side is the dynamic reassortment profile of each avian (H5N6 virus in the phylogenetic tree; colors represent gene segments. Colored boxes below the graph correspond to possible potential donor viruses. B) Novel avian and environmental origin H5N6 strains. Red circles represent human strains (Appendix Tables 13–17, https://wwwnc.cdc.gov/EID/article/28/8/21-2241-App1.pdf). HA, hemagglutinin; LPAIV, low-pathogenicity avian influenza virus; NA, neuraminidase; NS, nonstructural; M, matrix. PA, polymerase acidic; PB, polymerase basic.

clades (2.3.4.4a to 2.3.4.4h). The similarity between the HA genes of all 10 viruses was 99.1%–100%, and all belonged to clade 2.3.4.4b. The HA genes of all the strains had the typical HPAI virus amino acid sequence RRKR↓GLF at the cleavage site (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/28/8/21-2241-App1.pdf). All viruses contain the S137A and T192I mutations in the HA gene, which enable it to bind to the human α-2, 6-linked sialic acid receptor, thereby increasing human susceptibility to the virus (*8*,*13*). Mammalian adaptive mutations, such as T33K, L89V, and G309D (*14*), were detected in the polymerase basic (PB) 2 gene of all strains, which increases the virulence of H5 viruses in mice (Table 1). Those variants are uncommon in previously circulating H5N6 (clade 2.3.4.4h) viruses.

Because there was an epidemiologic correlation between the avian virus strains from Chongqing and the human infections in Chongqing, we used Chongqing avian strains as a representative virus for the control analysis with human-origin virus sequences (Table 2). We deemed this approach to be the appropriate way to reveal links between the human-origin and avian-origin viruses. We found many similarities between the PB2, polymerase acidic, and nonstructural genes of the Chongqing avian-origin strain and those in influenza A(H3N2) virus, which suggests that the novel virus reassorted with H3N2 virus. The PB1 gene is derived from influenza A(H1N2) virus. The HA gene of the Chongqing H5N6 virus was 99.2% similar to that of the H5N8 virus and the matrix genes were 99.9% similar to those of the H5N8 virus, which leads us to believe that both genes originated from the H5N8 virus. The neuraminidase gene was derived from the H5N6 virus, however, and we speculate that the novel H5N6 virus is a reassortment of the H5N8 and H5N6 viruses. The HA and matrix genes of all viruses were derived from H5N8, which suggests that the novel H5N6 virus may use the gene backbone of the H5N8 virus. Other low-pathogenicity avian influenza viruses have been found to be involved in reassortment, which makes the internal genes of all strains appear complex but inconsistent (Appendix Tables 2–11). Notably, >1 pattern of reassortment seems to be present. Some human-derived strains have internal genes that are close to known H5N6 HPAI virus genes, and others are less closely related (Figure 2, panel B).

Bayesian analysis indicated that the viruses in the Pearl River Delta region (Guangdong), the upper reaches of the Yangtze River (Sichuan/Chongqin), and the middle and lower reaches of the Yangtze River (Hunan/Hangzhou) formed 3 subclades according to geographic characteristics (Appendix Figure 1). Compared with the study of Gu et al. (*15*), we found that the domestic novel H5N6 virus initially formed 3 subclades and an additional 7 types of genomes (Figure 2, panel B). Current vaccine strains lack protection (hemagglutination inhibition test) against novel H5N6 circulating virus strains (Appendix Table 12). This result differs from that reported by Gu et al. (*15*), which may result from isolation of the virus from different regions. Our study suggests that the virus poses a high risk for further transmission, which can be reduced or avoided by updating the vaccine strains. In January 2022, the Chinese government introduced

Table 1. Mutation sites for novel influenza A(H5N6) avian influenza viruses detected from humans and birds, China, 2021*

Strain	HA gene	Function	PB2 gene	Function	Host
A/Chongqing/00013/2021/H5N6	S137A/T192I	Increased α-2,6 sialic acid receptor affinity	T33K/L89V/G309D	Enhanced virulence of H5 viruses in mice	Human
A/Sichuan/06681/2021/H5N6					Human
A/Sichuan/06689/2021/H5N6					Human
A/Hunan/09285/2021/H5N6					Human
A/Hunan/09911/2021/H5N6					Human
A/Chongqing/02/2021/H5N6					Human
A/chicken/Chongqing/001/2021/H5N6V†					Avian
A/goose/Guangdong/210520/2021/H5N6V†					Avian
A/chicken/Guangdong/210518/2021/H5N6†					Avian
A/duck/Chengdu/210462/2021/H5N6†					Avian
A/goose/Dongguan/D48/2021/H5N6†					Avian
A/duck/Dongguan/LD9/2021/H5N6†					Avian
A/ENV/Dongguan/ED3/2021/H5N6†					Environment
A/duck/Dongguan/IBC54/2021/H5N6†					Avian
A/duck/Dongguan/IBC59/2021/H5N6†					Avian
A/chicken/Guangxi/210539/2021/H5N6†					Avian
A/whooper swan/Xinjiang/13/2020/H5N6‡	137S/192T	None			Avian
A/chicken/Suzhou/j6/2019/H5N6‡	137S/192T	None			Avian
A/China/Original/2018/H5N6‡	137S/192T	None			Human

*HA, hemagglutinin; PB2, polymerase basic 2.
†Avian and environmental strains isolated in study of novel reassortant avian influenza A(H5N6) virus, China, in 2021. The remaining reference strains were downloaded from GISAID (http://www.gisaid.org). All strains isolated from humans in 2021 were novel A(H5N6) viruses.
‡Reference strain (clade 2.3.4.4h) that did not have HA gene mutation before 2021.

Table 2. Genomic similarity of influenza virus isolate A/chicken/Chongqing/001/2021/H5N6 from a bird in China, 2021, to previously detected influenza viruses from birds in China*

Gene	Name	Subtype	Similarity, %	Host	Year
PB2	A/chicken/Guangxi/165C7/2014(H3N2)	H3N2	96.90	Chicken	2014
PB1	A/duck/Guangxi/293D21/2017(H1N2)	H1N2	97.90	Duck	2017
PA	A/duck/China/322D22/2018(H3N2)	H3N2	96.51	Duck	2018
NP	A/Muscovy duck/China/H5N6/2020(H5N6)	H5N6	95.20	Duck	2020
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	H3N2	97.90	Chicken	2016
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	H5N8	99.90	Cygnus	2020
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	H5N8	99.20	Cygnus	2020
NA	A/Muscovy duck/China/H5N6/2020(H5N6)	H5N6	99.28	Duck	2020

*The virus isolate came from a dead chicken in the backyard of a patient with confirmed infection in Chongqing, China. The host, subtype, and similarity of reference sequences were obtained from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). HA, hemagglutinin; NA, neuraminidase; NS, nonstructural; M, matrix. PA, polymerase acidic; PB, polymerase basic.

new vaccine strains H5-Re14 and rHN5801 to prevent an epidemic of poultry infection with H5N6 virus.

