Highly Pathogenic Avian Influenza Virus (H5N1) Clade 2.3.4.4b Introduced by Wild Birds, China, 2021

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Highly pathogenic avian influenza (HPAI) subtype H5N1 clade 2.3.4.4b virus has spread globally, causing unprecedented large-scale avian influenza outbreaks since 2020. In 2021, we isolated 17 highly pathogenic avian influenza H5N1 viruses from wild birds in China. To determine virus origin, we genetically analyzed 1,529 clade 2.3.4.4b H5N1 viruses reported globally since October 2020 and found that they formed 35 genotypes. The 17 viruses belonged to genotypes G07, which originated from eastern Asia, and G10, which originated from Russia. The viruses were moderately pathogenic in mice but were highly lethal in ducks. The viruses were in the same antigenic cluster as the current vaccine strain (H5-Re14) used in China. In chickens, the H5/H7 trivalent vaccine provided complete protection against clade 2.3.4.4b H5N1 virus challenge. Our data indicate that vaccination is an effective strategy for preventing and controlling the globally prevalent clade 2.3.4.4b H5N1 virus.

The hemagglutinin (HA) gene of highly pathogenic avian influenza subtype H5 viruses has evolved into multiple clades (clades 0–9), and some clades are further divided into subclades. Of the 2 dominant clades, 2.3.2.1 has been further categorized into 7 (2.3.2.1a–g) and 2.3.4.4 have been further categorized into 8 (2.3.4.4a–h) subclades (1–3). The World Organisation for Animal Health reported that >8,000 outbreaks of highly pathogenic avian influenza (HPAI) subtype H5N1 clade 2.3.4.4b occurred in birds during October 2020–October 2022. Massive numbers of birds across 4 continents (Europe, Asia, Africa, and North America) were humanely killed directly or indirectly by infection with clade 2.3.4.4b H5 HPAI viruses (4).

H5 HPAI viruses of several clades have spread intercontinentally through global migration of wild birds. In 2005, clade 2.2 H5N1 virus spread by wild birds caused numerous outbreaks in wild birds and domestic poultry in countries throughout Asia, the Middle East, Europe, and West Africa (5,6). In 2009, clade 2.3.2 H5N1 virus caused problems mainly in Asia and eastern Europe (7,8). In 2014, both clade 2.3.4.4b H5N8 and clade 2.3.2.1c H5N1 viruses spread and circulated in Eurasia, the Middle East, and Africa (9–11). At the beginning of 2014, a clade 2.3.4.4c H5N8 virus emerged in South Korea and then circulated in Eurasia and Africa. In 2015, that same virus spread to North America and reassorted with local low pathogenicity avian influenza (LPAI) viruses to produce subtype H5N2, which circulated in the United States during 2015–2016 (12,13). At the beginning of 2020, clade 2.3.4.4b H5N8 virus caused disease outbreaks and destroyed poultry across Europe, after which it spread to many countries in Asia (14,15). The H5N8 virus reassorted with different viruses and formed several other subtypes of H5 viruses (e.g., H5N1, H5N2, H5N3, H5N4, H5N5, and H5N6) in different countries and regions. Among them, H5N1 became the globally predominant variant (16,17). In late 2021, the virus was carried across the Atlantic Ocean to North America (18). The viruses caused huge ongoing outbreaks in Europe and North America and led to massive destruction of poultry and wild bird populations (4,19,20).

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Clade 2.3.4.4b H5 viruses have crossed the bird–mammal barrier to infect humans and other mammals. Since the beginning of 2020, human infection with influenza A(H5N1) clade 2.3.4.4b viruses has been detected in Spain, the United Kingdom of Great Britain and Northern Ireland, the United States, China, and Vietnam and reported to the World Health Organization (*21*). Seven human infections caused by influenza A(H5N8) virus were reported in the Russian Federation (*22*), and some infections caused by H5N6 virus were reported in China in those 3 years (2020–2022) (*23*). In addition, fatal 2.3.4.4b H5N1 infection of some carnivorous mammals (e.g., foxes, otters, red foxes, skunks, coyotes, bobcats) and marine mammals (e.g., harbor seals, dolphins) has been reported in Europe and North America (*24*).

Clade 2.3.4.4b H5N1 virus has become a new threat to the global poultry industry and to public health. To learn more about its spatial transmission and biological properties, we performed extensive phylogeographic and epidemiologic analyses of the globally circulating H5N1 viruses detected during 2020–2022, evaluated the pathogenicity of H5N1 from China in mammalian and waterfowl hosts, compared H5N1 antigenicity with that of the updated vaccine candidate, and assessed the protective efficacy of the current H5-Re14 vaccine against challenge with H5N1 isolates.

All studies with live viruses were conducted in a Biosafety Level 3 laboratory approved for such use by the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. All experiments using animals were conducted in strict accordance with 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. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (approval nos.: duck, 211015-01; mouse, 211231-02; chicken, 211112-01).

Materials and Methods

Sample Collection and Virus Isolation
During 2020–2021, we collected 7,421 fresh fecal samples, 507 swab samples, and 6 tissue samples from wild birds in accordance with the regular surveillance of wild birds in China (Table). The samples were amplified in specific-pathogen–free (SPF) chicken embryos, and the HA subtype was identified by using the hemagglutinin inhibition (HI) test with a panel of H1–H16 reference serum. We verified the neuraminidase (NA) subtype by using reverse transcription PCR analysis with a panel of N1–N9 subtype primers (reference serum and primer sequences available on request). We identified host species by using DNA barcoding with the cytochrome C oxidase I mitochondrial gene (*25*).

Genome Sequencing and Phylogenic and Phylodynamic Analyses
We extracted total influenza A virus RNA from the allantoic fluid of virus-infected chicken embryos by using the QIAmp Viral RNA Mini Kit (QIAGEN, https://www.qiagen.com). We performed reverse transcription PCR by using a panel of gene-specific primers and sequenced the products by using an Applied Biosystems DNA analyzer (primers available on request). The genetic information collected from January 1, 2020, to October 17, 2022, was downloaded from GISAID (https://www.gisaid.org) on October 17, 2022 (Appendix 1, https://wwwnc.cdc.gov/EID/

Table. Avian influenza viruses isolated from wild bird samples collected in China, January 2020–December 2021*

Date	Province	Sample			Virus		
		Feces	Swab	Tissue	No. strains	Subtype	Pathotype†
2020							
Jan	Shanxi	700	0	0	2	H5N3	Low
Jan	Heilongjiang	980	394	2	0	NA	NA
Nov	Anhui	1,017	0	0	17	Multiple‡	Low
Nov	Ningxia	600	0	4	1	H5N2	Low
2021							
Mar	Anhui	700	0	0	0	NA	NA
Mar	Liaoning	600	0	0	0	NA	NA
Apr	Ningxia	450	0	0	0	NA	NA
Apr	Heilongjiang	750	0	0	0	NA	NA
Oct	Heilongjiang	324	113	0	2	H5N1	High
Dec	Shanxi	500	0	0	4	H5N1	High
Dec	Henan	800	0	0	11	H5N1	High

