

Outbreaks of Highly Pathogenic Avian Influenza (H5N6) Virus Subclade 2.3.4.4h in Swans, Xinjiang, Western China, 2020

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In January 2020, the subclade 2.3.4.4h of highly pathogenic avian influenza (H5N6) virus infected migratory whooper swans and mute swans in Xinjiang, western China. The virus is lethal to chickens and ducks but has low pathogenicity in mice. Antigenically, this subclade is similar to the H5N1 vaccine seed virus Re-11.

The H5 highly pathogenic avian influenza viruses (HPAIVs) of clade 2.3.4.4 are of great concern because of their global spread and circulation. Ample evidence indicates that clade 2.3.4.4 H5 viruses derived neuraminidase (NA) gene from other low-pathogenicity avian influenza viruses (LPAIVs) co-circulating in migratory birds, and new subtypes of H5N2, H5N5, H5N6, and H5N8 HPAIVs have been detected in wild bird species and poultry globally (1,2). To date, H5 viruses of clade 2.3.4.4 have evolved into 8 subclades (2.3.4.4a to 2.3.4.4h) according to the World Health Organization's (WHO) nomenclature system (1). Among them, H5N6 is the only subtype that has caused human infections. As of August 2019, a total of 24 human cases have been reported to WHO; the mortality rate is 67% (3,4).

H5N6 virus of subclade 2.3.4.4a was first detected in poultry in Laos in 2013, then spread to Vietnam and China and caused numerous cases in these ar-

reas. H5N8 virus of subclade 2.3.4.4b caused disease outbreaks in wild birds and poultry in Korea in 2014, then spread to North America through bird migration and established a new subclade, 2.3.2.4c. When the H5N8 virus of subclade 2.3.4.4b landed in Europe and Africa, it reassorted with the local LPAIV and produced H5N6 with a novel internal gene cassette in 2017 (5). Simultaneously, the H5N6 viruses of subclades 2.3.4.4d, 2.3.4.4e, 2.3.4.4f, 2.3.4.4g, and 2.3.4.4h established in poultry and wild birds in Southeast Asia (1,6–8). Among the 8 subclades of 2.3.4.4, only 3 (H5N6 2.3.4.4b, 2.3.4.4e, and 2.3.4.4f) had been previously detected in swans (1).

Since 2004, different vaccines have been developed and widely administered among poultry flocks in China and other countries for H5 avian influenza control, and the vaccine seed viruses used in China have been updated regularly to ensure antigenic match between the vaccine strain and the prevalent strains (9,10). After the H7N9 HPAIVs emerged in China in 2017, an H5/H7 combined inactivated vaccine was developed and used in poultry (11,12). Currently, the vaccine seed virus Re-11 is being used to control the clade 2.3.4.4 viruses (10). In our study, we analyzed the genetic evolution, antigenicity, and pathogenicity of the H5N6 HPAIVs isolated from migratory whooper swans (*Cygnus cygnus*) and mute swans (*C. olor*) in Xinjiang, western China, in January 2020.

The Study

The first sick whooper swan was found on December 29, 2019, in Sala Village, Samuyuzi Township, Yinling City, Xinjiang Uyghur Autonomous Region. The bird died on January 1, 2020. By January 17, deaths had been reported in 58 swans in 6 locations (Table 1; Figure 1, panel A). We received 5 batches of clinical

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Table 1. Avian influenza (H5N6) outbreaks among migratory whooper swans (*Cygnus cygnus*) and mute swans (*C. olor*), Xinjiang Province, China, January 2020

Time	Location description	Flock size*	Bird information	
			No. swans†	Dead
2019 Dec 29 to 2020 Jan 5	Small lake in Yining County, Ili Kazak City	>100	100	10
2020 Jan 1–6	Natural park in Yining County, Ili Kazak City	>2,300	40 (270)	6 (3)
2020 Jan 1–8	Natural park in Bole County, Botorla City	160	55	6
2020 Jan 8–10	Natural park in Hejing County, Bayingola City	1150	150	1
2020 Jan 12–14	Wetland in Manas County, Changji City	2,000	800	13
2020 Jan 17–20	Water reservoir in Maguan Chu County, Shihezi City	1,000	150	19

*Estimated number of total migratory birds at that location.
 †Numbers are whooper swans, except numbers in parentheses, which are mute swans.

samples from 13 dead birds (11 whooper swans and 2 mute swans), and 13 H5N6 viruses were isolated. The hemagglutinin (HA) subtypes were identified by a hemagglutinin-inhibition test with a panel of H1–H16 subtype antisera, whereas the NA subtypes were detected by reverse transcription PCR with a panel of N1–N9 subtype-specific primers (11).