Because reported cases in humans were concentrated in southern China within a short time and most of these cases happened during the noninfluenza season, we suspect that transmissibility or viral load of the novel H5N6 viruses has increased. Furthermore, the virus that was isolated from environmental swab specimens (sewage ditch swab in a live poultry market) in Dongguan indicates that the virus is already present in the surrounding environment, which could increase the likelihood that the virus will infect humans.

Conclusions

At the peak of human cases, we isolated a total of 10 novel reassortment H5N6 virus strains from local poultry and the environment that were highly similar to the H5N6 (human-origin) virus reported during the same period. The human and avian viruses belong to clade 2.3.4.4b. The initial epidemic strains clustered into 3 geographically characterized subclades, and each avian strain had the same mammalian susceptibility mutation. The apparent antigenic differences between the virus and vaccine antiserum suggest further transmission risk.

Acknowledgments

We acknowledge all contributors who submitted the sequence data on which this research is based to the GISAID EpiFlu Database. All submitters of data may be contacted directly through GISAID (http://www.gisaid.org). Human case reports and data were obtained from http://www.flu.org.cn/scn/news.

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About the Author

Dr. Chen is a postgraduate student at South China Agricultural University in Guangzhou, China. His research interests are the epidemiology and pathogenesis of emerging and re-emerging infectious diseases.

References

1. Bi F, Jiang L, Huang L, Wei J, Pan X, Ju Y, et al. Genetic characterization of two human cases infected with the avian influenza A (H5N6) viruses—Guangxi Zhuang Autonomous Region, China, 2021. China CDC Wkly. 2021;3:923–8. https://doi.org/10.46234/ccdcw2021.199
2. Xiao C, Xu J, Lan Y, Huang Z, Zhou L, Guo Y, et al. Five independent cases of human infection with avian influenza H5N6—Sichuan Province, China, 2021. China CDC Wkly. 2021;3:751–6. https://doi.org/10.46234/ccdcw2021.187
3. Zhu W, Li X, Dong J, Bo H, Liu J, Yang J, et al. Epidemiologic, clinical, and genetic characteristics of human infections with influenza A(H5N6) viruses, China. Emerg Infect Dis. 2022;28.
4. Jiang W, Dong C, Liu S, Peng C, Yin X, Liang S, et al. Emerging novel reassortant influenza A(H5N6) viruses in poultry and humans, China, 2021. Emerg Infect Dis. 2022;28:1064–6. https://doi.org/10.3201/eid2805.212163
5. Bai R, Sikkema RS, Li CR, Munnink BBO, Wu J, Zou L, et al. Antigenic variation of avian influenza A(H5N6) viruses, Guangdong Province, China, 2014–2018. Emerg Infect Dis. 2019;25:1932–45. https://doi.org/10.3201/2510.190274
6. Qin J, Zhang Y, Shen X, Gong L, Xue C, Cao Y. Biological characteristics and immunological properties in Muscovy ducks of H5N6 virus-like particles composed of HA-TM/HA-TM_{H3} and M1. Avian Pathol. 2019;48:35–44. https://doi.org/10.1080/03079457.2018.1546375
7. Chen J, Li X, Xu L, Xie S, Jia W. Health threats from increased antigenicity changes in H5N6-dominant subtypes, 2020 China. J Infect. 2021;83:e9–11. https://doi.org/10.1016/j.jinf.2021.06.013
8. Li J, Zhang C, Cao J, Yang Y, Dong H, Cui Y, et al. Re-emergence of H5N8 highly pathogenic avian influenza virus in wild birds, China. Emerg Microbes Infect. 2021;10:1819–23. https://doi.org/10.1080/22221751.2021.1968317
9. He G, Ming L, Li X, Song Y, Tang L, Ma M, et al. Genetically divergent highly pathogenic avian influenza A(H5N8) viruses in wild birds, eastern China. Emerg Infect Dis. 2021;27:2940–3. https://doi.org/10.3201/eid2711.204893
10. Zhou LC, Liu J, Pei EL, Xue WJ, Lyu JM, Cai YT, et al. Novel avian influenza A(H5N8) viruses in migratory birds, China,

2013-2014. Emerg Infect Dis. 2016;22:1121–3. https://doi.org/10.3201/eid2206.151754
11. Poen MJ, Venkatesh D, Bestebroer TM, Vuong O, Scheuer RD, Oude Munnink BB, et al. Co-circulation of genetically distinct highly pathogenic avian influenza A clade 2.3.4.4 (H5N6) viruses in wild waterfowl and poultry in Europe and East Asia, 2017-18. Virus Evol. 2019;5:vez004. https://doi.org/10.1093/ve/vez004
12. Zhang D, Gao F, Jakovlić I, Zou H, Zhang J, Li WX, et al. PhyloSuite: an integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Mol Ecol Resour. 2020;20:348–55. https://doi.org/10.1111/1755-0998.13096
13. Huang J, Wu S, Wu W, Liang Y, Zhuang H, Ye Z, et al. The biological characteristics of novel H5N6 highly pathogenic avian influenza virus and its pathogenesis in ducks. Front Microbiol. 2021;12:628545. https://doi.org/10.3389/fmicb.2021.628545
14. Wu Y, Lin J, Yang S, Xie Y, Wang M, Chen X, et al. The molecular characteristics of avian influenza viruses (H9N2) derived from air samples in live poultry markets. Infect Genet Evol. 2018;60:191–6.
15. Gu W, Shi J, Cui P, Yan C, Zhang Y, Wang C, et al. Novel H5N6 reassortants bearing the clade 2.3.4.4b HA gene of H5N8 virus have been detected in poultry and caused multiple human infections in China. Emerg Microbes Infect. 2022;11:1174–85. https://doi.org/10.1080/22221751.2022.2063076

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Appendix

Methods

Sample collection and virus isolation

For the farm poultry, we collected tissues from the organs, larynxes, and lungs, and we collected throat and cloacal swabs for the birds in the LPM. We isolated the viruses in SPF chicken embryos and conducted RT-PCR amplification and sequencing. Throat and cloacal swab samples of poultry from live poultry markets were collected in Guangdong and Guangxi provinces, China. Disease tissues were from Chongqing and Chengdu provinces of China, where Chongqing disease samples were from dead chickens at the rear of confirmed cases. Each sample was placed in 2 ml of the PBS supplemented with penicillin (5000 U/ml) and streptomycin (5000 U/ml). All the samples were inoculated in the allantoic cavities of 10-day-old SPF embryonated chicken egg at 37°C. The allantoic fluid was collected and tested for hemagglutinin (HA) assay with 1% chicken red blood cells and then used in this study.