*NA, not applicable.
†Pathotype is determined by the cleavage motif of its hemagglutinin gene.
‡The 17 low pathogenicity viruses are 1 H2N3, 1 H3N8, 9 H4N6, 3 H5N8, 1 H6N2, 1 H6N8, and 1 H12N2 strains.

article/29/7/22-1149-App1.pdf). We aligned sequences by using MAFFT version 7.475 with default settings (*26*) and ran neighbor-joining trees by using MEGA version 11 for 1,000 ultrafast bootstraps (*27*). To categorize the groups of each segment in the phylogenetic trees, we used a sequence identity cutoff of >95%. We constructed a maximum-clade credibility time-scaled phylogenetic tree of HA sequences from clade 2.3.4.4b H5N1 viruses by using the SRD06 nucleotide substitution model and an uncorrelated lognormal relaxed clock model (*28*). To investigate the transmission patterns of the clade 2.3.4.4b H5N1 viruses, we performed a phylogeographic analysis by using an asymmetric model with Bayesian stochastic search variable selection implemented in BEAST version 1.10.4 (*28,29*). We grouped the sequences from 1,529 isolates into 11 distinct geographic categories (Appendix 2 Table 3, https://wwwnc.cdc.gov/EID/article/29/7/22-1149-App2.pdf). To summarize the diffusion rates, we used the Bayesian stochastic search variable selection, and to estimate Bayes factors, we used SpreaD3 version 0.9.6 (https://rega.kuleuven.be/cev/ecv/software/SpreaD3) (Appendix 2 Table 4).

Animal Studies

We selected 2 representative isolates, A/mandarin duck/Heilongjiang/HL-1/2021 (MD/HLJ/HL-1/2021) and A/whooper swan/Henan/14/2021 (WS/HeN/14/2021), to test in mice and ducks. The 50% lethal dose (LD_{50}) for mice was tested in groups of five 6-week-old female BALB/c mice (Vital River, https://www.vitalriver.com). The mice were intranasally inoculated with a $10^{1.0}$ to $10^{6.0}$ 50% egg infectious dose (EID_{50}) of virus in a volume of 50 µL and then monitored daily for weight loss and death for 14 days. LD_{50} values for mice were calculated according to the Reed-Muench method (*30*). To evaluate virus replication, we tested 3 more mice in the $10^{6.0}$ EID_{50} group and humanely killed them on postinoculation day 3 to assess virus titers in their nasal turbinates, lungs, brains, kidneys, and spleens.

Among the ducks, we inoculated groups of eight 3-week-old SPF ducks (Jinding duck, a local breed; National Poultry Laboratory Animal Resource Center, Harbin, China) intranasally with $10^{6.0}$ EID_{50} of H5N1 virus in a volume of 0.1 mL; at 24 h after inoculation, 3 contact ducks were put in the same cage. On postinoculation day 3, we randomly selected 3 of 8 infected ducks, humanely killed them, and collected their organs (brain, spleen, kidneys, pancreas, cecal tonsil, bursa of Fabricius, thymus, lungs, and larynx) for virus titration. We observed the remaining 5 infected ducks and 3 contact ducks for 2 weeks and collected oropharyngeal and cloacal swab samples on postinoculation days 3 and 5 to detect virus shedding.

Antigenic Analysis

We used HI to perform antigenic analysis (*31*). We generated chicken antiserum of H5 vaccine seed viruses (H5-Re11, H5-Re12, H5-Re13, and H5-Re14) (Appendix 2 Table 7) by inoculating 5-week-old SPF chickens with 0.3 mL of the oil-emulsified inactivated viruses (*32*).

Challenge Study of Clade 2.3.4.4b H5N1 Virus in Chickens

We vaccinated groups of ten 3-week-old white leghorn SPF chickens (National Poultry Laboratory Animal Resource Center) with a 0.3-mL intramuscular injection of the trivalent H5/H7 vaccine previously reported by Zeng et al. (*32,33*) (Harbin Weike Biotechnology Co., http://www.hvriwk.com) or with phosphate-buffered saline as a control. Three weeks after vaccination, we challenged the chickens intranasally with 10^5 EID_{50} of MD/HLJ/HL-1/2021 in a volume of 0.1 mL. We collected oropharyngeal and cloacal swab samples on postchallenge days 3 and 5 to detect the virus and observed birds for disease and death for 2 weeks after challenge.

Results

Clade 2.3.4.4b H5N1 Virus Detection

As part of regular surveillance of wild birds during 2020–2021, we collected 7,934 samples, from which we detected 17 H5N1 HPAI viruses and 20 LPAI viruses of multiple subtypes (Table). To determine the evolution of the H5N1 viruses, we sequenced the whole genomes of the 17 HPAI viruses, deposited the sequences in the GISAID database (accession nos. EPI2070071–0206), and performed phylogenic analysis of these viruses together with global clade 2.3.4.4b HPAI H5 viruses submitted to GISAID from January 1, 2020, to October 17, 2022.

The HA genes of the 17 H5N1 viruses shared 98.3%–100% identity at the nucleotide level, and the NA genes shared 97.45%–100% identity (Appendix 2 Figure 2, Figure 3, panel A). The HA genes of the clade 2.3.4.4b H5 strains formed 2 branches—East Asia and Eurasia/Africa—in the phylogenetic tree (Appendix 2 Figures 1, 2); 2 H5N1 viruses isolated in Heilongjiang Province in this study belonged to the East Asia branch, whereas the other 15 viruses belonged to the Eurasia/Africa branch. The NA genes of the 17
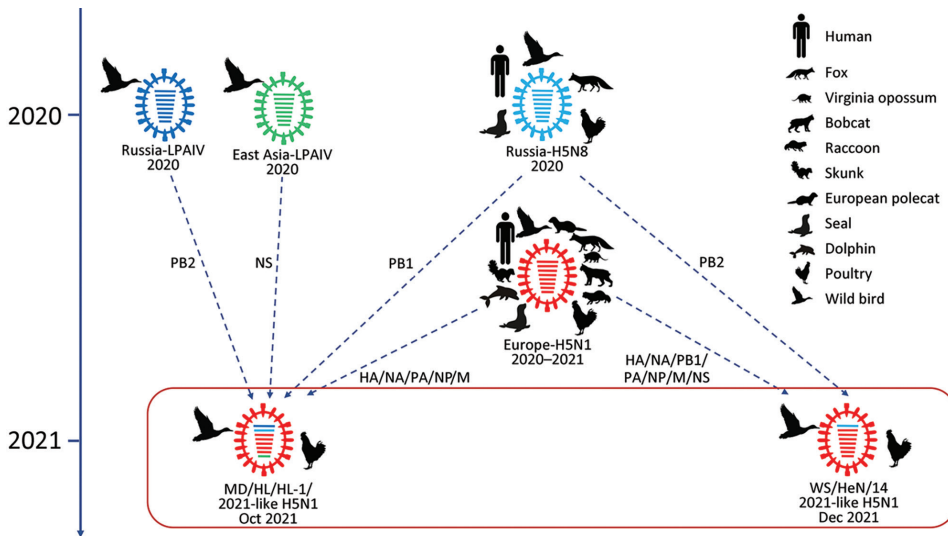
Figure 1. Formation of 2 representatives of avian influenza (H5N1) virus from China. Viral particles are represented by colored ovals containing horizontal bars representing the 8 gene segments (from top to bottom: PB2, PB1, PA, HA, NP, NA, M, and NS). Gene segments of the reassortants are colored according to their corresponding source virus. HA, hemagglutinin; LPAIV, low pathogenicity avian influenza virus; M, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural protein; PA, polymerase acidic; PB, polymerase basic.

