Highly Pathogenic Avian Influenza A(H5N1) Virus Clade 2.3.4.4b in Domestic Ducks, Indonesia, 2022

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Highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses were isolated from domestic ducks in South Kalimantan, Indonesia, during April 2022. The viruses were genetically similar to those detected in East Asia during 2021–2022. Molecular surveillance of wild birds is needed to detect potential pandemic threats from avian influenza virus.

The H5N1 subtype of the avian influenza virus A/goose/Guangdong/1/96 (Gs/GD/96) lineage has caused highly pathogenic avian influenza (HPAI) outbreaks in poultry since 1996. In 2008, various novel reassortant viruses were identified in domestic duck and live bird markets (LBMs) in China bearing the genetic backbone of Gs/GD/96 virus clade 2.3.4 hemagglutinin (HA) but different combinations of neuraminidase, such as H5N2, H5N5, H5N6, and H5N8 (*1*). Clade 2.3.4 continued to evolve into 5th order genetic groups (clades 2.3.4.4a–h); reassortment created different genotypes within those clades (*1*). H5N8 clade 2.3.4.4 viruses have predominantly spread across many countries in Asia to Europe, Africa, and North America (*1*,*2*); repeated outbreaks caused by H5N8 clade 2.3.4.4b viruses were reported during 2016 to mid-2020 (*3*,*4*). However, H5N1 clade 2.3.4.4b virus emerged in late 2020, which led to an increase in wild bird and poultry influenza outbreaks worldwide; this virus strain has almost entirely replaced H5N8 clade 2.3.4.4b globally since late 2021 (*5*). Moreover, the eastward movement of H5N1 clade 2.3.4.4b virus outbreaks from Europe to East Asia since late 2021 suggests that wild birds likely play a role in virus introduction (*5*,*6*).

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The Study

In April 2022, high numbers of poultry deaths were reported from 5 duck farms in Hulu Sungai Utara District, South Kalimantan Province, Indonesia (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/30/3/23-0973-App1.pdf). Approximately 4,430 of 5,770 (76.8%) ducks of different ages died; younger ducks manifested more severe disease. In July 2023, the deaths of 294 (135 adult and 159 young) of 450 ducks were reported in a Muscovy duck farm in Banjarbaru District of South Kalimantan Province. We collected oropharyngeal swab or tissue samples from ducks in Hulu Sungai Utara in 2022 and Banjarbaru in 2023 for necropsy and hematoxylin/eosin staining; gross and histologic pathology analyses were performed at the Disease Investigation Center Banjarbaru (Appendix). We also collected samples from ducks in LBMs within Banjar District (October 2022), which is located between the Hulu Sungai Utara and Banjarbaru districts where disease was reported (Appendix Figure 1). We sent all influenza A(H5) PCR–positive samples to the Disease Investigation Center Wates in Yogyakarta, where viruses were isolated by using the World Organisation for Animal Health protocol (*7*). However, viruses could only be isolated from 3 pooled swab samples from the initial cases in April 2022 in Hulu Sungai Utara, 1 tissue sample from the July 2023 case in Banjarbaru, and 1 pooled swab sample from LBMs in Banjar. We characterized the virus isolates antigenically by using hemagglutination inhibition assays and

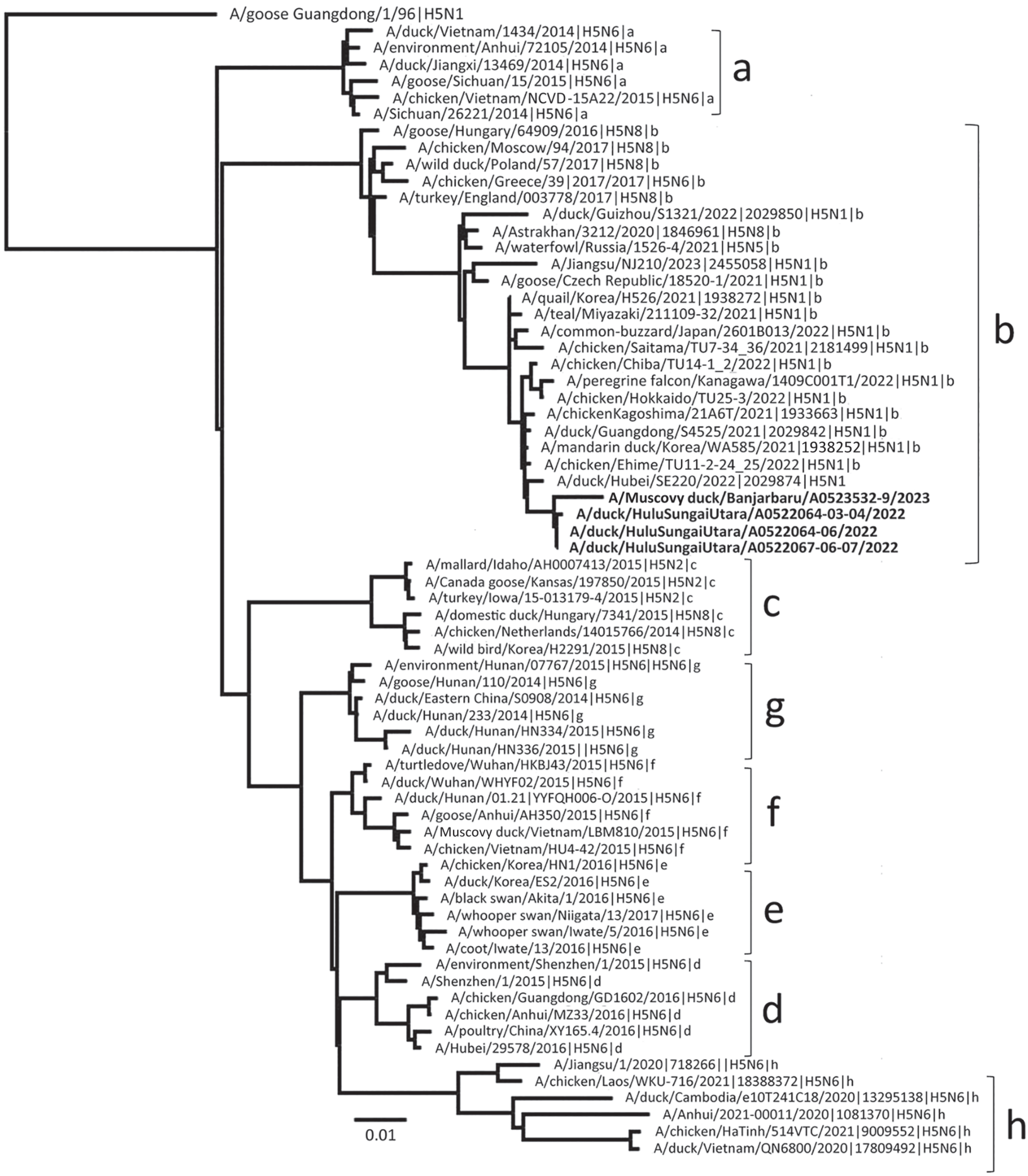

Figure. Phylogenetic analysis of the hemagglutinin gene of highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses isolated from domestic ducks during outbreaks in South Kalimantan, Indonesia, in April 2022 and July 2023 compared with reference sequences. Bold font indicates the viruses isolated from duck farms in this study. Letters at right indicate subclades. Evolutionary history was inferred by using the maximum-likelihood method and best-fit general time reversible plus gamma distribution 4 substitution model involving 67 hemagglutinin H5 sequences from the GISAID database (http://www.gisaid.org); a total of 1,656 positions were in the final dataset. Scale bar indicates nucleotide substitutions per site.

genetically by using whole-genome sequencing on an Illumina sequencing platform (https://www.illumina.com) (Appendix).