RNA extraction, RT-PCR, and DNA sequencing

RNA was extracted from the suspension of virus isolates with the RNeasy Mini Kit (Fastagen) as directed by the manufacturer. Two-step RT-PCR was conducted with universal primers as previously described (*1*), and the genome of H5N6 was amplified under standard conditions. PCR products were purified with a QIAamp Gel extraction kit (Qiagen) and sequenced with an ABI 3730 DNA Analyzer (Applied Biosystems).

Phylogenic analysis

All the available genomic sequences with the complete coding regions of influenza A(H5N6) viruses were downloaded from GISAID (http://www.gisaid.org/). The genome of H5N6 sequences data set (sequences alignment was available on request) was then created. The downloaded genomic sequences together with new 10 H5N6 strains were aligned using the MAFFT (version 7.149) program (*2*). The ML (Maximum likelihood) phylogenetic tree of each gene fragment was constructed by Phylosuite software (v 1.2.2) (*3*). Number of bootstrap with 5000 and Modle with GTR. The phylogenetic tree was visualized in the iTOL (version 6) website (https://itol.embl.de/).

Statistical analyses

Fluctuation curves of photoreceptors were generated by GraphPad Prism 5.0.

References

1. Bi Y, Chen Q, Wang Q, Chen J, Jin T, Wong G, et al. Genesis, Evolution and Prevalence of H5N6 Avian Influenza Viruses in China. Cell Host Microbe. 2016;20:810–21. PubMed https://doi.org/10.1016/j.chom.2016.10.022

2. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30:3059–66. PubMed https://doi.org/10.1093/nar/gkf436

3. Zhang D, Gao F, Jakovlić I, Zou H, Zhang J, Li WX, et al. PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Mol Ecol Resour. 2020;20:348–55. PubMed https://doi.org/10.1111/1755-0998.13096

Appendix Table 1. Information on the influenza A(H5N6) viruses in this study

Name	Location	Host	Date	Subtype	Cleavage site sequence
A_Duck_Chengdu_210462_2021_H5N6	Chengdu	Duck	2021/6/30	H5N6	RRKR↓GLF
A_Chicken_Chongqing_001_2021_H5N6	Chongqing	Chicken	2021/7/29	H5N6	RRKR↓GLF
A_Goose_Guangdong_210520_2021_H5N6	Guangxi	Chicken	2021/8/24	H5N6	RRKR↓GLF
A_Chicken_Guangdong_210518_2021_H5N6	Guangdong	Chicken	2021/8/24	H5N6	RRKR↓GLF
A_Chicken_Guangxi_210539_2021_H5N6	Guangdong	Chicken	2021/9/23	H5N6	RRKR↓GLF
A_Goose_Dongguan_D48_2021_H5N6	Guangdong	Chicken	2021/9/2	H5N6	RRKR↓GLF
A_Duck_Dongguan_LD9_2021_H5N6	Guangdong	Chicken	2021/9/15	H5N6	RRKR↓GLF
A_ENV_Dongguan_ED3_2021_H5N6	Guangdong	Chicken	2021/9/1	H5N6	RRKR↓GLF
A_Duck_Dongguan_IBC54_2021_H5N6	Guangdong	Chicken	2021/9/7	H5N6	RRKR↓GLF
A_Duck_Dongguan_IBC59_2021_H5N6	Guangdong	Chicken	2021/9/9	H5N6	RRKR↓GLF

Appendix Table 2. A_Duck_Donggaun_IBC59_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/goose/China/21FU008/2020(H5N8)	99.06%	H5N8
NA	A/China/Original/2018(H5N6)	98.91%	H5N6
PB2	A/Cygnus columbianus/Hubei/52/2020(H5N8)	100.00%	H5N8
PB1	A/duck/Guangxi/293D21/2017(H1N2)	100.00%	H1N2
PA	A/Cygnus columbianus/Hubei/116/2020(H5N8)	99.69%	H5N8
NP	A/chicken/Ganzhou/GZ43/2016(H3N2)	97.42%	H3N2
NS	A/duck/Mongolia/543/2015(H4N6)	97.90%	H4N6
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	100.00%	H5N8

Appendix Table 3. A_ENV_Dongguan_ED3_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/goose/China/21FU008/2020(H5N8)	99.59%	H5N8
NA	A/goose/Fujian/3.15_FZHX0007-C/2018(H5N6)	97.93%	H5N6
PB2	A/duck/Guangxi/293D21/2017(H1N2)	96.71%	H1N2
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.42%	H1N2
PA	A/wild bird /Shandong/11706/2019(H9N2)	97.95%	H9N2
NP	A/duck/Hunan/5.29_YYGK90P3-OC/2018(mixed)	95.97%	H5N6
NS	A/Duck/China/B1_NS/2018(H5N6)	99.15%	H5N6
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	100.00%	H5N8

Appendix Table 4. A_Duck_Dongguan_IBC54_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/goose/China/21FU008/2020(H5N8)	99.47%	H5N8
NA	A/China/Original/2018(H5N6)	98.91%	H5N6
PB2	A/Cygnus columbianus/Hubei/52/2020(H5N8)	99.79%	H5N8
PB1	A/duck/Guangxi/293D21/2017(H1N2)	100.00%	H1N2
PA	A/Cygnus columbianus/Hubei/116/2020(H5N8)	99.69%	H5N8
NP	A/duck/Mongolia/543/2015(H4N6)	99.04%	H4N6
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	97.42%	H3N2
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.81%	H5N8

Appendix Table 5. A_Goose_Dongguan_D48_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/goose/China/21FU008/2020(H5N8)	99.59%	H5N8
NA	A/duck/China/FJ1904/2019(H5N6)	98.48%	H5N6
PB2	A/Cygnus columbianus/Hubei/52/2020(H5N8)	99.69%	H5N8
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.61%	H1N2
PA	A/Cygnus columbianus/Hubei/116/2020(H5N8)	99.87%	H5N8
NP	A/duck/Mongolia/543/2015(H4N6)	99.10%	H4N6
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	97.30%	H3N2
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.81%	H5N8