viruses all belonged to the group Europe H5N1 2020 (Appendix 2 Figure 3, panel A). Identity at the nucleotide level was 94.12%–100% for the polymerase basic (PB) 2 genes, 92.96%–100% for the PB1 genes, 97.3%–100% for the polymerase acidic (PA) genes, 97.6%–100% for the nucleoprotein (NP) genes, 98.7%–100% for the matrix (M) genes, and 95.65%–100% for the nonstructural protein (NS) genes of the 17 H5N1 viruses, and they formed multiple groups in their phylogenetic trees (Appendix 2 Figure 3). Diversity of the internal genes of clade 2.3.4.4b H5N1 viruses was greater than that of the HA and NA genes. Of note, the 8 genes of the MD/HLJ/HL-1/2021 and MD/HLJ/HL-2/2021 viruses were similar, and the 8 genes of the other 15 viruses were similar, indicating that these 17 viruses formed 2 different genotypes.

Complex Reassortment of the H5N1 Wild Bird Viruses

To investigate the formation of the 17 H5N1 HPAI viruses, we used MD/HLJ/HL-1/2021 and WS/HeN/14/2021 as representatives of the 2 branches and conducted BLAST analysis (https://platform.epicov.org/epi3/frontend#27b74d) of the highest homology viruses to the 8 segments of 2 reference viruses from GISAID. The data showed that both representatives were reassortants (Figure 1; Appendix 2 Table 2). Seven genes of WS/HeN/14/2021 (PB1, HA, NA, PA, NP, M, and NS) were closely related to those of the H5N1 HPAI viruses in Europe during 2020–2021; the PB2 gene of WS/HeN/14/2021 was provided by the H5N8 HPAI virus in Russia in 2020. MD/HLJ/HL-1/2021 had 5 genes (HA, NA, PA, NP, and M) closely related to those of the H5N1

HPAI viruses in Europe during 2020–2021; its PB1 gene was closely related to the H5N8 HPAI virus in Russia during 2020, whereas its PB2 and NS genes were from the LPAI viruses that circulated in Russia during 2020. Our analysis thus demonstrates that clade 2.3.4.4b H5N1 viruses have undergone complex reassortment with LPAI viruses in different geographic sites since 2020.

Genotypic Analysis and Spread of Clade 2.3.4.4b H5N1 Viruses

Cui et al. analyzed 233 strains of clade 2.3.4.4b H5N1 viruses that were detected globally from October 1, 2020, to April 1, 2022, and revealed 16 genotypes (G01–16) (17). The 2 H5N1 viruses from Heilongjiang in this study belonged to G07, whereas the other 15 H5N1 viruses belonged to G10 (Appendix 2 Figure 1). Within 2 months of their detection in wild birds, viruses of these 2 genotypes spread to and were detected in domestic waterfowl in China (17).

Because the viruses are still spreading globally, we sought to gain further insight into the large-scale cross-continental transmission and reassortment patterns of clade 2.3.4.4b H5N1 viruses. To that end, we analyzed the sequences of 1,296 viruses that became available from April to October 2022 and detected an additional 19 genotypes (Appendix 1; Appendix 2 Table 3, Figure 4), including 5 in western Europe (G21, G28, G33, G34, and G35), 2 in eastern and central Europe (G19 and G20), 1 in Russia (G17), 1 in Africa (G18), and 10 in North America (G22, G23, G24, G25, G26, G27, G29, G30, G31, and G32) (Appendix 2 Table 3, Figure 5). We further grouped the available isolates into 11 distinct geographic

regions, performed a phylogeographic analysis of their HA genes (Appendix 2 Table 3, Figure 5), and identified 22 spread pathways of clade 2.3.4.4b H5N1 viruses. Of note, 4 of those pathways were decisively supported with Bayes factors >1,000: 2 pathways showed G01 spreading from eastern and central Europe to Russia and from western Europe to North America; 1 showed G04 spreading from western Europe to southern Europe; and the fourth showed G07 spreading from eastern Asia to China (Appendix 2 Table 4). Those data indicate that Europe was the epidemic source for the global spread of clade 2.3.4.4b H5N1 viruses.