We deposited whole-genome sequences of 4 virus isolates into the GISAID database (https://www.gisaid.org) under accession nos. EPI_ISL_17371282 (A/duck/Hulu Sungai Utara/A0522064-06/2022), EPI_ISL_17371283 (A/duck/Hulu Sungai Utara/A0522064-03-04/2022), EPI_ISL_17371284 (A/duck/Hulu Sungai Utara/A0522067-06-07/2022), and EPI_ISL_18438033 (A/Muscovy duck/Banjarbaru/A0523532-9/2023). All 5 identified virus isolates were H5N1 clade 2.3.4.4b viruses, but the virus isolate from LBMs in Banjar District was not included in further analysis or deposited in the GISAID database because of incomplete gene sequences (<50% full-length sequence for each gene segment).

Phylogenetic analysis of the HA gene segment showed that all 4 analyzed viruses clustered with recent HPAI H5 clade 2.3.3.4b viruses from Asia and Europe (Figure). However, they appeared to be more closely related to H5N1 clade 2.3.4.4b viruses from wild birds and poultry from Japan, China, and South Korea isolated during October 2021–February 2022. Phylogenetic trees for the other gene segments (polymerase basic 1, polymerase basic 2, polymerase acidic, nucleoprotein, neuraminidase, matrix protein, and nonstructural segments) also indicated that all 4 viruses were closely related to H5N1 clade 2.3.4.4b from Japan, China, and South Korea (Appendix Figures 2–5). The 3 viruses isolated from the influenza outbreak in April 2022 shared 99.8%–100% nucleotide sequence similarity for each viral segment; however, we observed a lower nucleotide sequence similarity between the viruses from April 2022 and the virus isolated in July 2023 (Table 1), indicating that H5N1 clade 2.3.4.4b continued to mutate resulting in genetic drift. We identified all virus isolates as HPAI on the basis of amino acid sequences within the HA cleavage site (REKRRKR|G); none of those isolates had molecular determinants associated with increased binding affinity or replication efficiency in mammals, including humans (Appendix Table 1) (*8*,*9*). A BLAST search (https://www.ncbi.nlm.nih.gov/blast) and pairwise distance analysis indicated all 8 gene segments from viruses isolated during the first outbreak in April 2022 had 98.4%–99.8% nucleic acid sequence identities to H5N1 clade 2.3.4.4b viruses from Japan, China, and South Korea, suggesting a close common ancestor.

The gross and histologic pathology of naturally infected ducks showed multiorgan hemorrhages

Table 1. DNA sequence homologies between highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses isolated from domestic ducks in Indonesia, 2022, and those from Banjarbaru and East Asia*

Virus name	GISAID no.†	Collection date	% Nucleic acid similarity for each gene segment							
			PB2	PB1	PA	HA	NP	NA	MP	NS
Viruses from first outbreak in Hulu Sungai Utara	EPI_ISL_17371282, EPI_ISL_17371283, EPI_ISL_17371284	2022 Apr	100	100	99.8–99.9	99.9–100	100	100	100	99.8–100
A/Muscovy duck/Banjarbaru/A0523532-9/2023	EPI_ISL_18438033	2023 Jul 7	99.4	99.3–99.4	99.1–99.2	98.9–99.0	99.1	98.8	99.8	98.7
A/mandarin duck/Korea/WA585/2021	EPI_ISL_6959592	2021 Oct 26	99.6	99.5–99.6	99.6–99.7	99.2–99.3	99.6	99.8	99.6	99.4
A/quail/Korea/H526/2021	EPI_ISL_6959593	2021 Nov 8	99.4	99.2	99.2–99.3	99.0–99.1	99.3	99.6	99.3	99.2
A/duck/Guangdong/S4525/2021	EPI_ISL_12572655	2021 Dec 8	99.6	99.6	99.3–99.4	99.2–99.3	99.5	99.7	99.7	99.2
A/duck/Hubei/SE220/2022	EPI_ISL_12572659	2022 Jan 10	99.6	99.5	99.3–99.4	99.0–99.2	99.5	99.5	99.6	99.2
A/duck/Guizhou/S1321/2022	EPI_ISL_12572656	2022 Feb 22	99.6	99.6	99.5–99.6	97.2–97.3	99.5	99.4	99.8	99.2
A/chicken/Kagoshima/21A6T/2021	EPI_ISL_6829533	2021 Nov 12	99.6	99.6	99.6–99.7	99.1–99.2	99.6	99.7	99.8	99.4
A/chicken/Saitama/TU7-34,36/2021	EPI_ISL_15063425	2021 Dec 7	99.6	99.3–99.4	99.1–99.2	98.4–98.6	99.1	99.6	99.3	99.0
A/chicken/Ehime/TU11-2-24 25/2022	EPI_ISL_15063431	2022 Jan 4	99.8	92.0	99.5–99.6	99.2–99.3	99.3	99.6	99.7	99.3
A/common buzzard/Japan/2601B013/2022	EPI_ISL_16831015	2022 Jan 27	99.6	99.2–99.3	99.3–99.4	98.6–98.7	99.3	99.6	99.3	99.2
A/teal/Miyazaki/211109-32/2021	EPI_ISL_15613494	2021 Nov 9	99.4	99.2–99.3	99.3	98.7–98.8	99.1	99.6	99.2	99.2

*H5N1 clade 2.3.4.4b viruses isolated from the initial poultry outbreak in Hulu Sungai Utara in April 2022 were compared with those isolated later from Banjarbaru, Indonesia, in July 2023 and H5N1 clade 2.3.4.b viruses from East Asia isolated during October 2021–February 2022. HA, hemagglutinin; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.
†GISAID database (https://www.gisaid.org).

Table 2. Hemagglutinin inhibition assay titers using 2-fold serial dilutions of virus-specific antiserum in study of highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b in domestic ducks, Indonesia, 2022*

Antiserum source, clade, GISAID no.†	Antigen source			
	A/duck/Hulu Sungai Utara/A0522064-06/2022	A/duck/Hulu Sungai Utara/A0522064-03-04/2022	A/duck/Hulu Sungai Utara/A0522067-06-07/2022	A/muscovy duck/ Banjarbaru/A05235 32-9/2023
A/chicken/West Java/PWT-WIJ/2006, H5N1 clade 2.1.3.2, EPI_ISL_12700530‡	<4	<4	<4	16
A/chicken/Barru/BBVM 41-13/2013, H5N1 clade 2.1.3.2a, EPI_ISL_17767706	16	16	16	16
A /duck/Sukoharjo/BBVW-1428-9/2012, H5N1 clade 2.3.2.1c, EPI_ISL_266808§	16	32	32	32
A/chicken/Tanggamus/031711076-65/2017, H5N1 clade 2.3.2.1c, EPI_ISL_17767763¶	16	32	16	32
A/duck/Laos/XBY004/2014, H5N6 clade 2.3.4.4b, EPI_ISL_168385	8	16	8	16
A/duck/Hulu Sungai Utara/A0522064-03-04/2022, H5N1 clade 2.3.4.4b#	512	512	128	128

*Viruses were isolated by using the World Organisation for Animal Health protocol (*7*). The 3 viruses isolated from Hulu Sungai Utara in April 2022 and the virus isolated from Banjarbaru in July 2023 were used as antigen sources.
†GISAID (https://www.gisaid.org).
‡H5N1 clade 2.1.3.2 vaccine-seed strain used in Indonesia since 2009.
§H5N1 clade 2.3.2.1c vaccine strain used during 2012–2020.
¶H5N1 clade 2.3.2.1c vaccine strain used since 2021.
#Homologous antiserum for H5N1 clade 2.3.4.4b viruses isolated from the initial outbreak in Hulu Sungai Utara, April 2022.

with prominent lesions in tissues and congestion and focal necrosis in parenchymal cells, often accompanied by inflammatory cell infiltrates (Appendix, Figure 6). Hemagglutination inhibition assays revealed the virus isolates from April 2022 had low reactivity with H5N1 antiserum derived from circulating viruses, including the H5N1 vaccine strains used for poultry (Table 2). Those results suggest that new vaccine candidates antigenically matched to circulating viruses might be needed in Indonesia, if H5N1 clade 2.3.4.4b viruses continue to infect poultry.