To trace the origin of the viruses and understand their genetic relationship, we sequenced the genome of the 13 viruses and performed comparative phylogenetic analysis with the representative H5 HPAIVs that were recommended by WHO (1). All 13 H5N6 viruses possess high identity with each other (99.5%–100%); 7 of 8 segments are closely related to the H5N6 virus isolated from environmental samples in Guangdong Province in 2017, whereas the other 1, nonstruc-

tural protein gene, is closest to A/chicken/Nghe An/01VTC/2018(H5N6) (Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/26/12/20-1201-App1.pdf>). The HA gene has the typical highly pathogenic amino acid sequence -RRKR- in its cleavage site, and a few mammalian adaptation mutations were detected in the genome (Table 2) (13). In the maximum clade credibility tree, the HA genes of the 13 H5N6 viruses are grouped into subclade 2.3.4.4h with the HA genes of the strains recently found in Vietnam, China, and Russia (1) (Figure 1, panel B). The neighbor-joining phylogenetic trees of the 8 gene segments are shown in Appendix Figure 1.

The hemagglutinin-inhibition test was performed with polyclonal antiserum generated from the SW/XJ/1/2020(H5N6) and the currently used H5N1

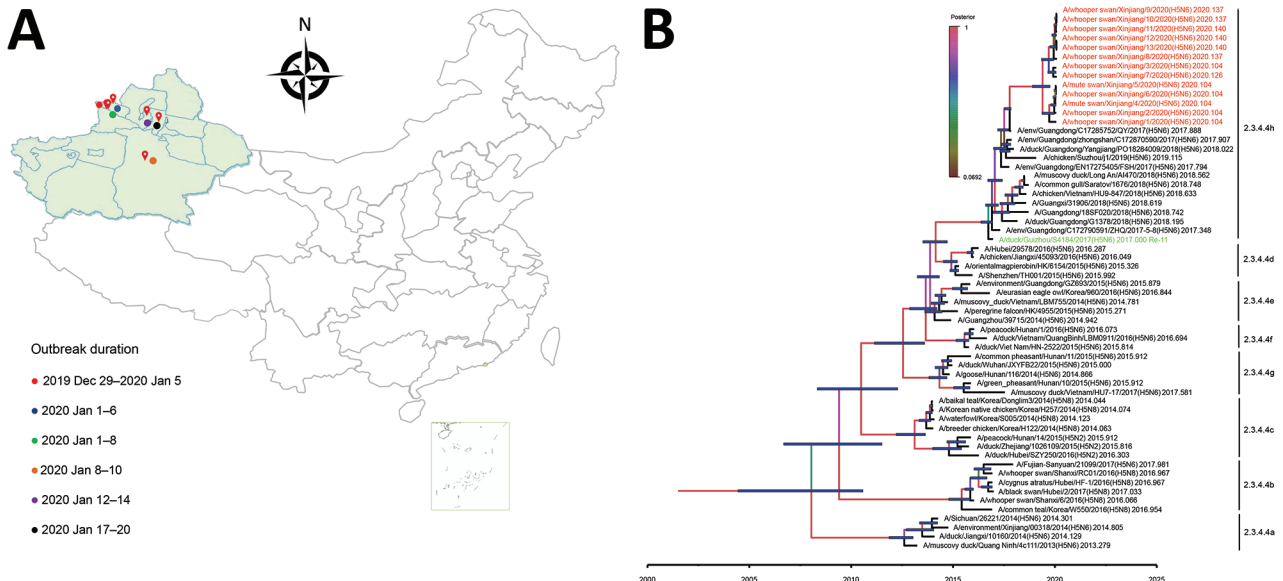


Figure 1. Geography and phylogeny of avian influenza (H5N6) outbreaks among migratory whooper swans (*Cygnus cygnus*) and mute swans (*C. olor*), Xinjiang Province, China, January 2020. A) Disease outbreak sites are marked with red drops, and dates of the outbreaks are indicated. Inset map shows islands in the South China Sea. B) Phylogenetic tree of the hemagglutinin (HA) genes of H5 viruses. The HA gene maximum clade credibility tree of the H5 viruses was constructed by using the BEAST 1.8.4 software package (<https://beast-dev.github.io/beast-mcmc>). Node bars indicate 95% highest posterior density of the node height. Each branch is colored by posterior probability: the 13 H5N6 viruses reported in this study are shown in red and the HA donor of the H5N1 vaccine Re-11 in green. The time to the most recent common ancestor is labeled at the bottom of the tree, which was estimated by using the Bayesian Markov chain Monte Carlo method in the BEAST 1.8.4 software package.