Amino Acid Residues of Clade 2.3.4.4b H5N1 Viruses

All 17 H5N1 viruses had residues 137A and 192I and did not have the glycosylation site at positions 158–160 in their HA (H3 numbering) (Appendix 2 Table 5), which have been reported to increase the affinity of avian influenza viruses for human-type receptors (*34,35*). The viruses also carried critical virulence-increasing residues, including 66S in PB1-F2 in 15 viruses; 30D, 43M, and 215A in M1 and 42S, 103F, and 106M in NS1 of all 17 viruses (*36–41*) (Appendix 2 Table 5). Therefore, clade 2.3.4.4b H5N1 viruses may have the capacity to infect and be virulent in humans.
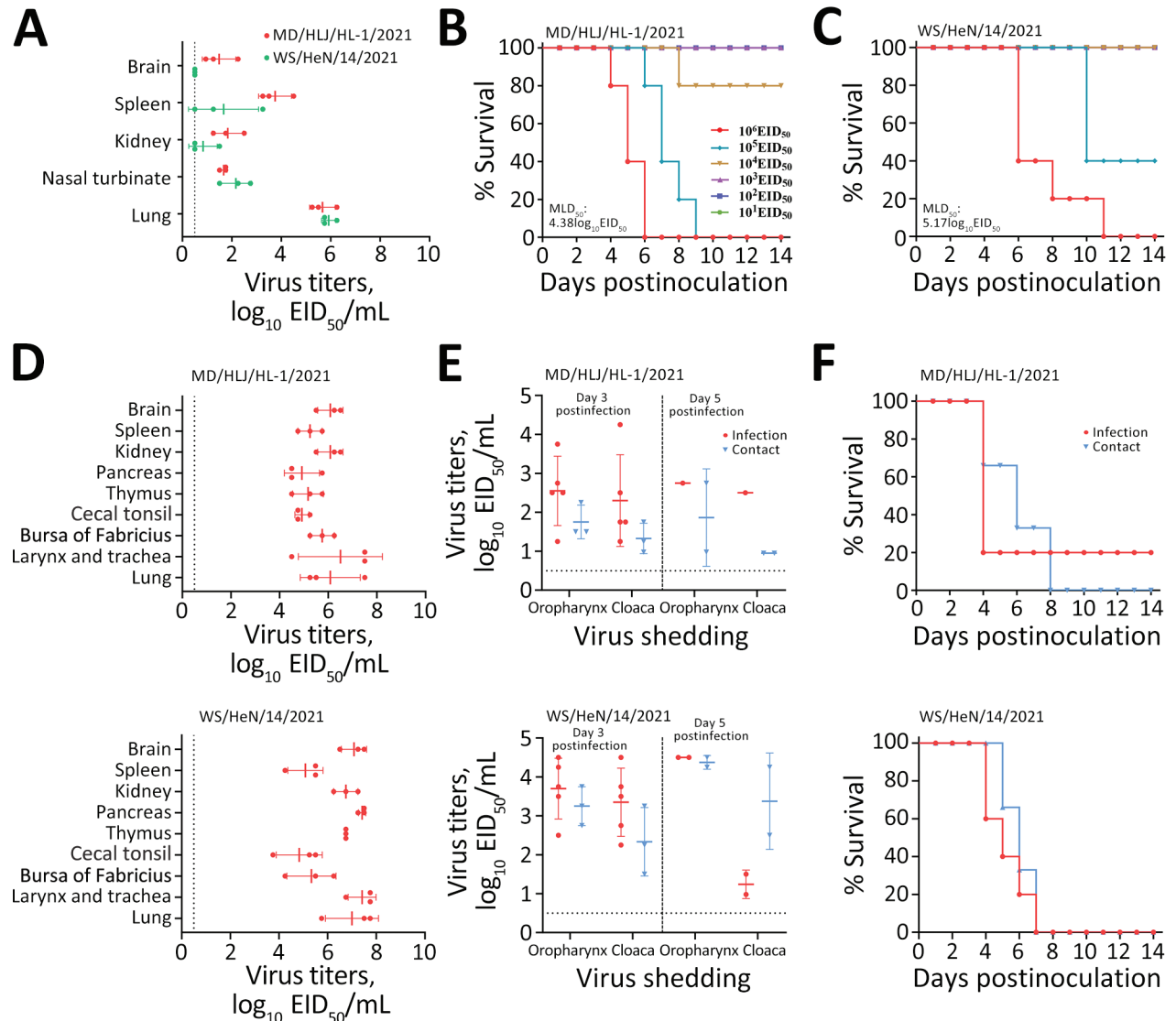
Figure 2. Replication and virulence of representative highly pathogenic avian influenza virus (H5N1) viruses in mice and ducks. A) Virus titers in organs of mice that were humanely killed on postinfection day 3 with 10^6 EID_{50} of the test viruses. B, C) MLD_{50} of the indicated viruses. D) Virus titers in organs of ducks that were killed on day 3 post inoculation with 10^6 EID_{50} in 0.1 mL of the indicated viruses. E) Virus shedding from ducks on the indicated days after inoculation. F) Death pattern of ducks in the infected and control groups. EID_{50}, 50% egg infectious dose; MLD_{50}, 50% lethal dose for mice.

Virulence of H5N1 Wild Bird Viruses in Mice

In several countries, clade 2.3.4.4 H5 viruses have caused infections in humans and other mammals, including terrestrial small carnivores and marine mammals (Appendix 1). To evaluate the replication and virulence of wild bird H5N1 viruses in mammals, we tested 2 representative strains in BALB/c mice: MD/HLJ/HL-1/2021 in G07 and WS/HeN/14/2021 in G10. We found that both viruses replicated systemically and were detected in the nasal turbinates, lungs, spleens, and kidneys of the mice; MD/HLJ/HL-1/2021 was also detected in the brains of the mice (Figure 2, panel A). The LD_{50} value of MD/HLJ/HL-1/2021 in mice was 4.38 $\log_{10}$ EID_{50} and of WS/HeN/14/2021 was 5.17 $\log_{10}$ EID_{50} (Figure 2, panels B, C), indicating that H5N1 wild bird viruses are moderately pathogenic in mice (1,5,7,9).

Lethality of H5N1 Wild Bird Viruses in Ducks

Many H5 viruses that are highly pathogenic to galliformes (e.g., chickens, quail, and turkeys) may still be of low pathogenicity to waterfowl (1,15). To investigate the replication and virulence of clade 2.3.4.4b H5N1 virus in waterfowl, we tested MD/HLJ/HL-1/2021 and WS/HeN/14/2021 in SPF ducks. We found that the 2 viruses replicated efficiently in ducks and were detected in all 9 investigated organs of ducks that were humanely killed on postinoculation day 3 (Figure 2, panel D). We detected virus shedding in oropharyngeal and cloacal swab samples from the infected ducks and contact ducks on postinoculation days 3 and 5 (Figure 2, panel E). MD/HLJ/HL-1/2021 led to the death of 4 of 5 inoculated and 3 contact ducks, whereas WS/HeN/14/2021 led to the death of all 5 inoculated and 3 contact ducks (Figure 2, panel F). Those data indicate that the 2 clade 2.3.4.4b H5N1 viruses we isolated from wild birds are lethal to ducks.

Antigenic Matching of the H5 Vaccine Seed Virus Re14 with the H5N1 Wild Bird Viruses

To evaluate the antigenic difference between the emerged clade 2.3.4.4b of H5N1 viruses and the H5 vaccine strains, we generated antiserum in SPF chickens against 4 H5 vaccine strains (H5-Re11 [clade 2.3.4.4h], H5-Re12 [clade 2.3.2.1f], H5-Re13 [clade 2.3.4.4h], and H5-Re14 [clade 2.3.4.4b]) and tested their cross-reactive HI antibody titers to 4 representative viruses detected in this study: A/mandarin duck/Heilongjiang/HL-1/2021, A/whooper swan/Shanxi/608/2021, A/whooper swan/Henan/14/2021, and A/mandarin duck/Henan/426/2021 (Appendix 2 Table 7). We found that the 4 viruses reacted well with the antiserum of H5-Re14, with titers ranging from 64 to 128, less than 4-fold to the homologous titer, but reacted poorly with the antiserum of the other 3 vaccine strains (Figure 3, panel A; Appendix 2 Table 7).

We further evaluated the protective efficacy of the H5/H7 trivalent vaccine in chickens against challenge with a clade 2.3.4.4b H5N1 virus. Three weeks
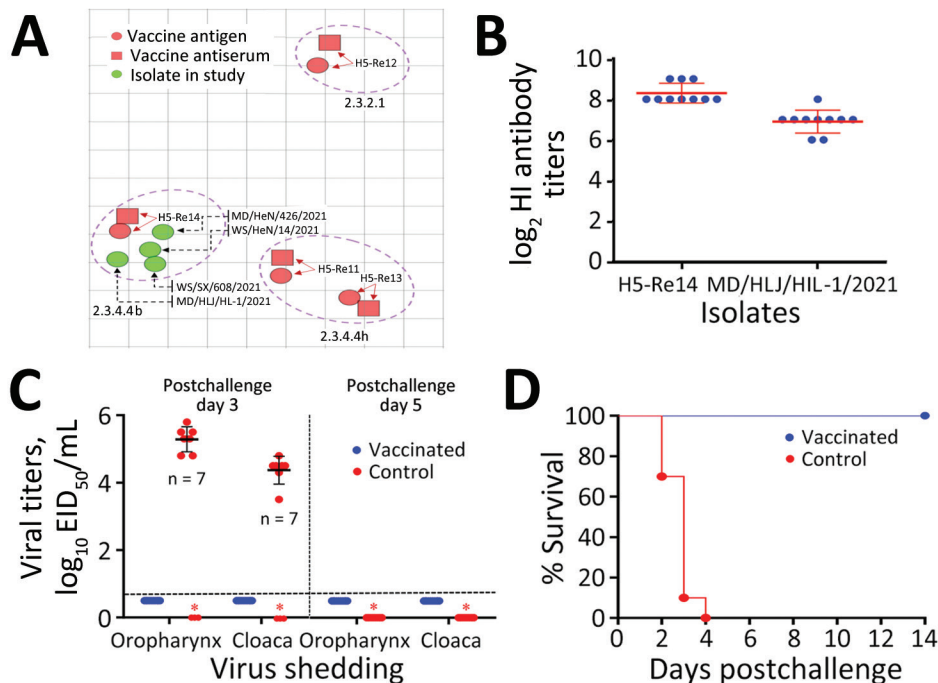