Wild migratory birds might play a role in the intercontinental spread of HPAI H5Nx clade 2.3.4.4 viruses (*1*,*10*,*11*). Indonesia is situated within the East Asian Flyway's island or oceanic routes linking eastern Russia and Japan to the Philippines and eastern Indonesia (*12*). One stopover site is on the west coast of South Kalimantan, where 23 migratory bird species have been identified and observed (*13*). Migratory birds often use stopover sites for 1 day to several weeks to rest and refuel (*12*), providing opportunities for virus transmission through direct or indirect contacts with local wild birds or aquatic poultry within their shared habitats.

During April 2022–July 2023, we conducted molecular surveillance through a network for influenza virus monitoring in Indonesia (*14*) and did not detect other H5N1 clade 2.3.4.4b outbreaks outside of South Kalimantan. Similar to an earlier virus incursion of H5N1 clade 2.3.2.1c in Java in 2012, which initially also affected ducks (*15*), we could not determine the exact origin of virus incursion. However, genetic evidence and bird migration patterns suggest that migratory birds contributed to the introduction of H5N1 clade 2.3.4.4b into Indonesia.

Conclusion

We identified HPAI H5N1 clade 2.3.4.4b viruses in ducks in South Kalimantan, Indonesia. The role of migratory birds in virus introduction cannot be ruled out because South Kalimantan is situated within the East Asia Flyway corridor, and the infected farms were connected to marshes that provided opportunity for direct or indirect contacts with migratory birds. Limited wild bird surveillance and genome sequence data for avian influenza viruses impeded our ability to determine further transmission and spread of H5N1 clade 2.3.4.4b in Indonesia. Both epidemiologic studies and molecular surveillance of wild birds are needed to better prepare for pandemic threats caused by continued avian influenza virus evolution in Indonesia and elsewhere.

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Highly Pathogenic Avian Influenza A(H5N1) Virus Clade 2.3.4.4b in Domestic Ducks, Indonesia, 2022

Appendix

Material and Methods

Samples

In April 2022, we collected 18 oropharyngeal swab and 2 tissue samples from ducks within 3 of 5 duck farms located close to marshes in 2 villages (Sungai Malang and Mamar) in Hulu Sungai Utara District, South Kalimantan Province, Indonesia, that reported duck deaths with neurologic signs. In addition, 35 oropharyngeal swab and 7 tissues samples were collected from domestic ducks for sale in live bird markets (LBMs) in Banjar District in October 2022; 8 tissue and 5 oropharyngeal swab samples were also collected from ducks at a Muscovy duck farm in Banjarbaru Distict in July 2023. Oropharyngeal swab samples were pooled; pooled samples comprised 4–5 swab samples that were placed into viral transfer media in the field. Those samples were collected by the Disease Investigation Center (DIC) Banjarbaru; samples that were influenza A H5–positive by PCR were sent to the National Avian Influenza Reference Laboratory at DIC Wates for virus isolation in 9–10-day-old specific pathogen-free chicken embryonic eggs by using the WOAH protocol (*1*). Viruses could be isolated from only 3 pooled swab samples from the initial duck cases in April 2022 in Hulu Sungai Utara, 1 pooled swab sample from an LBM in Banjar, and 1 tissue sample from July 2023 in Banjarbaru. Viruses were characterized antigenically by using hemagglutination inhibition assays and genetically by whole-genome sequencing for avian influenza virus.

Whole-Genome Sequencing and Phylogenetic Analysis

We performed RNA extraction by using the QIAamp Viral RNA Mini Kit (Qiagen, https://www.qiagen.com) and multisegment reverse transcription PCR with specific primers, MBTuni-12 and MBTuni-13, to amplify all 8 gene segments of avian influenza virus (*2*). DNA libraries were prepared by using the Nextera-XT DNA Library Preparation Kit (Illumina, https://www.illumina.com) according to the manufacturer`s instructions. Whole-genome sequencing was performed by using the MiSeq next-generation sequencing instrument and MiSeq Reagent Kit v3 (both Illumina). Validation and assembly of nucleotide sequences were performed by using Geneious Prime version 2022.2.1 (Geneious, https://www.geneious.com).

Complete genome sequences of A/duck/Hulu Sungai Utara/A0522064–06/2022 (GISAID accession no. EPI_ISL_17371282), A/duck/Hulu Sungai Utara/A0522064–03–04/2022 (no. EPI_ISL_17371283), A/duck/Hulu Sungai Utara/A0522067–06–07/2022 (no. EPI_ISL_17371284), and A/Muscovy duck/ Banjarbaru/A0523532–9/2023 (no. EPI_ISL_18438033) have been deposited in the GISAID database (https://www.gisaid.org). The virus isolate from 1 duck sampled in an LBM in Banjar (A/duck/Banjar/A0522477–74/2022) was identified as avian influenza A(H5N1) clade 2.3.4.4b, but was not included in downstream analyses because of incomplete sequences (partial genes, <50% full-length sequences for each segment). The PhyML Maximum Likelihood method in Unipro UGENE v.46 (*3*) was used for phylogenetic analysis of each gene segment; the general time-reversible nucleotide substitution model with 4 discrete gamma categories was used. Final dendrograms of Newick trees were generated and visualized in FigTree v.1.4.4 (https://github.com/rambaut/figtree/releases). We performed BLAST tool searches (https://www.ncbi.nlm.nih.gov/blast) and nucleotide identity analyses, which calculated identities from the output of pairwise distance analysis for each gene segment in MEGA X (*4*).

Hemagglutination Inhibition Test

We performed hemagglutination inhibition assays by using the WOAH standard method (*1*) to test the reactivity of 3 virus isolates against representative antiserum derived from H5N1 clade 2.1.3.2 virus strains (very few still detected in poultry), clade 2.3.2.1c virus strains (dominant circulating virus clade in poultry), H5N6 clade 2.3.4.4b (A/duck/Laos/XBY004/2014), and the homologous H5N1 clade 2.3.4.4b strain (A/duck/Hulu Sungai Utara/A0522064–03–04/2022).

Gross and Histologic Pathology

Carcasses from dead ducks were necropsied by veterinary pathologists within the postmortem facility in DIC Banjarbaru. Tissue samples included brain, lungs, heart, liver, spleen, pancreas, intestines, and kidney and were processed and embedded into wax by using routine histologic laboratory processes. Embedded tissues were sliced by using standard microtomy methods, and consecutive 4-μm-thick sections were stained with hematoxylin and eosin.

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Appendix Table 1. Amino acid changes in protein segments from highly pathogenic avian influenza A(H5N1) viruses related to increased binding activity and replication in mammal cells or increased virulence in mammals in study of outbreak in domestic ducks, Indonesia, 2022*

	Virus protein segments																
	PB2			PB1	PB1-F2	PA	HA					NA	M2	NS		
Virus, clade	Q591K	E627K	D701N	N105S	N66S	T515I	Cleavage site	Q192R	Q222L	S223N	G224S	Stalk deletion	S31N	Deletion aa 80−84	P42S	PDZ motif
A/Vietnam/1203/2004, clade 3A.1	Q	K	D	N	N	T	RERRRKKR|G	Q	Q	S	G	Yes	N	Yes	S	ESEV
A/Jiangsu/NJ210/2023, clade 2.3.3.4b	Q	E	D	N	N	T	REKRRKR|G	K	Q	R	G	No	S	No	S	ESEV
A/duck/Hulu Sungai Utara/A0522064-06/2022	Q	E	D	N	N	T	REKRRKR|G	K	Q	R	G	No	S	No	S	ESEV
A/duck/Hulu Sungai Utara/A0522064-03-04/2022	Q	E	D	N	N	T	REKRRKR|G	K	Q	R	G	No	S	No	S	ESEV
A/duck/Hulu Sungai Utara/A0522067-06-07/2022	Q	E	D	N	N	T	REKRRKR|G	K	Q	R	G	No	S	No	S	ESEV
A/muscovy duck/Banjarbaru/A0523532-9/2023	Q	E	D	N	N	T	REKRRKR|G	K	Q	R	G	No	S	No	S	EPEV

*HA, hemagglutinin; M2, matrix protein 2; NA, neuraminidase; NS, nonstructural; PA, polymerase acidic; PB1, polymerase basic 1; PB1-F2, polymerase basic 1-frame 2; PB2, polymerase basic 2; PDZ, postsynaptic density protein-95/discs large/zonula occludens-1.