Appendix Table 6. A_Duck_Dongguan_LD9_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	99.47%	H5N8
NA	A/China/Original/2018(H5N6)	98.77%	H5N6
PB2	A/Cygnus columbianus/Hubei/52/2020(H5N8)	99.70%	H5N8
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.65%	H1N2
PA	A/Cygnus columbianus/Hubei/116/2020(H5N8)	99.60%	H5N8
NP	A/duck/Mongolia/543/2015(H4N6)	99.16%	H4N6
NS	A/duck/Zhejiang/10.26_HZBX001-C/2018(mixed)	97.09%	H5N6
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.71%	H5N8

Appendix Table 7. A_Chicken_Chongqing_001_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	99.18%	H5N8
NA	A/Muscovy duck/China/H5N6/2020(H5N6)	99.28%	H5N6
PB2	A/chicken/Guangxi/165C7/2014(H3N2)	96.88%	H3N2
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.82%	H1N2
PA	A/duck/China/322D22/2018(H3N2)	96.51%	H3N2
NP	A/Muscovy duck/China/H5N6/2020(H5N6)	95.19%	H5N6
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	97.85%	H3N2
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.81%	H5N8

Appendix Table 8. A_Goose_Guangdong_210520_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	99.55%	H5N8
NA	A/Muscovy duck/China/H5N6/2020(H5N6)	99.13%	H5N6
PB2	A/Cygnus columbianus/Hubei/52/2020(H5N8)	99.70%	H5N8
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.39%	H1N2
PA	A/Cygnus columbianus/Hubei/116/2020(H5N8)	99.55%	H5N8
NP	A/duck/Mongolia/543/2015(H4N6)	99.22%	H4N6
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	97.53%	H3N2
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.71%	H5N8

Appendix Table 9. A_Chicken_Guangdong_210518_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	99.77%	H5N8
NA	A/duck/China/FJ1904/2019(H5N6)	97.97%	H5N6
PB2	A/Duck/China/B6_PB2/2019(H5N6)	98.33%	H5N6
PB1	A/duck/Mongolia/MN18-1/2018(H3N6)	99.05%	H3N6
PA	A/duck/Laos/XBY004/2014(H5N6)	98.22%	H5N8
NP	A/duck/Guangdong/8.30_DGCP030-C/2017(mixed)	98.40%	H5N6
NS	A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)	98.74%	H5N6
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.32%	H5N8

Appendix Table 10. A_Chicken_Guangzhou_210539_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	99.55%	H5N8
NA	A/Muscovy duck/China/H5N6/2020(H5N6)	99.13%	H5N6
PB2	A/chicken/Guangxi/165C7/2014(H3N2)	96.71%	H3N2
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.74%	H1N2
PA	A/duck/China/322D22/2018(H3N2)	97.76%	H3N2
NP	A/duck/Hunan/4.26_YYGK90R3-OC/2018(mixed)	98.66%	H5N6
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	98.09%	H3N2
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.71%	H5N8

Appendix Table 11. A_Chicken_Guangzhou_210539_2021_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)	97.46%	H5N6
NA	A/duck/Hainan/12.29_HKPL002-C/2017(H5N6)	97.82%	H5N6
PB2	A/chicken/Qingyuan/zd201601/2016(H9N2)	98.85%	H9N2
PB1	A/Muscovy duck/Vietnam/HN4856/2018(H5N6)	97.82%	H5N6
PA	A/goose/Fujian/3.15_FZHX0007-O/2018(H5N6)	97.98%	H5N6
NP	A/goose/Fujian/3.15_FZHX0010-O/2018(H5N6)	98.78%	H5N6
NS	A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)	98.40%	H5N6
M	A/Muscovy duck/Vietnam/HN5135/2018(H5N6)	99.42%	H5N6

Appendix Table 12. Hemagglutinin inhibition tests of antigen differences between influenza A (H5N6) virus and vaccine in China in 2021

	Antibody			
Antigen	Re-11	Re-12	rFJ56	rSD57
A_Chicken_Chongqing_001_2021_H5N6	2	0	2	0
A_Goose_Guangdong_210520_2021_H5N6	2	0	2	0
A_Chicken_Guangxi_210539_2021_H5N6	2	0	2	0
A_Goose_Dongguan_D48_2021_H5N6	5	3	3	2
A_Duck_Dongguan_LD9_2021_H5N6	3	2	3	0
A_ENV_Dongguan_ED3_2021_H5N6	0	0	0	0
A_Duck_Dongguan_IBC54_2021_H5N6	1	0	0	0
A_Duck_Dongguan_IBC59_2021_H5N6	3	0	3	1
Re-11	8	-	-	-
Re-12	-	9	-	-
rFJ56	-	-	10	-
rSD57	-	-	-	9

Re-11, Re-12, rFJ56 and rSD57 are vaccine strains widely used in China. Both antigen and antiserum of Re-11 and Re-12 were purchased from the Harbin Weike Biotechnology Development Company (www.hvriwk.com). Both antigen and antiserum of rFJ56 and rSD57 were from Guangzhou South China Biologic Medicine (http://www.gzscbm.com).

Appendix Table 13. A_Chongqing_00013_202_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)	97.46%	H5N6
NA	A/duck/Hainan/12.29_HKPL002-C/2017(H5N6)	97.82%	H5N6
PB2	A/chicken/Qingyuan/zd201601/2016(H9N2)	98.85%	H9N2
PB1	A/Muscovy duck/Vietnam/HN4856/2018(H5N6)	97.82%	H5N6
PA	A/goose/Fujian/3.15_FZHX0007-O/2018(H5N6)	97.98%	H5N6
NP	A/goose/Fujian/3.15_FZHX0010-O/2018(H5N6)	98.78%	H5N6
NS	A/goose/Guangdong/7.20_DGCP010-C/2017(H5N6)	98.40%	H5N6
M	A/Muscovy duck/Vietnam/HN5135/2018(H5N6)	99.42%	H5N6

Appendix Table 14. A_GX-guilin_11151_202_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnus columbianus/Hubei/53/2020(H5N8)	99.66%	H5N8
NA	A/Muscovy duck/Vietnam/HN6606/2020(H5N6)	99.02%	H5N6
PB2	A/duck/Guangxi/293D21/2017(H1N2)	96.50%	H1N2
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.18%	H1N2
PA	A/duck/Vietnam/HN5958/2019(H3N8)	97.58%	H3N8
NP	A/chicken/Shandong/2.28_TAWM016-C/2017(H5N6)	98.72%	H5N6
NS	A/Muscovy duck/Vietnam/HN6610/2020(H5N6)	99.54%	H5N6
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.61%	H5N8