Table 2. Virulence related molecular markers detected in the WS/XJ/1/2020 (H5N6) virus detected among migratory whooper swans (*Cygnus cygnus*) and mute swans (*C. olor*), Xinjiang Province, China, January 2020

Protein	Amino acid/motif	Phenotypic consequences
Hemagglutinin	Cleavage site motif: -RRKR ⁻ G-	Polybasic cleavage motif sequence required for high pathogenicity of avian influenza viruses in chickens
Neuraminidase	Stalk deletion 58–68	Increased virulence in mice
Polymerase acidic protein	515T	Increased polymerase activity in mammalian cells
Matrix protein 1	30D 215A	Increased virulence in mice Increased virulence in mice
Nonstructural protein 1	80–84 deletion 42S 98F 101M 222–225 ESEV (PDZ domain)	Increased virulence in mice Increased virulence in mice Increased virulence in mice Increased virulence in mice Increased virulence in mice

inactivated vaccine Re-11, which carries the HA gene from A/duck/Guizhou/S4184/2017(H5N6) virus (10). We found that the SW/XJ/1/2020(H5N6) cross-reacted well with Re-11 antisera, and vice versa (Appendix Table 2), yielding a cross-reactivity R value of 0.26.

We conducted an intravenous pathogenicity index test in chickens with the index virus, WS/XJ/1/2020(H5N6), by following the protocol of the World Organisation for Animal Health (OIE) (14). Ten 6-week-old specific-pathogen-free chickens were inoculated with 0.2 mL of virus intravenously, and all the birds died within 3 days postinoculation, yielding an intravenous pathogenicity index test value of 2.59.

We tested the virulence and transmission of the WS/XJ/1/2020(H5N6) in ducks as previously described (2). Eight 3-week-old specific-pathogen-free ducks were intranasally inoculated with 10^6 50% egg infective dose (EID_{50}) WS/XJ/1/2020(H5N6), and 3 uninfected ducks were put in the same cage 24 hours later for monitoring transmission. Three virus-inoculated ducks were euthanized on day 3 postinoculation, and high titers of virus were detected in the tested organs (Figure 2, panel A). Virus was also detected in the oropharyngeal and cloacal swabs of the surviving virus-inoculated ducks and the contact ducks on days 3 and 5 postinoculation (Figure 2, panel B). All