Appendix Table 2. Sequence acknowledgment table for using GISAID EpiFlu database*

No.	Isolate name	GISAID no.	Country	Collection date	Originating Laboratory	Submitting Laboratory	Authors
1	A/Goose/Guangdong/1/96	EPI_ISL_1254	China	1996-Jan-01	NA	Import from public domain	NA
2	A/duck/Vietnam/1434/2014	EPI_ISL_177703	Vietnam	2014-Nov-18	NA	Import from public domain	Hatamachi, J.; Ogaswara, K.; Chu, D.H.; Okamatsu, M.; Sakoda, Y.; Kida, H.; Ogasawara, K.
3	A/Environment/Anhui/72105/2014	EPI_ISL_219801	China	2014-May-20	NA	WHO Chinese National Influenza Center	Wang, Dayan; Li, Xiaodan; Zou, Shumei; Zhang, Ye; Bo, Hong; Li, Xiyan; Chen, Wenbing; Yang, Lei; Shu, Yuelong
4	A/duck/Jiangxi/13469/2014	EPI_ISL_173478	China	2014-Mar-30	NA	Import from public domain	Ma, C.; Lam, T.T.Y.; Chai, Y.; Wang, J.; Fan, X.; Hong, W.; Zhang, Y.; Li, L.; Liu, Y.; Smith, D.K.; Webby, R.J.; Peiris, J.S.M.; Zhu, H.; Guan, Y.
5	A/chicken/Yangzhou/YD1/2014	EPI_ISL_295144	China	2014-Sep-01	NA	Import from public domain	Li, J.; Gu, M.; Sun, W.; Liu, K.; Gao, R.; Liu, D.; Hu, J.; Wang, X.; Hu, S.; Liu, X.

No.	Isolate name	GISAID no.	Country	Collection date	Originating Laboratory	Submitting Laboratory	Authors
6	A/Sichuan/26221/2014	EPI_ISL_163493	China	2014-Apr-21	NA	WHO Chinese National Influenza Center	NA
7	A/chicken/Vietnam/NCVD-15A22/2015	EPI_ISL_244487	Vietnam	2015-Apr-02	NA	Import from public domain	Davis, T.; Jang, Y.
8	A/goose/SiChuan/15/2015	EPI_ISL_255850	China	2015-Apr-07	NA	Wuhan Institute of Virology	NA
9	A/Goose/Hungary/64909/2016	EPI_ISL_271713	Hungary	2016-Dec-14	National Food Chain Safety Office Veterinary Diagnostic Directorate Laboratory for Molecular Biology	DaNAm.Vet. Molbiol	Adam, Dan
10	A/chicken/Moscow/94/2017	EPI_ISL_17767843	Russian Federation	2017-Feb-28	N.F. Gamaleya Research Center for Epidemiology and Microbiology	Import from public domain	Voronina, O.L.; Ryzhova, N.N.; Aksenova, E.I.; Kunda, M.S.; Sharapova, N.A.N.E.; Fedyakina, I.T.; Chvala, I.A.; Borisevich, S.V.; Loguniv, D.Y.; Gintsburg, A.L.
11	A/wild duck/Poland/57/2017	EPI_ISL_300745	Poland	2017-Jan-27	NA	National Veterinary Research Institut Poland, PIWet-PIB	Swieton, E.; Smietanka, K.
12	A/chicken/Greece/39_2017/2017	EPI_ISL_288362	Greece	2017-Feb-06	Thessalonica Veterinary Centre (TVC)	Animal and Plant Health Agency (APHA)	Seekings, James; Ellis, Richard; Brookes, Sharon M.; Reid, Stephen; Lewis, Nicola; Brown, Ian H.; Dovas, C.; Georgiades, D.
13	A/turkey/England/003778/2017	EPI_ISL_253036	United Kingdom	2017-Jan-15	Animal and Plant Health Agency (APHA)	Animal and Plant Health Agency (APHA)	Seekings, James; Ellis, Richard; Brookes, Sharon M.; Reid, Scott; Essen, Stephen; Brown, Ian H.
14	A/duck/Guizhou/S1321/2022	EPI_ISL_12572656	China	2022-Feb-22	Harbin Veterinary Research Institute (CAAS)	Harbin Veterinary Research Institute (CAAS)	Pengfei Cui; Congcong Wang
15	A/Astrakhan/3212/2020	EPI_ISL_1038924	Russian Federation	2020-Dec-12	Center of Hygiene and Epidemiology in Astrakhan Region	State Research Center of Virology and Biotechnology (VECTOR)	Pyankova, O.; Susloparov, I.; Marchenko, V.; Ryzhikov, A.
16	A/goose/Czech Republic/18520-1/2021	EPI_ISL_17767177	Czech Republic	2021-Sep-27	State Veterinary Institute Prague	Import from public domain	Nagy, A.; Cernikova, L.; Stara, M.
17	A/chicken/Saitama/TU7-34,36/2021	EPI_ISL_15063425	Japan	2021-Dec-07	NA	Import from public domain	Soda, K.; Usui, T.; Ito, H.; Yamaguchi, T.; Ito, T.
18	A/teal/Miyazaki/211109-32/2021	EPI_ISL_15613494	Japan	2021-Nov-09	NA	Import from public domain	Soda, K.; Mekata, H.; Yamada, K.; Ito, H.; Usui, T.; Yamaguchi, T.; Ito,T.
19	A/common buzzard/Japan/ 2601B013/2022	EPI_ISL_16831015	Japan	2022-Jan-27	NA	Import from public domain	Soda, K.; Ito, H.; Usui, T.; Yamaguchi, T.; Ito, T.