Appendix Table 15. A_Hangzhou_01_202_H5N6 genomic similarity

Gene	Name	Similarity	Subtype
HA	A/Cygnuscolumbianus/Hubei/53/2020(H5N8)	99.16%	H5N8
NA	A/Muscovy duck/Vietnam/HN6606/2020(H5N6)	98.77%	H5N6
PB2	A/duck/Guangxi/293D21/2017(H1N2)	96.53%	H1N2
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.05%	H1N2
PA	A/wild bird /Shandong/11706/2019(H9N2)	97.77%	H9N2
NP	A/chicken/Anhui/8.28_YHZGS017-O/2018(H5N6)	98.19%	H5N6
NS	A/Muscovy duck/Vietnam/HN6609/2020(H5N6)	99.03%	H5N6
M	A/eurasian coot/Shandong/W5611/2020(H5N8)	99.49%	H5N8

Appendix Table 16. A_Sichuan_06681_2021_H5N6 genomic similarity

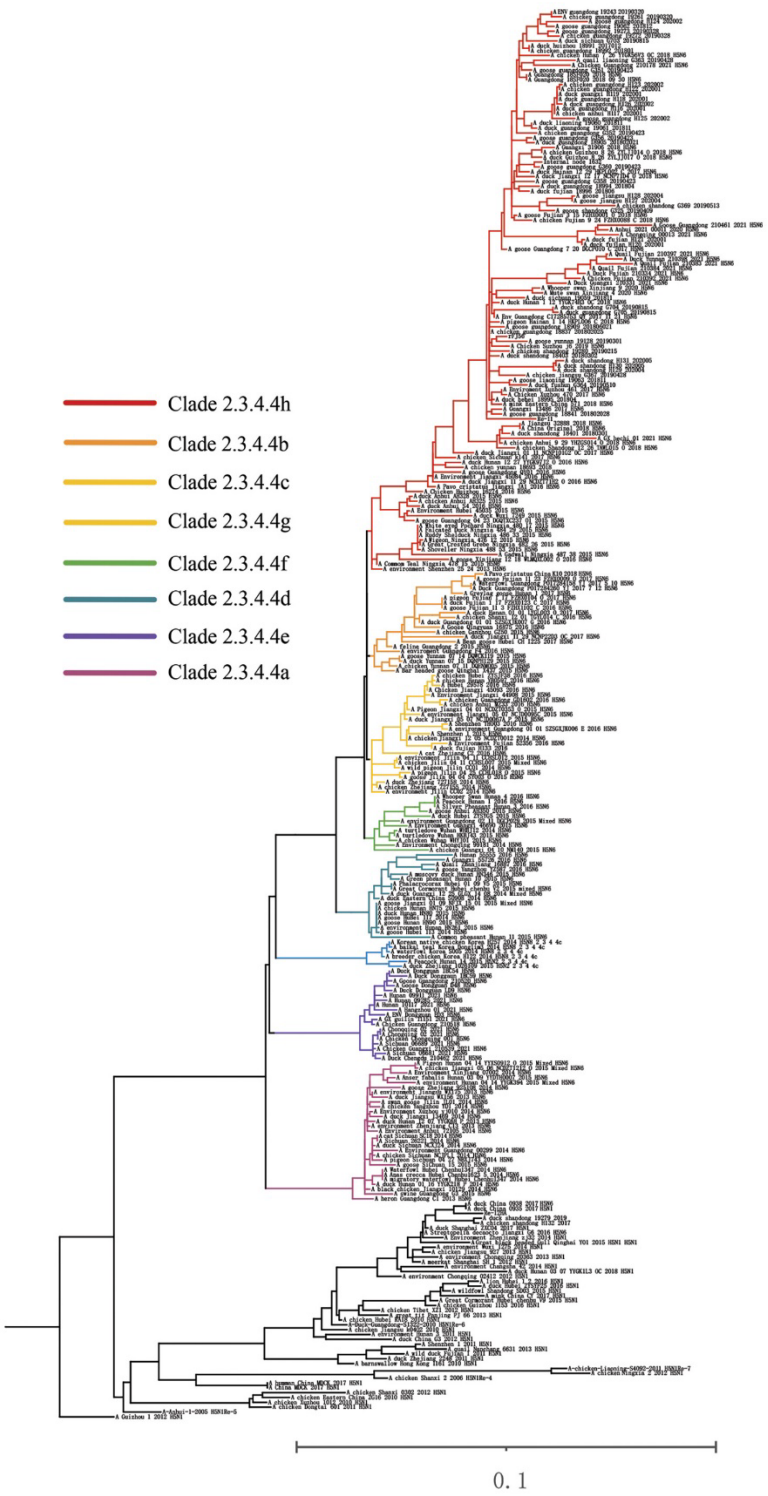
Gene	Name	Similarity	Subtype
HA	A/environment sample/China/TZ001/2021(H5N8)	99.44%	H5N8
NA	A/duck/Hainan/12.29_HKPL002-C/2017(H5N6)	97.54%	H5N6
PB2	A/chicken/Guangxi/165C7/2014(H3N2)	96.63%	H3N2
PB1	A/duck/Guangxi/293D21/2017(H1N2)	97.86%	H1N2
PA	A/duck/China/322D22/2018(H3N2)	97.76%	H3N2
NP	A/duck/Hunan/4.26_YYGK90R3-OC/2018(mixed)	98.66%	H5N6
NS	A/chicken/Ganzhou/GZ43/2016(H3N2)	98.09%	H3N2
M	A/Cygnus columbianus/Hubei/56/2020(H5N8)	99.90%	H5N8