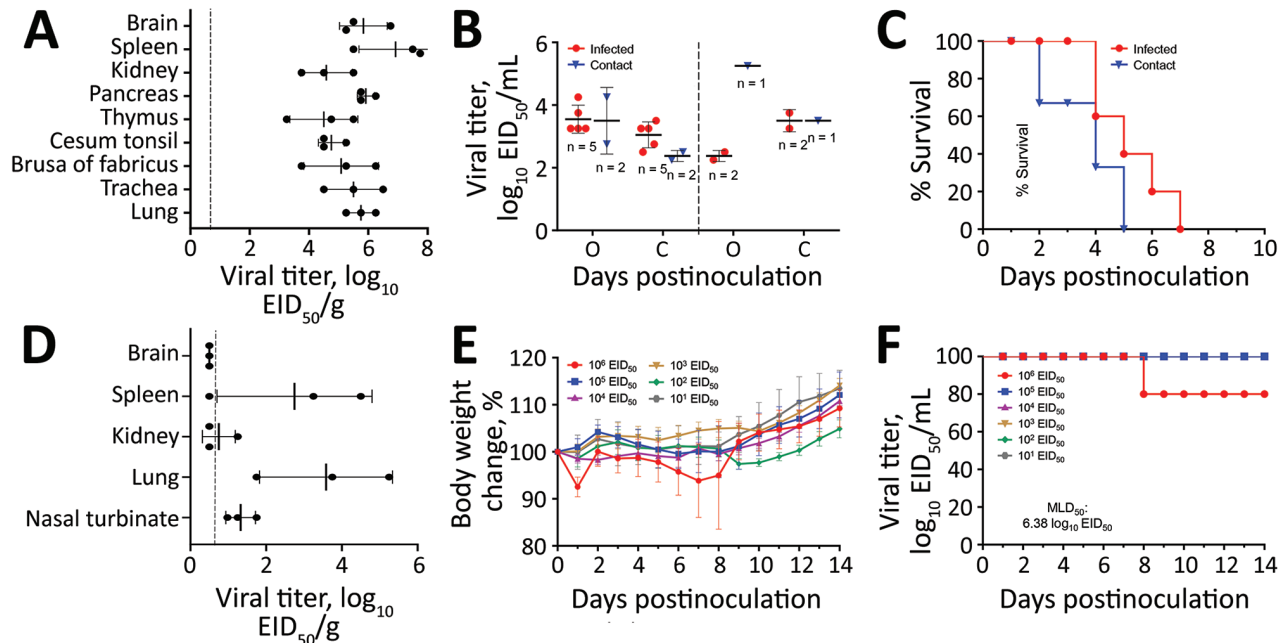


Figure 2. Replication and virulence of the WS/XJ/1/2020(H5N6) isolate in ducks and mice in a laboratory test performed after H5N6 avian influenza (H5N6) outbreaks among migratory whooper swans (*Cygnus cygnus*), Xinjiang Province, China, January 2020. A) Viral titer in organs of ducks that were euthanized on day 3 postinoculation. B) Viral titers in oropharyngeal and cloacal swabs from all surviving ducks were collected on days 3 and 5 postinoculation. C) Lethality of the virus in ducks. D) Viral titer in organs of mice that were euthanized on day 3 postinoculation. E) Bodyweight change of mice after inoculation with different doses of the virus. F) MLD_{50} of the virus. Viral titers in panels A, B, and D are shown as the mean \pm SD. The dashed lines indicate the lower limit of detection. EID_{50} , 50% egg infective dose; MLD_{50} , 50% mouse lethal dose.

5 virus-inoculated ducks and 3 contact ducks died within 7 days postinoculation (Figure 2, panel C).

The replication and 50% mouse lethal dose (MLD₅₀) of the WS/XJ/1/2020(H5N6) were evaluated in BALB/c mice as previously reported (2). Three mice were intranasally inoculated with 10⁶ EID₅₀ of WS/XJ/1/2020(H5N6) in a volume of 50 μL and were euthanized on day 3 postinoculation to assess virus replication in organs, and we found the virus in the brain of 1 mouse, the spleens of 2 mice, and the nasal turbinates and lungs of all 3 mice, but not in the kidneys of any mouse (Figure 2, panel D). To test the MLD₅₀, groups of five 6-week-old mice were intranasally inoculated with 10¹ to 10⁶ EID₅₀ of WS/XJ/1/2020(H5N6) in a volume of 50 μL and were monitored for bodyweight loss and death for 14 days. Only 1 of 5 mice that received the highest dose of 10⁶ EID₅₀ died on day 8 postinoculation; all other mice survived the 14-day observation period, yielding an MLD₅₀ value of 6.38 log₁₀ EID₅₀ (Figure 2, panel E, F).

Conclusions

A total of 58 swans died from H5N6 virus infection in 6 wild bird habitats in Xinjiang in January 2020, and we isolated 13 similar H5N6 HPAIVs from the swan specimens. These viruses bear the HAs of subclade 2.3.4.4h, which were previously detected in other bird species but not in swans.

The WS/XJ/1/2020(H5N6) is highly pathogenic to chickens and ducks, and antigenically close to the H5N1 vaccine seed virus Re-11. Although the virus is low pathogenic in mice, it bears multiple residues that can increase its virulence in mammals, and thus might pose a potential threat to public health.

Wild birds carry and spread the H5 HPAIV, as evidenced by the dissemination of the clade 2.2 viruses from Asia to Europe and Africa in 2005, and the intercontinental distribution of the clade 2.3.4.4b viruses in 2014 (5,15). The prospect of these H5N6 viruses detected in swans being distributed widely by wild birds is worrisome. Therefore, with the migratory season coming, surveillance and preventive measures should be implemented in poultry raised on the migration routes of wild birds.