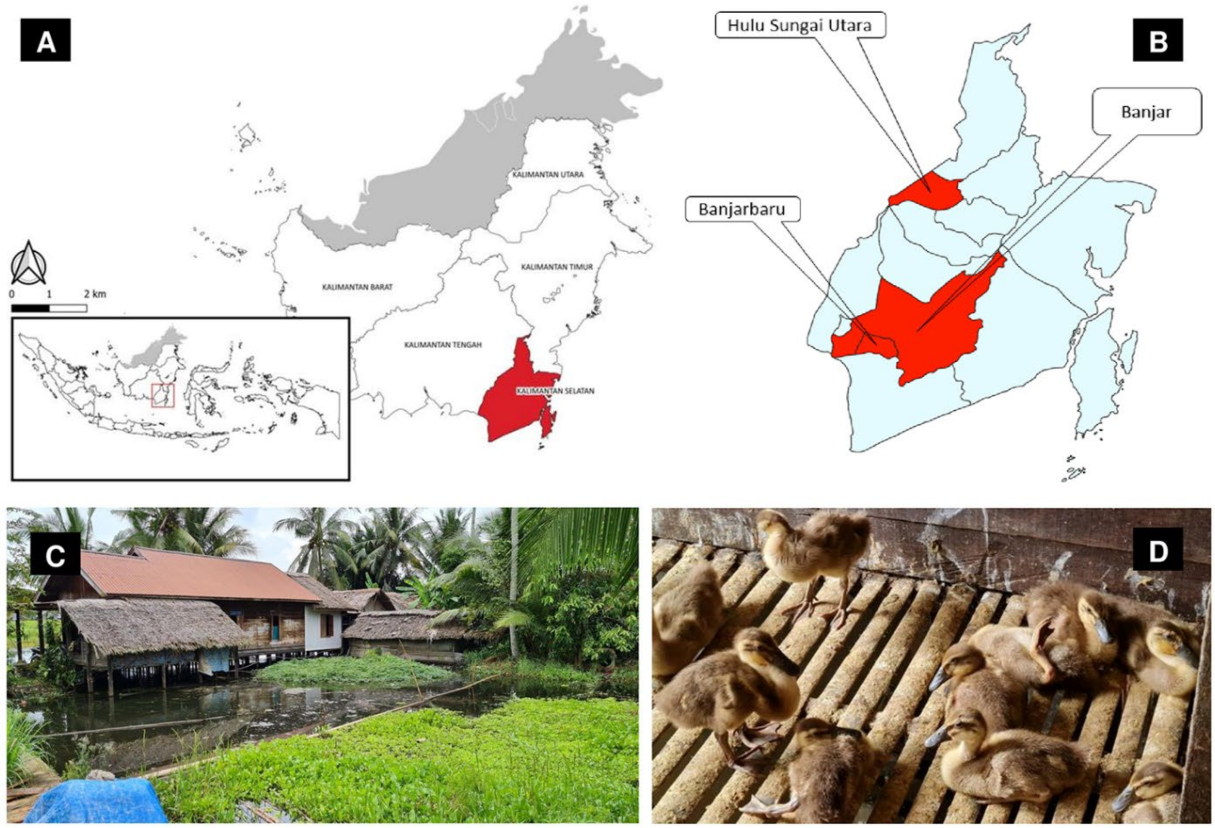
No.	Isolate name	GISAID no.	Country	Collection date	Originating Laboratory	Submitting Laboratory	Authors
20	A/quail/Korea/H526/2021	EPI_ISL_6959593	Korea, Republic of	2021-Nov-08	Animal and Plant Quarantine Agency (O-2144)	Animal and Plant Quarantine Agency (APQA)	NA
21	A/peregrine falcon/Kanagawa/1409C001T1/2022	EPI_ISL_15923322	Japan	2022-Sep-25	National Institute for Environmental Studies	National Institute of Animal Health	Manabu, Onuma; Kei, Nabeshima; Atsushi, Haga; Hisako, Honjo; Misako, Yokoyama; Yuko, Uchida; Kohtaro, Miyazawa; Ryota, Tsunekuni; Junki, Mine; Saki,Sakuma; Asuka, Kumagai; Yoshihiro Takadate
22	A/chicken/Ehime/TU11-2-24,25/2022	EPI_ISL_15063431	Japan	2022-Jan-04	NA	Import from public domain	Soda, K.; Ito, H.; Hisada, R.; Usui, T.; Yamaguchi, T.; Ito, T.
23	A/chicken/Kagoshima/21A6T/2021	EPI_ISL_6829533	Japan	2021-Nov-12	National Institute of Animal Health	National Institute of Animal Health	NA
24	A/mandarin duck/Korea/WA585/2021	EPI_ISL_6959592	Korea, Republic of	2021-Oct-26	Animal and Plant Quarantine Agency (O-2144)	Animal and Plant Quarantine Agency (APQA)	NA
25	A/duck/Guangdong/S4525/2021	EPI_ISL_12572655	China	2021-Dec-08	Harbin Veterinary Research Institute (CAAS)	Harbin Veterinary Research Institute (CAAS)	Pengfei Cui; Congcong Wang
26	A/duck/Hubei/SE220/2022	EPI_ISL_12572659	China	2022-Jan-10	Harbin Veterinary Research Institute (CAAS)	Harbin Veterinary Research Institute (CAAS)	Pengfei Cui; Congcong Wang
27	A/duck/Hulu Sungai Utara/A0522064-06/2022	EPI_ISL_17371282	Indonesia	2022-Apr-04	Disease Investigation Centre Regional V Banjarbaru (BPPVRV)	Balai Besar Veteriner Wates	Wibawa, Hendra; Wibowo, Putut Eko; Lestari; Irianingsih, Sri Handayani; Supriyadi, Arif; Fiqri, Anna Januar; Fahmia, Zaza; Silaban, Jesiaman; Mulyawan, Herdiyanto
28	A/duck/Hulu Sungai Utara/A0522064-03-04/2022	EPI_ISL_17371283	Indonesia	2022-Apr-04	Disease Investigation Centre Regional V Banjarbaru (BPPVRV)	Balai Besar Veteriner Wates	Wibawa, Hendra; Wibowo, Putut Eko; Lestari; Irianingsih, Sri Handayani; Supriyadi, Arif; Fiqri, Anna Januar; Fahmia, Zaza; Silaban, Jesiaman; Mulyawan, Herdiyanto
29	A/duck/Hulu Sungai Utara/A0522067-06-07/2022	EPI_ISL_17371284	Indonesia	2022-Apr-04	Disease Investigation Centre Regional V Banjarbaru (BPPVRV)	Balai Besar Veteriner Wates	Wibawa, Hendra; Wibowo, Putut Eko; Lestari; Irianingsih, Sri Handayani; Supriyadi, Arif; Fiqri, Anna Januar; Fahmia, Zaza; Silaban, Jesiaman; Mulyawan, Herdiyanto
30	A/muscovy duck/Banjarbaru/A0523532-9/2023	EPI_ISL_18438033	Indonesia	2022-Apr-04	Disease Investigation Centre Regional V Banjarbaru (BPPVRV)	Balai Besar Veteriner Wates	Wibawa, Hendra; Wibowo, Putut Eko; Lestari; Irianingsih, Sri Handayani; Supriyadi, Arif; Fiqri, Anna Januar; Fahmia, Zaza; Silaban, Jesiaman; Mulyawan, Herdiyanto
31	A/domestic duck/Hungary/7341/2015	EPI_ISL_177584	Hungary	2015-Feb-23	Danam.Vet.Molbiol	Danam.Vet. Molbiol	Krisztian, Banyai; Szilvia, Farkas; Adam, Dan
32	A/chicken/Netherlands/14015766/2014	EPI_ISL_174349	Netherlands	2014-Nov-19	Wageningen Bioveterinary Research	Wageningen Bioveterinary Research	Heutink, Rene; Harders, Frank; Verschuren-Pritz, Sylvia; Bossers, Alex; Koch, Guus; Bouwstra, Ruth

No.	Isolate name	GISAID no.	Country	Collection date	Originating Laboratory	Submitting Laboratory	Authors
33	A/wild bird/Korea/H2291/2015	EPI_ISL_234336	Korea, Republic of	2015-Jan-30	NA	Animal and Plant Quarantine Agency (APQA)	NA
34	A/mallard/Idaho/AH0007413/2015	EPI_ISL_206408	United States	2015-Jan-17	NA	Import from public domain	Killian, M.L.
35	A/Canada goose/Kansas/197850/2015	EPI_ISL_206450	United States	2015-Mar-13	NA	Import from public domain	Killian, M.L.; Ip, H.S.; Griffin, K.; Messer, J.; McMullen, K.; Long, R.; Hesting, S.
36	A/turkey/Iowa/15-013179-4/2015	EPI_ISL_301110	United States	2015-Jan-01	NA	Import from public domain	Lee, D.-H.; Torchetti, M.; Hicks, J.; Killian, M.; Bahl, J.; Pantin-Jackwood, M.; Swayne, D.
37	A/Environment/Shenzhen/1/2015	EPI_ISL_205314	China	2015-Dec-21	NA	WHO Chinese National Influenza Center	Fang, Shisong; Yang, Lei
38	A/Shenzhen/1/2015	EPI_ISL_205313	China	2015-Dec-28	Shenzhen center for disease control and prevention	WHO Chinese National Influenza Center	Fang, Shisong; Yang, Lei
39	A/poultry/China/XY165.4/2016	EPI_ISL_17767176	China	2016-Sep-01	Chinese Academy of Medical Sciences	Import from public-domain	Zhao, Z.
40	A/chicken/Guangdong/GD1602/2016	EPI_ISL_282397	China	2016-Mar-22	NA	Import from public domain	Sun, W.
41	A/chicken/Anhui/MZ33/2016	EPI_ISL_297930	China	2016-Feb-01	NA	Import from public domain	Liu, K.; Gu, M.; Gao, R.; Li, J.; Liu, D.; Sun, W.; Hu, J.; Xu, X.; Wang, X.; Liu, X.
42	A/black swan/Akita/1/2016	EPI_ISL_243058	Japan	2016-Nov-19	NA	Import from public domain	Okamatsu, M.; Hiono, T.; Matsuno, K.; Kida, H.; Sakoda, Y.
43	A/whooper swan/Iwate/5/2016	EPI_ISL_17767791	Japan	2016-Dec-18	Graduate School of Veterinary Medicine, Hokkaido University	Import from public domain	Sakoda, Y.; Okamatsu, M.; Matsuno, K.
44	A/whooper swan/Niigata/13/2017	EPI_ISL_17767801	Japan	2017-Jan-27	Graduate School of Veterinary Medicine, Hokkaido University	Import from public domain	Sakoda, Y.; Okamatsu, M.; Matsuno, K.
45	A/coot/Iwate/13/2016	EPI_ISL_256512	Japan	2016-Dec-22	NA	Hokkaido University	NA
46	A/chicken/Korea/HN1/2016	EPI_ISL_239261	Korea, Republic of	2016-Nov-16	NA	Animal and Plant Quarantine Agency (APQA)	NA
47	A/duck/Korea/ES2/2016	EPI_ISL_239262	Korea, Republic of	2016-Nov-16	NA	Animal and Plant Quarantine Agency (APQA)	NA
48	A/turtledove/Wuhan/HKBJ43/2015	EPI_ISL_205140	China	2015-Jan-01	NA	Import from public domain	Chen, L.-J.; Lin, X.-D.; Guo, W.-P.; Tian, J.-H.; Zhang, Y.-Z.
49	A/duck/Wuhan/WHYF02/2015	EPI_ISL_205115	China	2015-Jan-01	NA	Import from public domain	Chen, L.-J.; Lin, X.-D.; Guo, W.-P.; Tian, J.-H.; Zhang, Y.-Z.
50	A/duck/Hunan/01.21 YYFQH006-O/2015	EPI_ISL_199079	China	2015-Jan-21	NA	Institute of Microbiology, Chinese	NA