Appendix Table 17. A_GX_hechi_01_2021_H5N6 genomic similarity

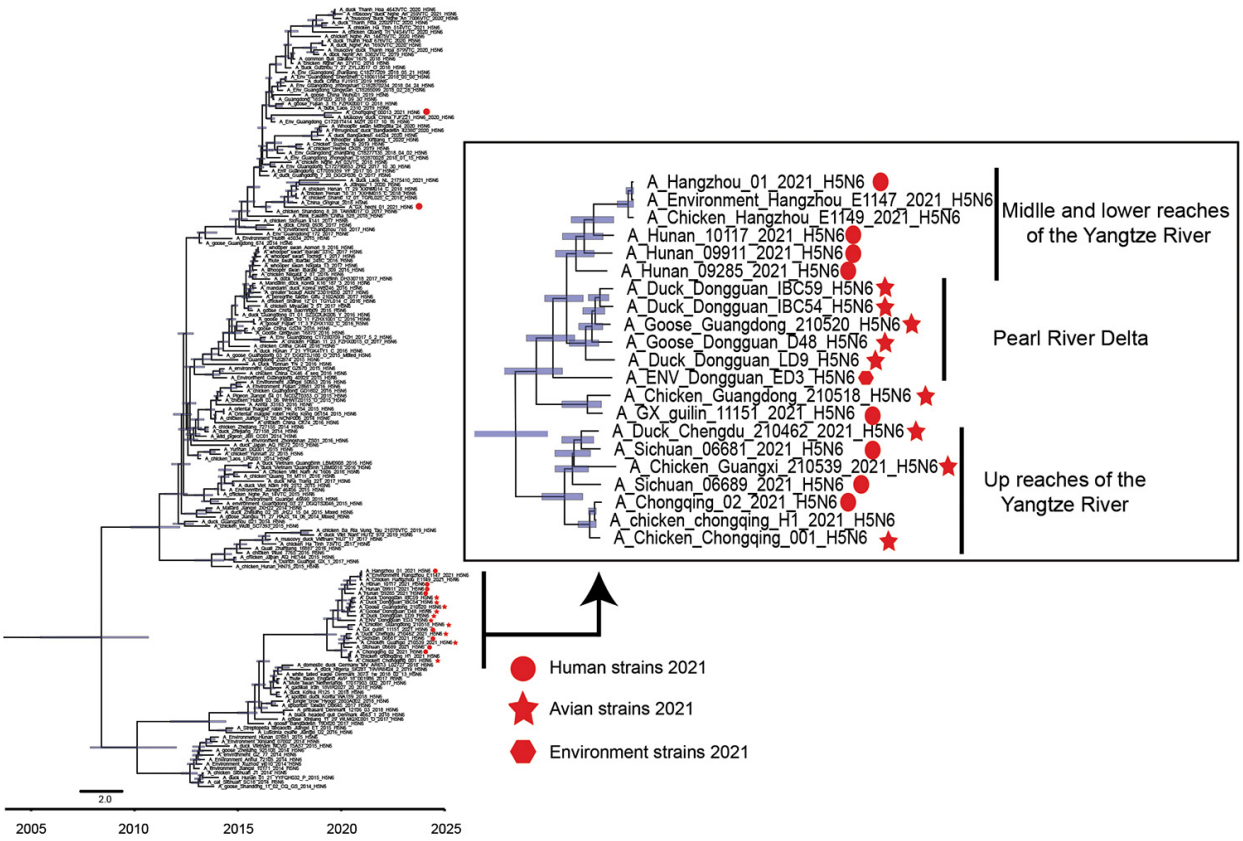
Gene	Name	Similarity	Subtype
HA	A/environment/Anhui/11.29_YHZGS003-E/2018	97.74%	H5N8
NA	A/Muscovy duck/Vietnam/HN6606/2020(H5N6)	99.23%	H5N6
PB2	A/goose/Fujian/3.15_FZHX0011-O/2018(H9N2)	92.18%	H9N2
PB1	A/duck/China/322D22/2018(H3N2)	94.79%	H3N2
PA	A/duck/Vietnam/HN5958/2019(H3N8)	97.04%	H3N8
NP	A/duck/Vietnam/HN6611/2020(H5N6)	99.62%	H5N6
NS	A/Muscovy duck/Vietnam/HN6610/2020(H5N6)	99.43%	H5N6
M	A/chicken/Hunan/4.26_YYGK37R3-OC/2018(mixed)	98.64%	H5N8

Appendix Table 18. The accession numbers in GISAID of the influenza A(H5N6) viruses in this study

Name	ID sequence	Time
A_Duck_Chengdu_210462_2021_H5N6	EPI_ISL_5797454	2021/6/30
A_Chicken_Chongqing_001_2021_H5N6	EPI_ISL_5797453	2021/7/29
A_Goose_Guangdong_210520_2021_H5N6	EPI_ISL_5797280	2021/8/24
A_Chicken_Guangdong_210518_2021_H5N6	EPI_ISL_5797279	2021/8/24
A_Chicken_Guangxi_210539_2021_H5N6	EPI_ISL_5797272	2021/9/23
A_Goose_Dongguan_D48_2021_H5N6	EPI_ISL_5797238	2021/9/2
A_Duck_Dongguan_LD9_2021_H5N6	EPI_ISL_5797237	2021/9/15
A_ENV_Dongguan_ED3_2021_H5N6	EPI_ISL_5797236	2021/9/1
A_Duck_Dongguan_IBC54_2021_H5N6	EPI_ISL_5797235	2021/9/7
A_Duck_Dongguan_IBC59_2021_H5N6	EPI_ISL_5795871	2021/9/9