Acknowledgments

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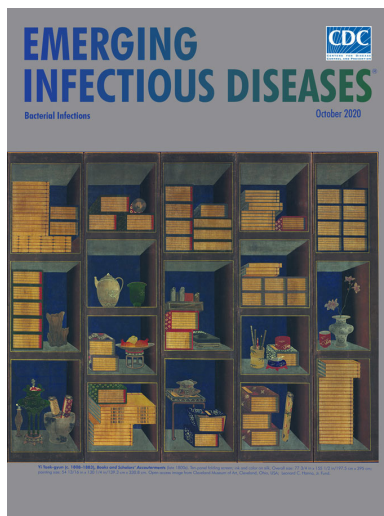
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**EMERGING
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Appendix

Biosafety Statement and Facility

The diagnosis of H5N6 and all experiments with live H5N6 viruses were conducted in the enhanced animal biosafety level 3 (ABSL3+) facility in the HVRI of the CAAS, which is approved for such use by the Ministry of Agriculture and Rural Affairs of China. All animal studies were approved by the Review Board of the HVRI, CAAS.

Animal Testing

All experiments using animals were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. All protocols were approved by the Committee on the Ethics of Animal Experiments of the HVRI of the CAAS.

SPF Eggs

SPF embryonated chicken eggs (CEEs) were obtained from Harbin Weike Biotechnology Development Company, Harbin, China.

Chickens and Ducks

Six-week-old specific-pathogen-free (SPF) chickens (White Leghorn) and 3-week-old SPF ducks (Shaoxin shelduck, a local bred) were obtained from the Experimental Animal Division of HVRI and housed in ventilated isolators (maximum 8 birds per isolator) in the enhanced animal biosafety level 3 (ABSL3+) facility at the HVRI of the CAAS.

Mice

Five-week-old female BALB/c mice were purchased from Vital River Laboratories (Beijing, China) and housed in ventilated cages (maximum 8 mice per cage) in the enhanced animal biosafety level 3 (ABSL3+) facility at the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS).

Virus Isolation and Identification

The 13 H5N6 viruses used in this study were isolated from samples of dead wild birds sent to the National Avian Influenza Reference Laboratory for the diagnosis of suspected cases of influenza A virus infection in January, 2020 (Table 1, <https://wwwnc.cdc.gov/EID/article/26/12/20-1201-T1.htm>). The samples were inoculated in 9–11 days SPF CEEs, identification of the HA subtype in the allantoic fluid of HA positive chicken eggs was performed using the hemagglutinin inhibition (HI) test with a panel of H1-H16 reference sera, while the NA subtype was verified by RT-PCR analysis using a panel of N1-N9 subtype primers (1,2).

Genome Sequencing and Phylogenetic Analysis

The RNA of influenza A(H5N6) viruses were extracted from the allantoic fluid of virus-infected chicken eggs by using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany). Reverse transcription PCR was performed with a set of gene-specific primers, and the products were sequenced by using an Applied Biosystems DNA analyzer. Primer sequences are available upon request. The nucleotide sequences were edited by using the Seqman module of the DNASTar package. Phylogenetic analysis was performed by employing the neighbor-joining method by using the Mega 6.0.6 ClustalW software package. The tree topology was evaluated by 1,000 bootstrap analyses, and a 97% sequence identity cutoff was used to categorize the groups of each gene segment in the phylogenetic trees (3).

A Bayesian time-resolved phylogenetic tree was also created for the hemagglutinin gene of group 1 and 2 viruses using BEAST 1.8.4 (4). The SRD06 nt substitution model, the uncorrelated relaxed clock with a log-normal distribution, and the Skygrid flexible effective

population-size tree prior were selected for the analysis. A Markov Chain Monte Carlo (MCMC) chain was run for 30,000,000 steps, with sampling every 3,000 steps. The first 10% of the samples were discarded as burn-in. The above MCMC settings were chosen to achieve a post burn-in effective sample size of at least 200 in all parameters, as recommended by the BEAST program.

The data were evaluated by using the Akaike information criterion in Tracer 1.6 (5). The sequences of 13 H5N6 isolates were deposited in the Global Initiative on Sharing Avian Influenza Data database (<http://www.gisaid.org>) under accession nos. EPI1718935-EPI1719038.

Chicken Test

Intravenous Pathogenicity Index (IVPI)

The pathogenicity of the representative A(H5N6) virus was determined in chicken by means of the IVPI test in accordance with the IVPI protocol of the World Organisation for Animal Health (6). Ten 6-week-old SPF chickens were inoculated with 0.2 mL of a 1:10 dilution of $10^{7.0}$ EID₅₀/mL of the virus through the intravenous route, examine the chickens for clinical signs at intervals of 24 hours over a ten-day period (6,7).