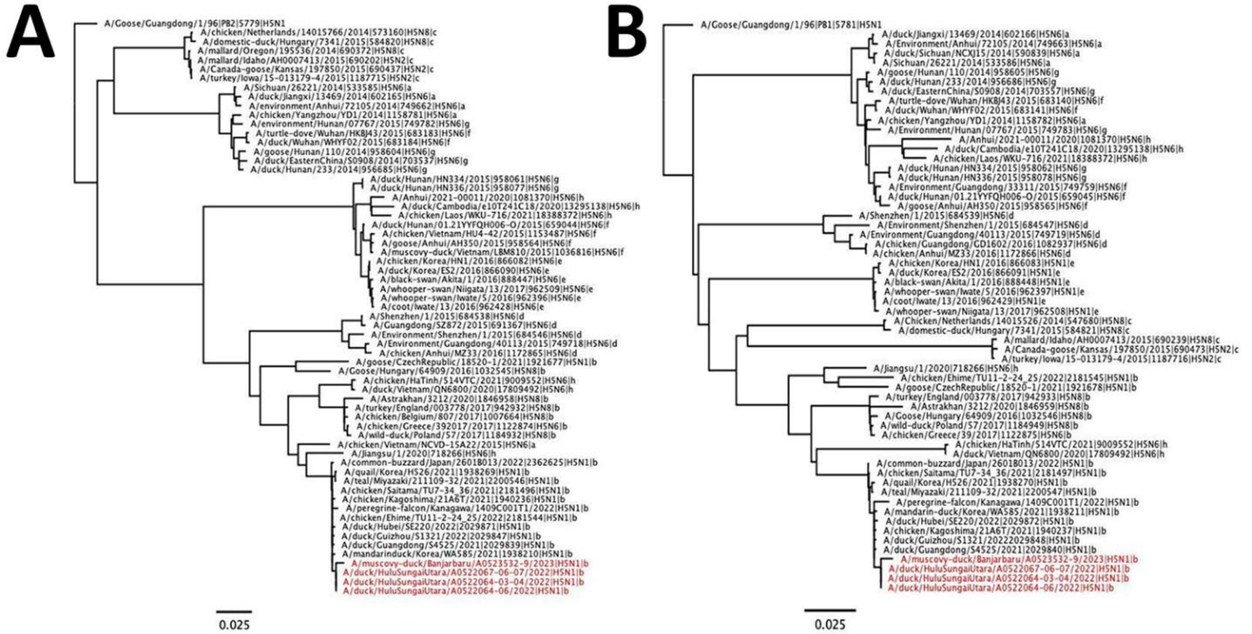
No.	Isolate name	GISAID no.	Country	Collection date	Originating Laboratory	Submitting Laboratory	Authors
51	A/goose/Anhui/AH350/2015	EPI_ISL_255822	China	2015-Dec-19	NA	Wuhan Institute of Virology	NA
52	A/muscovy duck/Vietnam/ LBM810/2015	EPI_ISL_230756	Vietnam	2015-Nov-10	NA	Import from public domain	Soda, K.; Nguyen, K.H.; Le, Q.M.; Ito, T.; Maegaki, S.
53	A/chicken/Vietnam/HU4-42/2015	EPI_ISL_293989	Vietnam	2015-Nov-07	NA	Import from public domain	Sakoda, Y.; Okamatsu, M.; Matsuno, K.; Jizou, M.
54	A/Environment/Hunan/07767/2015	EPI_ISL_219816	China	2015-Jan-08	NA	WHO Chinese National Influenza Center	Wang, Dayan; Li, Xiaodan; Zou, Shumei; Zhang, Ye; Bo, Hong; Li, Xiyan; Chen, Wenbing; Yang, Lei; Shu, Yuelong
55	A/goose/Hunan/110/2014	EPI_ISL_255827	China	2014-Nov-13	NA	Wuhan Institute of Virology	NA
56	A/duck/Eastern China/S0908/2014	EPI_ISL_208838	China	2014-Sep-08	NA	Import from public domain	Sun, H.; Sun, Y.; Pu, J.; Liu, L.; Li, C.; Xu, G.; Qin, M.; Zhang, Y.; Zhao, H.; Wei, K.; Liu, J.
57	A/duck/Hunan/233/2014	EPI_ISL_255493	China	2014-Nov-13	NA	Wuhan Institute of Virology	NA
58	A/duck/Hunan/HN334/2015	EPI_ISL_255757	China	2015-Dec-18	NA	Wuhan Institute of Virology	NA
59	A/duck/Hunan/HN336/2015	EPI_ISL_255759	China	2015-Dec-18	NA	Wuhan Institute of Virology	NA
60	A/duck/Sichuan/NCXJ15/2014	EPI_ISL_179647	China	2014-Apr-27	NA	Import from public domain	NA
61	A/Environment/Guangdong/40113/2015	EPI_ISL_219808	China	2015-May-11	NA	WHO Chinese National Influenza Center	Wang, Dayan; Li, Xiaodan; Zou, Shumei; Zhang, Ye; Bo, Hong; Li, Xiyan; Chen, Wenbing; Yang, Lei; Shu, Yuelong
62	A/Jiangsu/1/2020	EPI_ISL_718266	China	2020-Nov-28	Nanjing Municipality Center for Disease Control and Prevention	Nanjing Municipality Center for Disease Control and Prevention	NA
63	A/Anhui/2021–00011/2020	EPI_ISL_1081370	China	2020-Dec-22	Anhui Provincial Center for Disease Control and Prevention	WHO Chinese National Influenza Center	NA
64	A/chicken/Ha Tinh/514VTC/2021	EPI_ISL_9009552	Vietnam	2021-Feb-04	State Research Center of Virology and Biotechnology (VECTOR)	State Research Center of Virology and Biotechnology (VECTOR)	NA
65	A/chicken/Laos/WKU-716/2021	EPI_ISL_18388372	Lao, People's Democratic Republic	2021-Apr-20	NA	NA	Duong, B.T.; Than, D.; Yeo, J.S.; Theppangna, W.; Park, H.
66	A/duck/Cambodia/e10T241C18/2020	EPI_ISL_13295138	Combodia	2020-Sep-12	Institut Pasteur du Cambodia	The University of Hong Kong	NA