Figure 3. Antigenic difference of avian influenza (H5N1) virus isolates with H5 vaccine strains and protective efficacy of H5 vaccine against the challenge of H5N1 virus. A) Antigenic cartography of H5N1 viruses. The map was generated by using Antigenic Cartography software (https://acmacs-web.antigenic-cartography.org); 1 unit (grid) represents a 2-fold change in the HI assay results (Appendix 2 Table 7, https://wwwnc.cdc.gov/EID/article/29/7/22-1149-App2.pdf). B) HI antibody titers of vaccinated chickens against the vaccine seed virus and the challenge strain. C) Virus shedding from chickens on postchallenge days 3 and 5. D) Survival patterns of chickens in the vaccinated and control groups after challenge with the indicated H5N1 virus. EID_{50}, 50% egg infectious dose; HI, hemagglutination inhibition.

after vaccination, high titers of HI antibody to vaccine strain H5-Re14 and the H5N1 wild bird virus MD/HLJ/HL-1/2021 developed in the chickens (Figure 3, panel B). The vaccinated chickens were completely protected against MD/HLJ/HL-1/2021, with no virus shedding, and all chickens survived during the 14-day observation period (Figure 3, panels C, D); however, all control chickens died within 4 days of challenge, and the 7 chickens that were alive on post-challenge day 3 shed high titers of virus (Figure 3, panels C, D). Those data indicate that the vaccine currently used in China could provide solid protection against the clade 2.3.4.4b H5N1 viruses.

Discussion

Clade 2.3.4.4b H5N8 virus caused massive outbreaks in poultry and wild birds in Europe, Africa, and Asia from January 2020 to October 2021 (*15,17*). During that time, the virus reassorted in countries or regions with different viruses and generated H5N1, H5N2, H5N3, H5N4, H5N5, and H5N6 viruses bearing the clade 2.3.4.4b HA gene (*4,14,16,17,23*). Among those new subtypes, only H5N1 viruses were widely spread by the movement of migratory birds and have caused thousands of outbreaks in Europe, Africa, Asia, and North America since their generation in the Netherlands in October 2020 (*4,14,17*). Our study revealed that the widely circulating H5N1 viruses have undergone complex reassortment with other LPAI viruses and formed ≥35 genotypes. Of those novel genotypes of H5N1 viruses, 10 were generated in North America since December 2021, when clade 2.3.4.4b H5N1 virus was first introduced to that region from western Europe (*18*). Given that the H5N1 reassortants found in Europe and North America have infected multiple wild mammals, their threat to public health should be carefully monitored and assessed.

Many countries in Europe and North America control HPAI by culling infected and suspected domestic birds, whereas China controls HPAI by using a massive vaccination strategy. The key to the success of the vaccination strategy is timely updates of vaccine strains (*15,32,33*). The currently used H5/H7 trivalent inactivated vaccine was updated and has been applied since January 2022; it is produced with 3 seed viruses: H5-Re13, targeting clade 2.3.4.4h H5 viruses; H5-Re14, targeting clade 2.3.4.4b H5 viruses; and H7-Re4, targeting H7N9 viruses (*33*). The antigenic assay in our study demonstrated that the H5-Re14 seed virus antigenically matches well with the recently emerged H5N1 wild bird viruses in China. The vaccine protective efficacy test also demonstrated that the novel H5/H7

trivalent vaccine provides solid protection against challenge with an emerging H5N1 isolate. The success of the mandatory vaccination policy for preventing and controlling HPAI virus in China is an example of a high-risk country using a vaccine to protect the poultry industry and of how vaccinating poultry can prevent and eliminate human infections with avian influenza virus, as evidenced by the successful control of H7N9 influenza (*32,33,42*). Therefore, we strongly recommend the use of vaccines to protect poultry from globally circulating 2.3.4.4b H5N1 viruses.

About the Author

Dr. Tian works at the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China, with a research focus on avian influenza virus surveillance.

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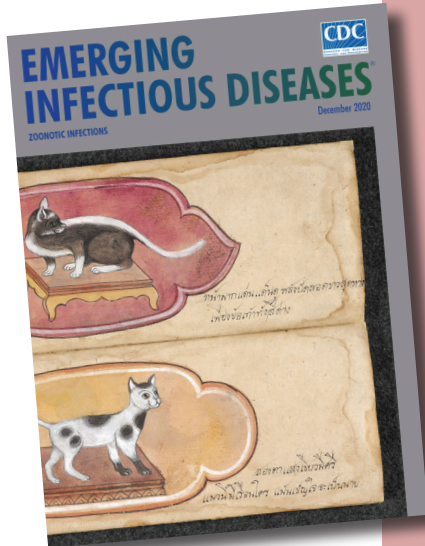the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog. 2009;5:e1000709. https://doi.org/10.1371/journal.ppat.1000709

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etymologia revisited

Salmonella
[sal''mo-nel'ə]

Named in honor of Daniel Elmer Salmon, an American veterinary pathologist, *Salmonella* is a genus of motile, gram-negative bacillus, nonspore-forming, aerobic to facultatively anaerobic bacteria of the family Enterobacteriaceae. In 1880, Karl Joseph Eberth was the first to observe *Salmonella* from specimens of patients with typhoid fever (from the Greek *typhōdes* [like smoke; delirious]), which was formerly called *Eberthella typhosa* in his tribute. In 1884, Georg Gaffky successfully isolated this bacillus (later described as *Salmonella* Typhi) from patients with typhoid fever, confirming Eberth's findings. Shortly afterward, Salmon and his assistant Theobald Smith, an American bacteriologist, isolated *Salmonella* Choleraesuis from swine, incorrectly assuming that this germ was the causative agent of hog cholera. Later, Joseph Lignières, a French bacteriologist, proposed the genus name *Salmonella* in recognition of Salmon's efforts.