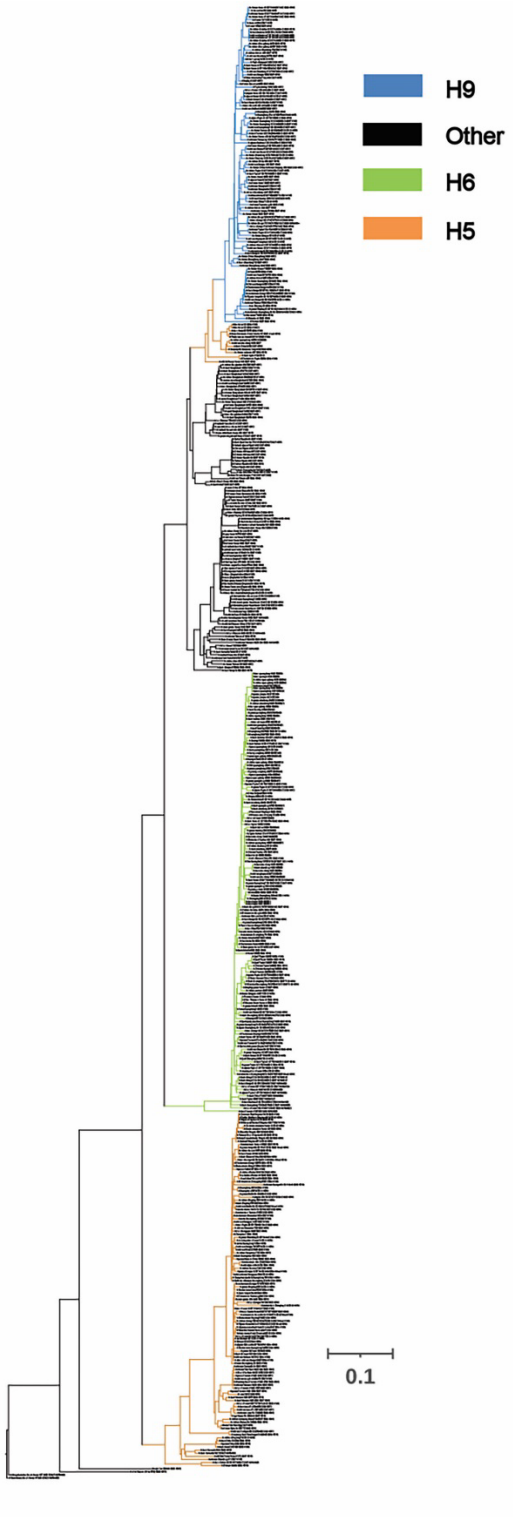


Appendix Figure 1. Phylogenic tree of HA gene sequences. The total HA genes (n=342) of H5 viruses collected from 2000-2021 in China were analyzed. The tree is rooted to A/Guizhou/1/2012/H5N6. The red color indicates the novel H5N6 isolates in this study.

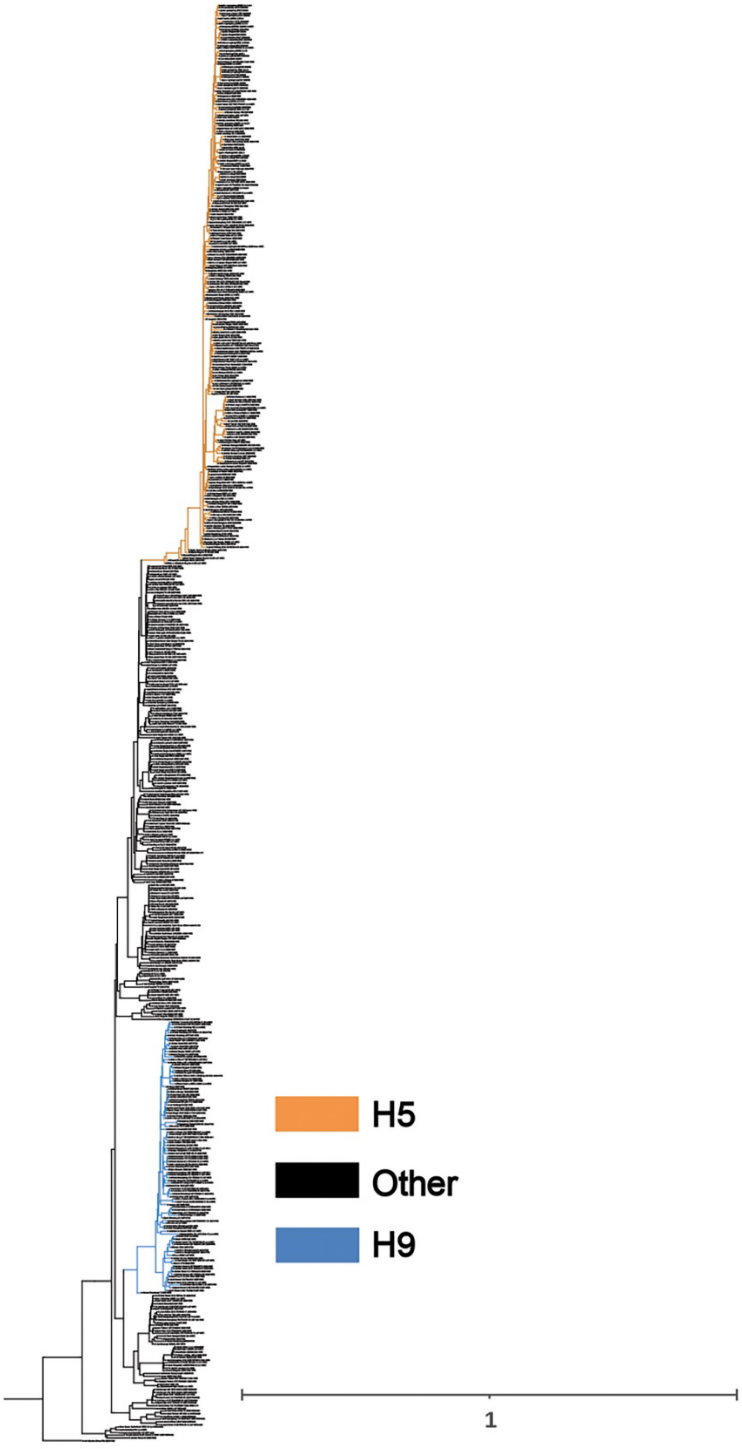


Appendix Figure 2. Maximum confidence tree of the HA gene sequence. The total number of HA genes (n = 174) from H5N6 viruses collected in 2014-2021 was analyzed. The red circle represents the human origin H5N6 isolate in this study, and the Red Pentagram represents the avian origin isolate in this study. The MCMC chain was run for 100 million steps with tree sampling every 1000 steps, and a 10% burn-in fraction was used when calculating the final MCC tree with common ancestor node heights in TreeAnnotator v.1.10.4.

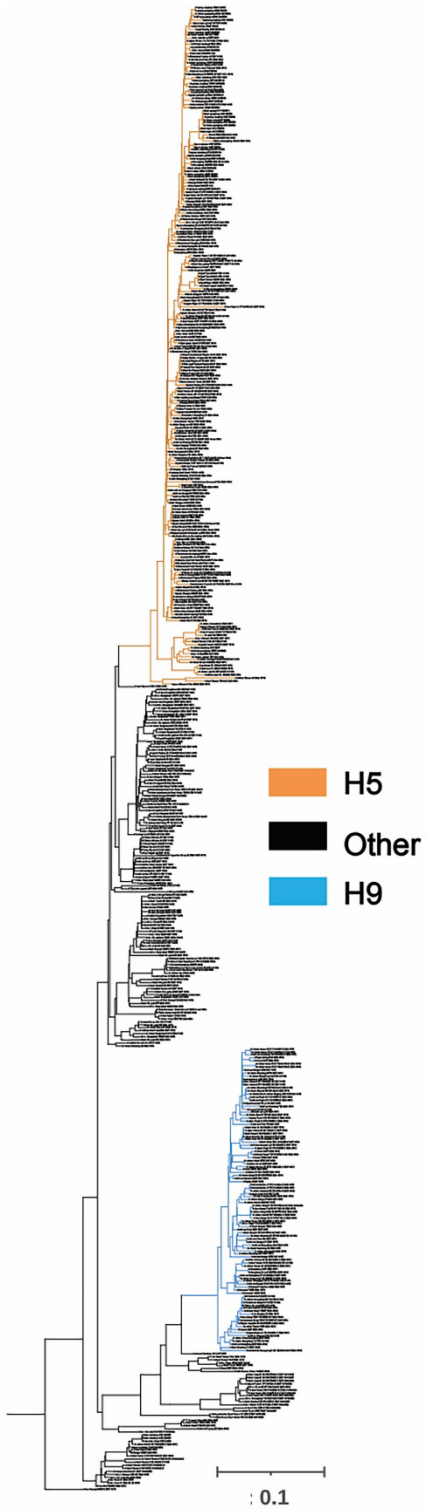
Appendix Figure 3. Phylogenic tree of NA gene sequences. The total NA genes (n=242) of H5 viruses collected from 2000-2021 in China were analyzed.