No.	Isolate name	GISAID no.	Country	Collection date	Originating Laboratory	Submitting Laboratory	Authors
67	A/duck/Vietnam/QN6800/2020	EPI_ISL_17809492	Vietnam	2020-Dec-25	Icahn School of Medicine at Mount Sinai	Icahn School of Medicine at Mount Sinai	Lizheng Guan; Gabriele Neumann; Yoshihiro Kawaoka

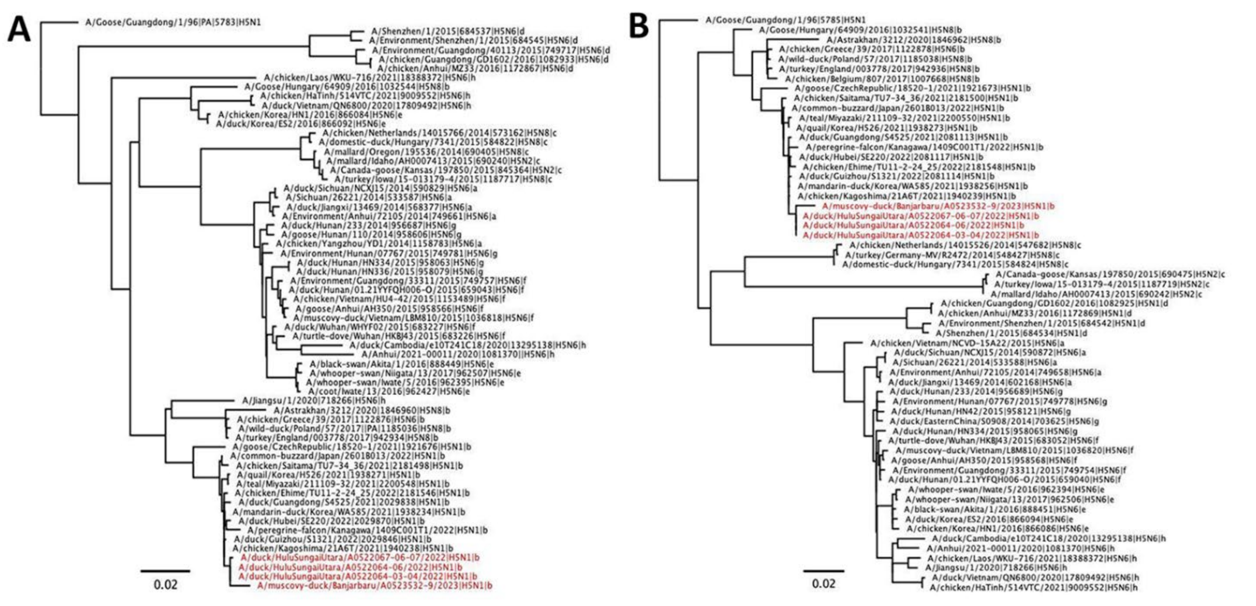
*We gratefully acknowledge the authors and originating and submitting laboratories for the sequences from GISAID's EpiFlu™ database (https://www.gisaid.org) on which this research is based. NA, not applicable; WHO, World Health Organization.



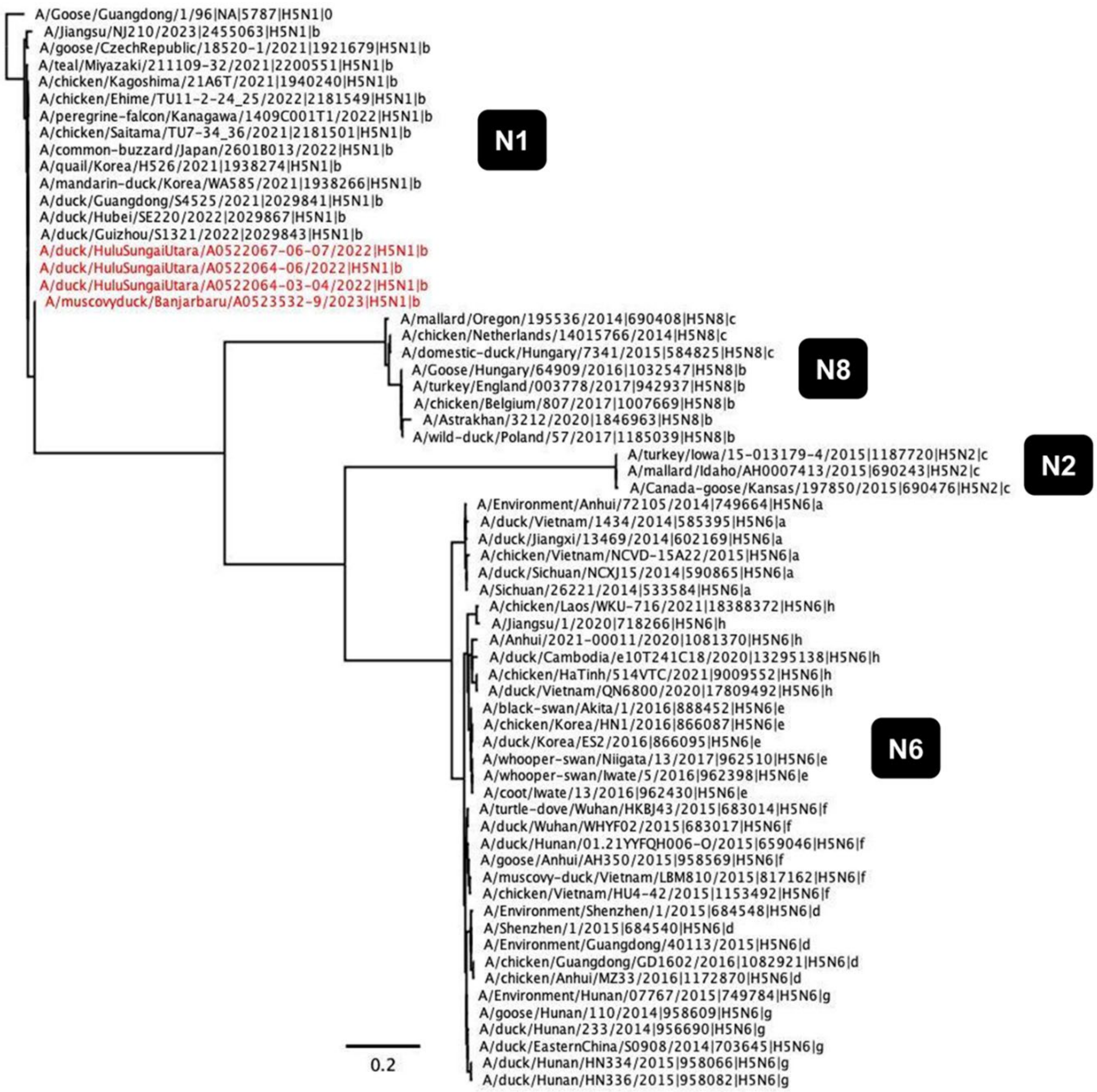
Appendix Figure 1. Duck farms in South Kalimantan Province, Indonesia, infected with highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b. A) Map showing location of South Kalimantan Province, Indonesia (highlighted in red). Inset map shows the islands of Indonesia; red square indicates South Kalimantan Province. B) Map showing districts where H5N1 clade 2.3.4.4b–positive samples were collected from infected duck farms in Hulu Sungai Utara District (April 2022), in Banjarbaru District (July 2023), and from live bird markets in Banjar District (October 2022). C) Photo showing farms identified in the initial outbreak (April 2022) were located above a water flow from marshes. D) High mortality rate found in ducks infected with H5N1 clade 2.3.4.4b; younger ducks showed more severe disease with neurologic signs, such as torticollis and paralysis.