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Highly Pathogenic Avian Influenza Virus (H5N1) Clade 2.3.4.4b Introduced by Wild Birds, China, 2021

Appendix 2

Appendix 2 Table 1. Clade 2.3.4.4b H5N1 HPAI viruses isolated from wild birds in China in 2021

Name	Abbreviation	Order	Time	Sample type (no.)	Total	Positive
A/mandarin duck/Heilongjiang/HL-1/2021	MD/HLJ/HL-1/2021	Anseriformes	Oct.2021	feces (324) swabs (113)	437	2
A/mandarin duck/Heilongjiang/HL-2/2021	MD/HLJ/HL-2/2021	Anseriformes				
A/whooper swan/Shanxi/14/2021	WS/SX/14/2021	Anseriformes	Dec.2021	feces (500)	500	4
A/whooper swan/Shanxi/20/2021	WS/SX/20/2021	Anseriformes				
A/whooper swan/Shanxi/601/2021	WS/SX/601/2021	Anseriformes				
A/whooper swan/Shanxi/608/2021	WS/SX/608/2021	Anseriformes				
A/whooper swan/Henan/2/2021	WS/HeN/2/2021	Anseriformes	Dec.2021	feces (800)	800	11
A/whooper swan/Henan/6/2021	WS/HeN/6/2021	Anseriformes				
A/whooper swan/Henan/8/2021	WS/HeN/8/2021	Anseriformes				
A/whooper swan/Henan/14/2021	WS/HeN/14/2021	Anseriformes				
A/whooper swan/Henan/15/2021	WS/HeN/15/2021	Anseriformes				
A/whooper swan/Henan/28/2021	WS/HeN/28/2021	Anseriformes				
A/whooper swan/Henan/44/2021	WS/HeN/44/2021	Anseriformes				
A/whooper swan/Henan/45/2021	WS/HeN/45/2021	Anseriformes				
A/whooper swan/Henan/46/2021	WS/HeN/46/2021	Anseriformes				
A/whooper swan/Henan/424/2021	WS/HeN/424/2021	Anseriformes				
A/mandarin duck/Henan/426/2021	MD/HeN/426/2021	Anseriformes				

Appendix 2 Table 2. The highest nucleotide homology of the whole genome for two H5N1 representative viruses.

Virus	Genotype	Segment	Phylogenic group	Closest viruses	Nucleotide identity (%)
MD/HL/HL-1/2021(H5N1)	G07	HA	Europe-H5N1 2020-2021	A/goose/Korea/H449/2020 (H5N8)	99.65
		NA	Europe-H5N1 2020-2021	A/turkey/Italy/21VIR8540-82/2021 (H5N1)	99.00
		PB2	Russia-LPAIV-2020	A/mallard/Novosibirsk region/3445k/2020 (H1N1)	99.30
		PB1	Russia-H5N8-2020	A/chicken/Czech Republic/1175-1/2020 (H5N8)	98.00
		PA	Europe-H5N1 2020-2021	A/Mallard/Netherlands/14/2021 (H5N1)	98.00
		NP	Europe-H5N1 2020-2021	A/turkey/England/057679/2021 (H5N1)	98.00
		M	Europe-H5N1 2020-2021	A/Bar-headed Goose/Tibet/XZ901/2021 (H5N1)	99.80
		NS	East Asia-LPAIV 2020	A/mallard/Novosibirsk region/3541k/2020 (H12N5)	99.40
WS/HeN/14/2021(H5N1)	G10	HA	Europe-H5N1 2020-2021	A/eurasian teal/Netherlands/20016896-013/2020 (H5N1)	99.00
		NA	Europe-H5N1 2020-2021	A/duck/Bangladesh/19D1819/2021(H5N1)	99.00
		PB2	Russia-H5N8-2020	A/goose/Hunan/SE284/2022(H5N1)	99.00
		PB1	Europe-H5N1 2020-2021	A/chicken/Tyumen/27-40V/2021(H5N1)	99.00
		PA	Europe-H5N1 2020-2021	A/Cygnus columbianus/Hubei/121/2021(H5N1)	99.00
		NP	Europe-H5N1 2020-2021	A/duck/Bangladesh/19D1819/2021(H5N1)	99.00
		M	Europe-H5N1 2020-2021	A/turkey/Italy/21VIR8585-1/2021(H5N1)	99.00
		NS	Europe-H5N1 2020-2021	A/duck/Saratov/29-11V/2021(H5N1)	99.00

Appendix 2 Table 3. Geographic definitions for the regions used in this study

Continents	Regions	Countries	Virus (Total)	Genotype (virus number)
Europe	South Europe	Greece, Italy, Portugal, Spain	430	G01 (303), G04 (85), G05 (02), G08 (07), G12 (32), G11 (01)
	West Europe	Belgium, France, Germany, Ireland, Luxemburg, The Netherlands, United Kingdom	361	G01 (141), G02 (01), G04 (187), G08 (01), G11 (03), G15 (01), G16 (07), G21 (01), G28 (01), G33 (16), G34 (01), G35 (01)
	East and Central Europe	Albania, Belarus, Bulgaria, Croatia, Czech Republic, Hungary, Moldova, Poland, Romania, Russia (west of Ural Mountains), Slovenia	135	G01 (78), G04 (19), G05 (11), G08 (14), G11 (01), G12 (02), G13 (07), G19 (01), G20 (02)
	North Europe	Denmark, Estonia, Finland, Lithuania, Sweden	53	G01 (44), G04 (09)
Asia	East Asia	Japan, South Korea	23	G01 (20), G07(03),
	China		37	G01 (01), G07 (09), G09 (06), G10 (21)
	North Asia	Russia (east of Ural Mountains)	36	G01 (24), G03 (07), G05 (01), G06 (01), G10 (02), G17 (01),
	South Asia	Bangladesh	6	G14 (06)
	West Asia	Israel	2	G01 (02)
Africa	Africa	Niger, Benin, Senegal, Nigeria, Mali, Lesotho, Ghana, Burkina Faso	27	G01 (21), G18 (06)
North America	North America	USA, Canada	419	G01 (172), G22 (19), G23 (15), G24 (02), G25 (01), G26 (148), G27 (17), G29 (09), G30 (23), G31 (03), G32 (10)

Appendix 2 Table 4. Bayes factor and posterior probability for geographic spread

From	To	Bayes factor*	Posterior probability	Genotype
East and Central Europe	North Asia	6015.419813	0.994988167	G01
West Europe	South Europe	2062.449073	0.98552137	G04
East Asia	China	1783.415845	0.983293888	G07
West Europe	North America	1502.417593	0.980231101	G01
West Europe	East and Central Europe	359.7463069	0.922316581	G04
West Europe	North Europe	289.7673852	0.905332034	G04
East and Central Europe	South Europe	266.6247127	0.897953501	G01
South Europe	West Europe	202.2275488	0.869692329	G01
East and Central Europe	North Europe	158.1378828	0.839203675	G01
Africa	East and Central Europe	141.8880848	0.824028957	G01
West Europe	North Europe	116.4604235	0.793540303	G01
North Europe	East and Central Europe	69.99751646	0.697897814	G01
East and Central Europe	North Asia	67.8712242	0.691354587	G05
East and Central Europe	West Europe	58.39012651	0.658360017	G08
East and Central Europe	South Europe	57.35427561	0.654322706	G12
East and Central Europe	West Asia	43.9563981	0.591953223	G01
South Europe	East and Central Europe	41.98345781	0.580815815	G11
East and Central Europe	China	41.60138395	0.578588334	G01
South Europe	East and Central Europe	38.35787664	0.558680217	G08
East and Central Europe	East Asia	38.16350037	0.557427259	G01
West Europe	Africa	33.4508595	0.524711123	G01
North Asia	China	30.69927481	0.503271614	G10