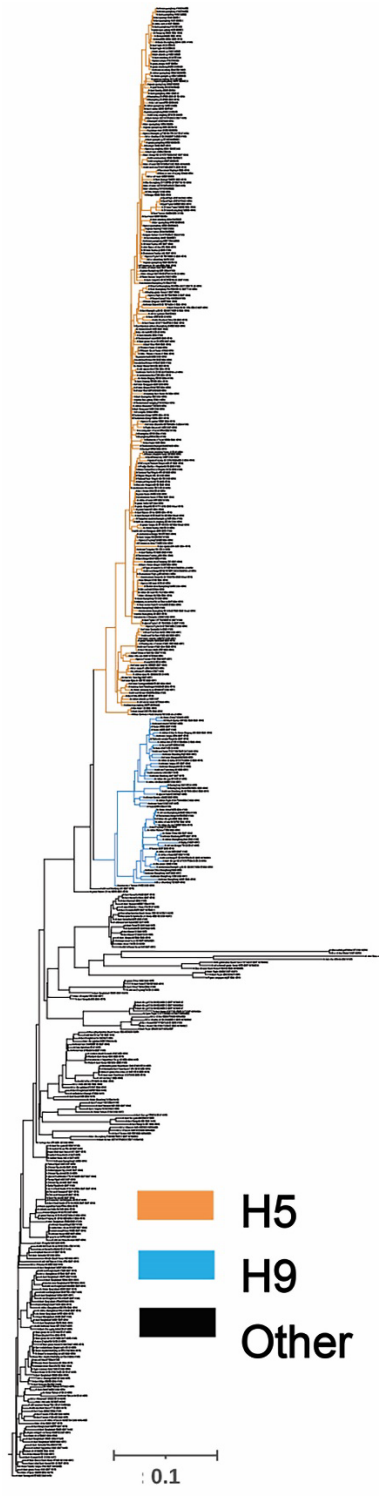
Appendix Figure 4. Phylogenic tree of PB2 gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.



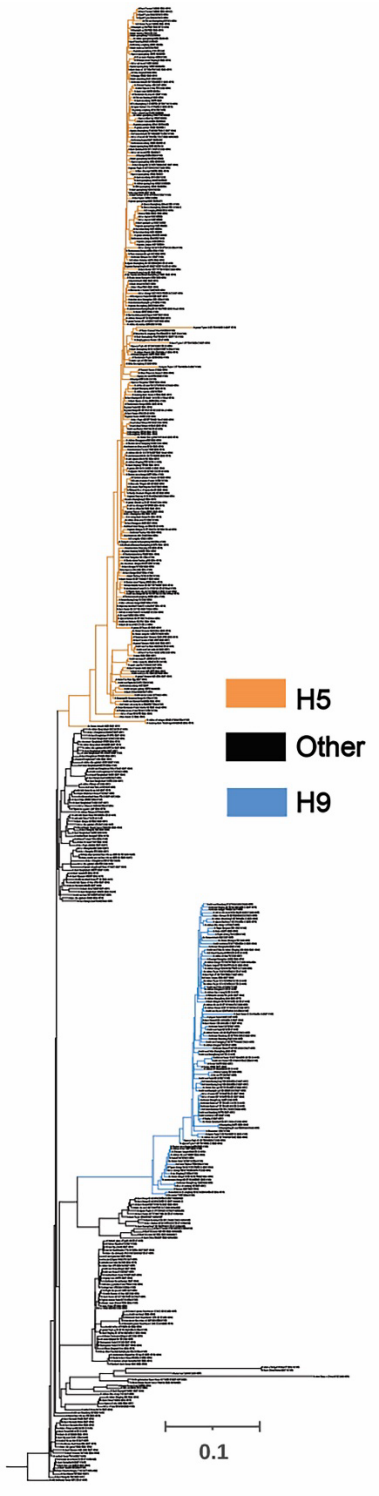
Appendix Figure 5. Phylogenic tree of PB1 gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.



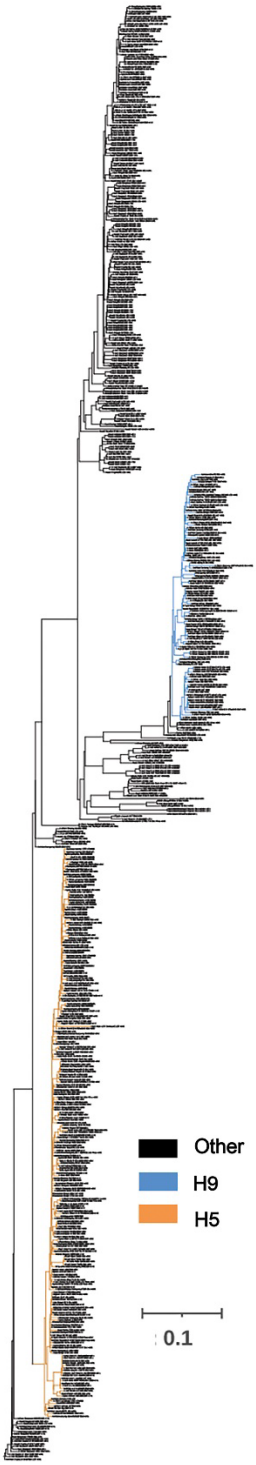
Appendix Figure 6. Phylogenic tree of PA gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.



Appendix Figure 7. Phylogenic tree of NP gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.



Appendix Figure 8. Phylogenic tree of NS gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.



Appendix Figure 9. Phylogenic tree of M gene sequences. The phylogenetic tree was drawn with the multiple subtype sequences possible for H5N6, which were considered to be highly similar to the subtype when it was in the same branch as the corresponding other subtype sequences.