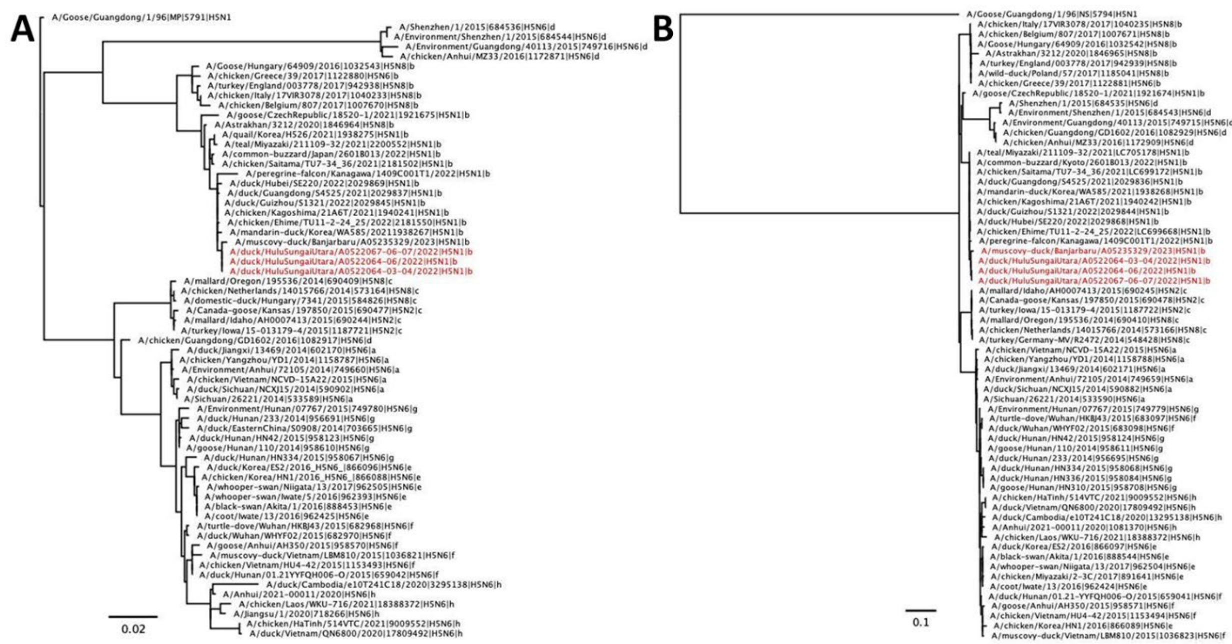
Appendix Figure 2. Phylogenetic analysis of polymerase basic 2 (A) and polymerase basic 1 (B) gene segments from highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses isolated during poultry outbreaks in South Kalimantan, Indonesia. Trees were constructed by using the maximum-likelihood method. Red font indicates viruses isolated from domestic ducks in this study compared with other H5 virus sequences from the GISAID database (https://www.gisaid.org). Scale bar indicates nucleotide substitutions per site.



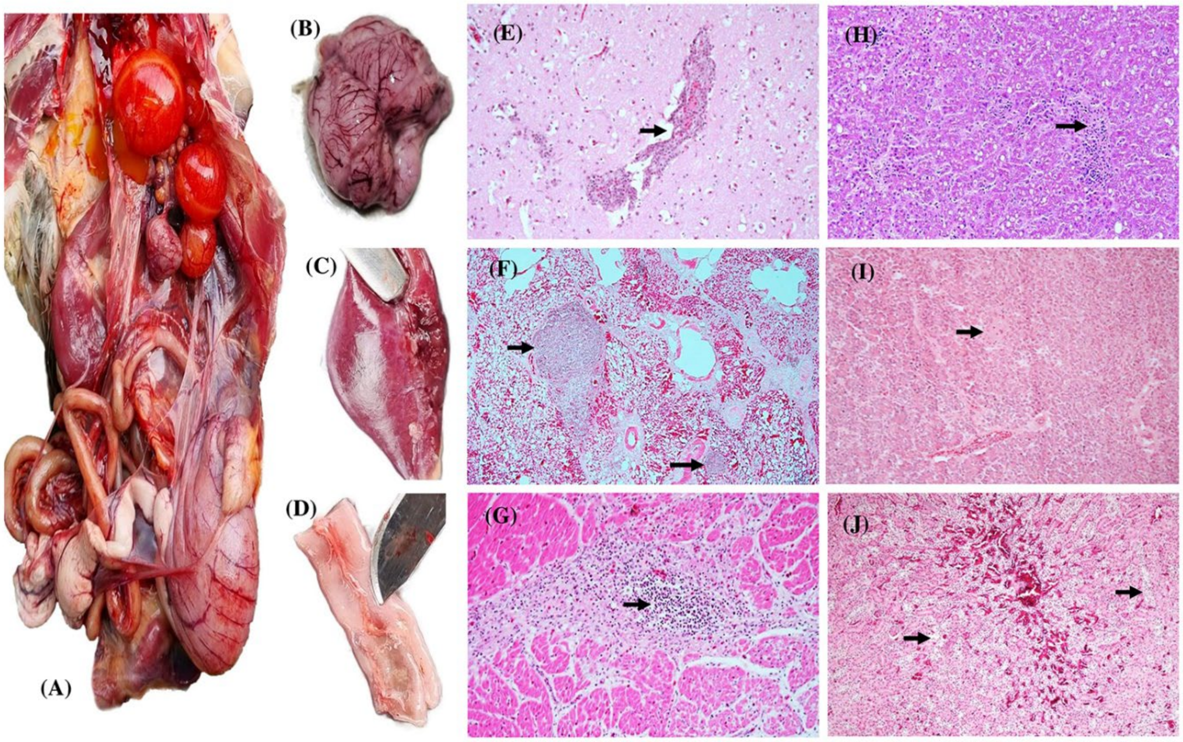
Appendix Figure 3. Phylogenetic analysis of polymerase acidic (A) and nucleoprotein (B) gene segments from highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses isolated during poultry outbreaks in South Kalimantan, Indonesia. Trees were constructed by using the maximum-likelihood method. Red font indicates viruses isolated from domestic ducks in this study compared with other H5 virus sequences from the GISAID database (https://www.gisaid.org). Scale bar indicates nucleotide substitutions per site.



Appendix Figure 4. Phylogenetic analysis of neuraminidase gene segment from highly pathogenic avian influenza A(H5Nx) clade 2.3.4.4b viruses isolated during poultry outbreaks in South Kalimantan, Indonesia. Tree was constructed by using the maximum-likelihood method. Red font indicates viruses isolated from domestic ducks in this study compared with other virus sequences from the GISAID database (https://www.gisaid.org). Numbers in black boxes indicate viruses with neuraminidase subtypes N1, N2, N6, and N8. Scale bar indicates nucleotide substitutions per site.



Appendix Figure 5. Phylogenetic analysis of matrix protein (A) and nonstructural protein (B) gene segments from highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses isolated during poultry outbreaks in South Kalimantan, Indonesia. Trees were constructed by using the maximum-likelihood method. Red font indicates viruses isolated from domestic ducks in this study, which were compared with other H5 virus sequences from the GISAID database (https://www.gisaid.org). Scale bar indicates nucleotide substitutions per site.



Appendix Figure 6. Gross and histologic pathology of ducks naturally infected with highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses in South Kalimantan, Indonesia in April 2022. A–D) Hemorrhages and acute necrosis observed in visceral organs (A) and in brain (B), spleen (C), and intestine (D). E–J) Tissue sections were stained with hematoxylin/eosin. E) Brain section shows congestion, edema, and vasculitis (arrow). F) Lung section shows congestion, perivascular edema, and lymphoid follicles (arrows). G) Heart section shows focal necrosis and inflammatory cell infiltrates (arrow). H) Liver section shows mild focal necrosis and inflammatory cell infiltrates (arrow) and lipid degeneration. I) Pancreas section shows hemorrhages and mild focal necrosis (arrow). J) Kidney section shows interstitial hemorrhages, congestion, and focal necrosis (arrow). E–J) Original magnification ×200.