Duck Test

Eight ducks of 3-week-old were intranasally inoculated with 0.1 mL of $10^{6.0}$ EID₅₀/mL of WS/XJ/1/2020(H5N6). At 24 h p.i., three naive ducks from the same flock were co-housed with the infected ducks to monitor the transmission of the A(H5N6) virus (contact group). The ducks were observed for clinical signs daily over 14 days.

Three birds in the infection group were euthanized randomly on day 3 p.i., and the brain, lungs, kidneys, spleen, bursa, thymus, trachea, cecal tonsil, and pancreas were collected for titrating the virus present in these organs in SPF CEEs. Oropharynxgeal and cloacal swabs were collected from the infection and contact groups on days 3, 5, 7, and 9 p.i. and used for titrating the virus in SPF CEEs. On days 14 p.i., serum samples were collected from the surviving birds for detecting homologous hemagglutination-inhibiting antibodies (6,8).

Mouse Test

To investigate the virulence of WS/XJ/1/2020(H5N6) in mice, six groups of 6-week-old female BALB/c mice (five mice per group) were lightly anesthetized with dry ice. The mice were then separately inoculated intranasally with $10^{1.0}$ to $10^{6.0}$ EID₅₀ of the virus in a volume of 50 μ L. The control group (five mice) was mock-infected with phosphate-buffered saline. Each group was monitored daily for weight loss and mortality for 14 days. In addition, another group of three mice was inoculated intranasally with $10^{6.0}$ EID₅₀ of the A(H5N6) virus in a volume of 50 μ L. These mice were euthanized by dry ice on day 3 p.i., and the brain, lungs, kidneys, spleen, and nasal turbinate were collected for virus titration (6).

Antigenic Analysis

The HI assay was used to antigenically characterize the A(H5N6) viruses isolated in swan in Xinjiang, western China in 2020 with the current vaccine candidate, Re-11, A/duck/Guizhou/S4184/2017(H5N6). Antisera were generated in 6-week-old white Leghorn SPF chickens, chickens were subcutaneously vaccinated once with 2.0 ml Freund's-adjuvanted inactivated whole virus vaccines (HA content, 9log₂). Sera from vaccinated chickens were collected 4 weeks after vaccination (9). The HI test was performed using a 1% SPF chicken red blood cell suspension as previously described (10). The HI titer was expressed as the reciprocal of the highest serum dilution in which hemagglutination was inhibited.

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Appendix Table 1. The closest relatives to WS/XJ/1/2020(H5N6) in the GISAID database.

Segment	Position	Homology of 13 H5N6 viruses (%)	Closest relatives	Segment ID (EPI_ISL)	Homology (%)
HA	29–1729	99.5–100	A/Env/Guangdong/C17285752-QY/2017(H5N6)	340844	98.8
NA	19–1409	99.9–100	A/Env/Guangdong/C17285752-QY/2017(H5N6)	278026	99.3
PB2	28–2307	99.9–100	A/Duck/Guangdong/PO17281256/MZH/2017(H5N6)	340789	98.9
PB1	25–2298	99.8–100	A/Env/Guangdong/Foshan/C182750085/2018(H5N6)	340824	98.7
PA	25–2175	100	A/Env/Guangdong/Jieyang/C18289059/2018(H5N6)	340825	99.4
NP	46–1542	99.9–100	A/Env/Guangdong/C17285753/QY/2017(H5N6)	340845	98.6
M	26–1007	99.9–100	A/Env/Guangdong/C17285752/QY/2017(H5N6)	340844	99.2
NS	27–855	99.8–100	A/chicken/Nghe_An/01VTC/2018(H5N6)	389022	99.4