Appendix 2 Table 5. Mutations detected in the clade 2.3.4.4b H5N1 viruses that contributed to increased binding to human-type receptors and virulence in mammals

| Virus | Genotype | Amino acids in HA that may increase the affinity to human-type receptor (H3 numbering) | | | | Mutations in different genes that may increase virulence in mice | | | | | | |
		S137A	N158D	T160A	T192I	PB1-F2 N66S	N30D	I43M	T215A	P42S	L103F	I106M
MD/HLJ/HL-1/2021	G07	A	N	A	I	N	D	M	A	S	F	M
MD/HLJ/HL-2/2021	G07	A	N	A	I	N	D	M	A	S	F	M
WS/SX/14/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/SX/20/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/SX/601/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/SX/608/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/2/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/6/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/8/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/14/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/15/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/28/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/44/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/45/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/46/2021	G10	A	D	A	I	S	D	M	A	S	F	M
WS/HeN/424/2021	G10	A	D	A	I	S	D	M	A	S	F	M
MD/HeN/426/2021	G10	A	D	A	I	S	D	M	A	S	F	M

Appendix 2 Table 6. Clade 2.3.4.4b H5N1 outbreaks. Data downloads from WOAH (https://wahis.woah.org/#/dashboards/qd-dashboard)

Year	Semester	World region	Animal category	Outbreaks	Cases	Killed and disposed of	Deaths
2020	Jan-Jun	Africa	Wild	0	0	0	0
			Domestic	9	12,178	178,030	0
		Asia	Wild	11	217	0	217
			Domestic	16	20,597	25,075	18,074
	Jul-Dec	Africa	Wild	0	0	0	0
			Domestic	5	171,100	167,222	58,241
		Asia	Wild	0	0	0	0
			Domestic	11	2,957	9,930	1,896
		Europe	Wild	13	22	5	11
			Domestic	2	167	27,534	167
2021	Jan-Jun	Africa	Wild	56	1,585	578	1,482
			Domestic	63	762,858	2,740,292	355,189
		Asia	Wild	1	1	0	1
			Domestic	65	21,662	199,426	15,439
		Europe	Wild	82	262	1	261
			Domestic	4	30,507	56,209	9,326
	Jul-Dec	Africa	Wild	16	22,740	621	16,915
			Domestic	121	1,118,830	1,325,752	435,672
		Asia	Wild	39	8,513	1	8,438
			Domestic	65	142,464	3,144,530	80,090
		Europe	Wild	777	2,593	776	1,980
			Domestic	663	4,424,915	12,892,088	964,652
		Americas	Wild	11	18	8	8
			Domestic	2	344	109	328
2022	Jan-Jun	Africa	Wild	14	5,016	33	6,479
			Domestic	156	1,388,427	1,414,322	608,374
		Asia	Wild	143	560	0	547
			Domestic	248	206,996	5,606,772	242,085
		Europe	Wild	1,660	13,251	2,298	13,103
			Domestic	1,798	15,609,649	23,075,022	1,605,322
		Americas	Wild	225	1,038	230	1,144
			Domestic	487	94,597	42,181,259	274,446
	Jul-Dec	Africa	Wild	1	2	1	1
			Domestic	24	100,472	76,839	53,813
		Asia	Wild	4	275	0	275
			Domestic	81	86,838	1,280,501	72,685
		Europe	Wild	671	1,853	2,369	1,700
			Domestic	382	1,286,247	8,034,970	341,365
		Americas	Wild	43	40	8	35
			Domestic	285	288	8,790,840	277,716
Total				8,254	25,540,079	111,233,651	5,467,477

Appendix 2 Table 7. Cross-reactive hemagglutinin inhibition (HI) antibody titers of different H5N1 viruses with antisera of different vaccine strains

		Sera			
		Vaccine candidate			
		Clade 2.3.2.1f	Clade 2.3.4.4h	Clade 2.3.4.4b	
		Re12 CK/LN/SD007/2017 (H5N1)	Re13 DK/FJ/S1424/2020 (H5N6)	Re11 DK/GZ/S4184/2017 (H5N6)	Re14 WS/SX/4-1/2020 (H5N8)
Clade	Antigen				
2.3.2.1f	CK/LN/SD007/2017(H5N1) Re12	512	2	4	2
2.3.4.4h	DK/FJ/S1424/2020(H5N6) Re13	1*	1024	64	4
	DK/GZ/S4184/2017 (H5N6) Re11	1	128	256	8
2.3.4.4b	WS/SX/4-1/2020(H5N8) Re14	1	16	8	256
	MD/HLJ/HL-1/2021(H5N1)	1	8	16	64
	WS/HeN/14/2021(H5N1)	1	16	32	128
	WS/SX/608/2021(H5N1)	1	16	32	128
	MD/HeN/426/2021(H5N1)	2	16	32	128
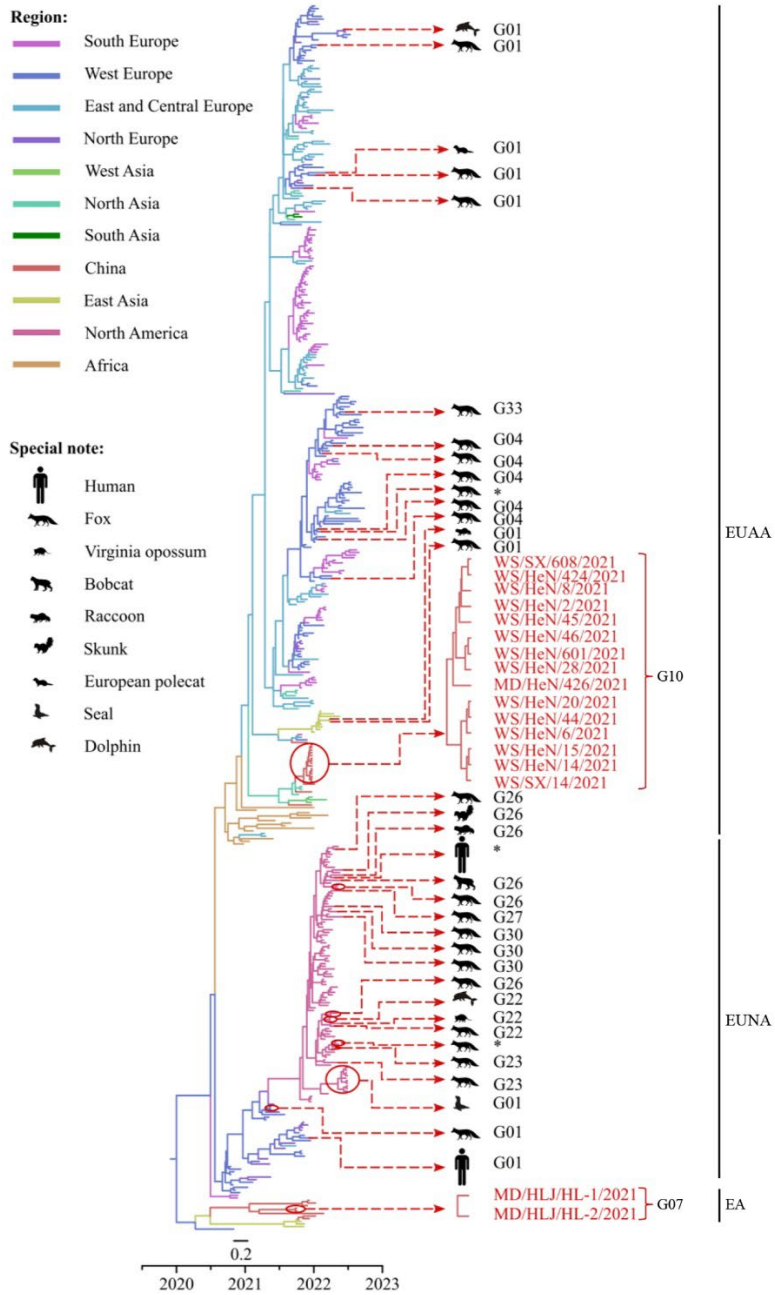
Appendix 2 Figure 1. Maximum-clade credibility time-scaled phylogenetic tree of the hemagglutinin