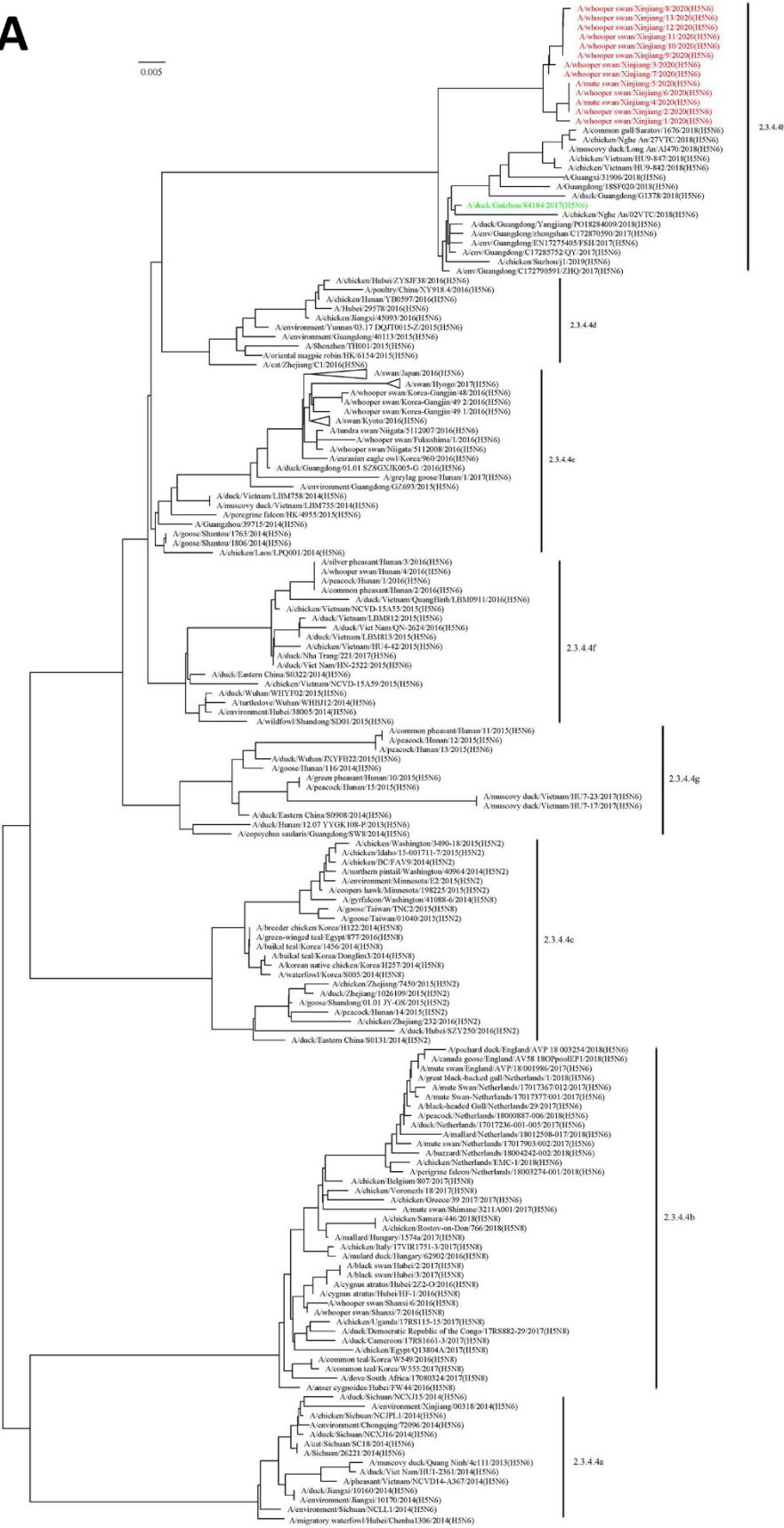
Appendix Table 2. Antigenic analysis by chicken sera for clade 2.3.4.4 H5 HPAIV.

Virus	Cross-reactive HI antibody titers of chicken antiserum	
	H5N1 vaccine seed virus Re-11	WS/XJ/1/2020(H5N6)
H5N1 vaccine seed virus Re-11*	512	256
WS/XJ/1/2020(H5N6)	128	1024

*H5 vaccine seed virus Re-11 bears the HA gene from DK/GZ/S4184/17 (H5N6) virus.

A

0.005



B
0.005



SEA-2

SEA-1

ER

C

0.005



SEA-2

ER

SEA-1

D

0.005



SEA-3

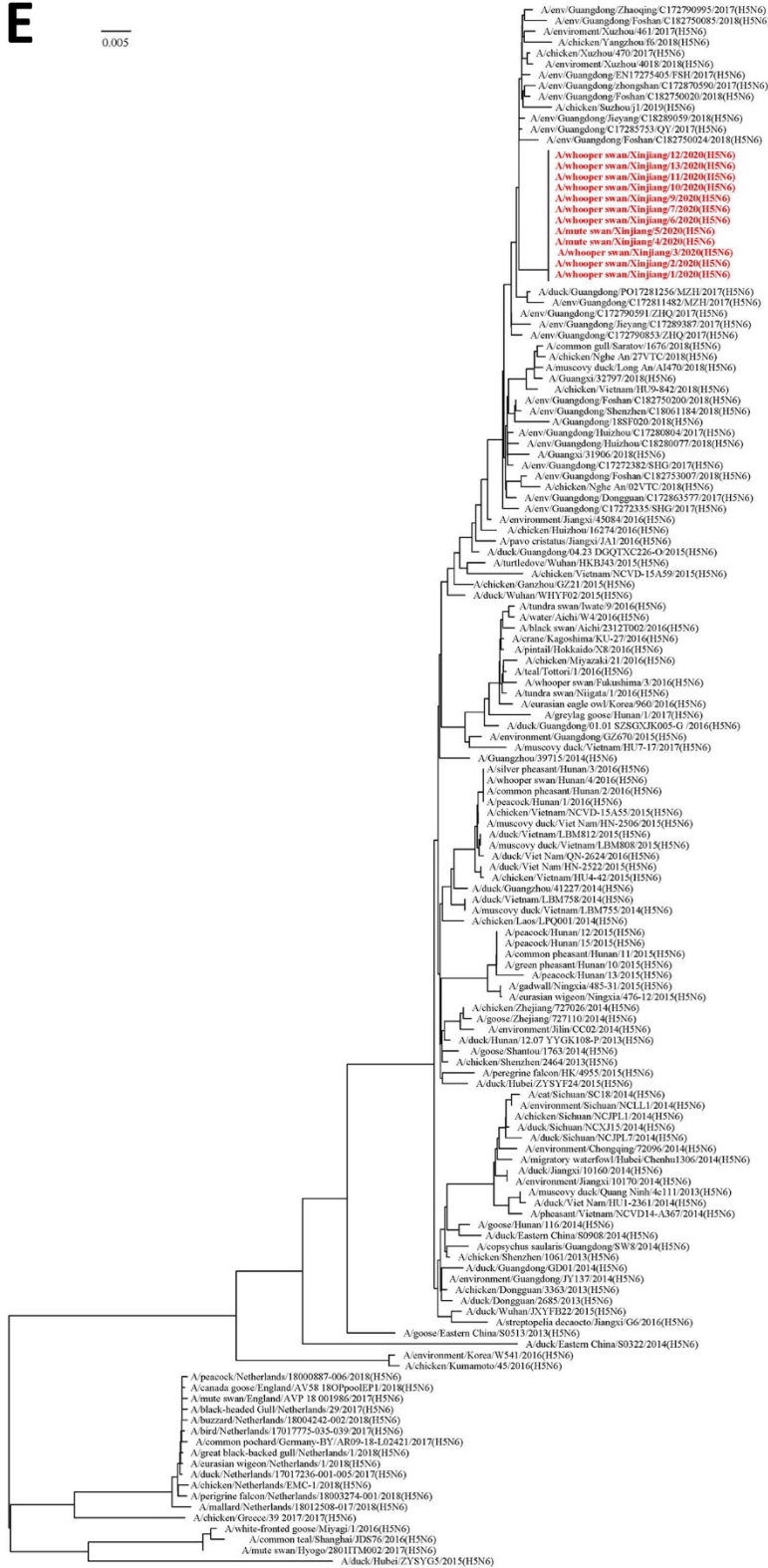
SEA-1

ER

SEA-2

E

0.005



SEA-2

SEA-1

ER

F

0.005



SEA-2

SEA-1

ER

G

0.005



SEA-2

SEA-1

ER

H

0.005



SEA-2

SEA-1

ER

Appendix Figure. Phylogenetic analysis of SW/XJ/2020(H5N6). The neighbor joining trees of SW/XJ/2020(H5N6) virus genes were built by using the Mega 6.0.6 ClustalW software package. Segments shown are: hemagglutinin (HA) (A); neuraminidase (NA) (B); polymerase basic (PB2) (C); polymerase basic (PB1) (D); polymerase (PA) (E); nucleoprotein (NP) (F); matrix (M) (G); Nonstructural

protein (NS) (H). The unrooted trees were based on nucleotide positions 29 to 1701 for HA, 29 to 1409 for N6 NA, 28 to 2307 for PB2, 25 to 2298 for PB1, 25 to 2175 for PA, 46 to 1542 for NP, 26 to 1007 for M, and 27 to 855 for NS. The 13 H5N6 from the swans in this study are shown in red and bold, the HA donor of Re-11 vaccine candidate is shown in green and bold in panel A. SEA, Southeast Asia; ER, Europe.