genes of clade 2.3.4.4b H5N1 viruses. The trunk is colored according to geographic location. The detailed

viral information used to generate the tree is shown in Appendix 1

(https://wwwnc.cdc.gov/EID/article/29/7/22-1149-App1.xlsx) and Appendix 2 Figure 2. The names of the

17 viruses isolated and sequenced in this study are shown in red. *, genotype is unknown because its

complete genomic sequence is unavailable.
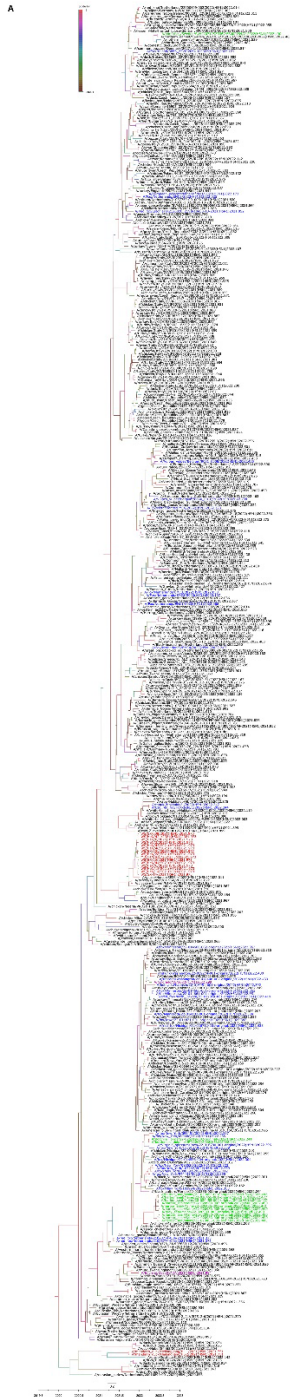
Appendix 2 Figure 2. The maximum-clade credibility tree of the HA gene was constructed by using the package BEAST (v1.10.2). A) Tree branch is colored for posterior probability; B) Tree branch is colored for genotype. The H5N1 viruses isolated in this study are colored in red. The H5N1 viruses isolated from humans, mammals, and marine mammals are shown in purple, blue, and green, respectively.

Appendix 2 Figure 3. Phylogenic analysis of the NA and internal genes of H5N1 viruses by using the neighbor-joining method. (A) NA, (B) PB2, (C) PB1, (D) PA, (E) NP, (F) M, (G) NS. The H5N1 viruses isolated in this study are shown in red.

AF: Africa, WEU: West Europe, NEU: North Europe, ECEU: East and Central Europe, SEU: South Europe, CN: China, NAS: North Asia, EAS: East Asia, WAS: West Asia, SAS: South Asia, NA: North America, RU: Russia, EU: Europe, LP: low pathogenic avian influenza.

Appendix 2 Figure 4. The genotypes of the 1529 H5N1 viruses analyzed in this study. The groups for each segment are indicated by different colored bars.
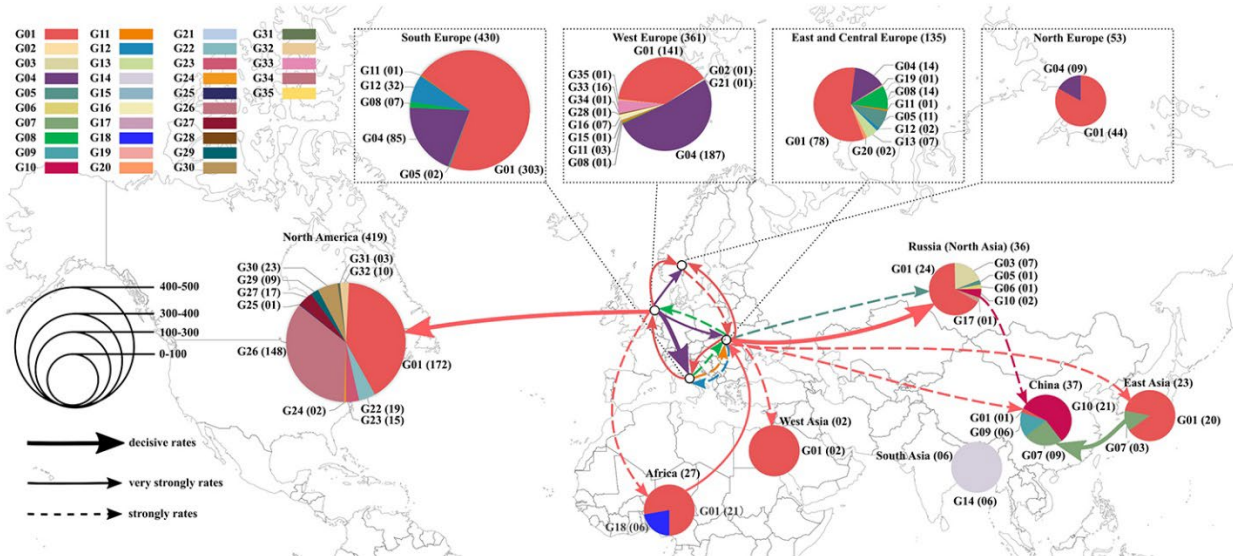
Appendix 2 Figure 5. Spatiotemporal spread and distribution of different genotypes of highly pathogenic avian influenza virus (H5N1) viruses bearing the clade 2.3.4.4b HA gene. The arrows in boldface indicate decisively supported diffusion (Bayes factor [BF] $\geq$1,000), solid arrows represent strongly supported diffusions (100 $\leq$BF <1,000), and dashed arrows show supported diffusions (30 $\leq$BF <100). The circle size is proportional to the number of isolates, and the numbers of strains of each genotype reported in the indicated region are shown in the parentheses.