Detection of Clade 2.3.4.4b Avian Influenza A(H5N8) Virus in Cambodia, 2021

Kimberly M. Edwards, Jurre Y. Siegers, Xiaoman Wei, Ammar Aziz, Yi-Mo Deng, Sokhoun Yann, Chan Bun, Seng Bunnary, Leonard Izzard, Makara Hak, Peter Thielen, Sothyra Tum, Frank Wong, Nicola S. Lewis, Joe James, Filip Claes, Ian G. Barr, Vijaykrishna Dhanasekaran,[1] Erik A Karlsson[1]

In late 2021, highly pathogenic avian influenza A(H5N8) clade 2.3.4.4b viruses were detected in domestic ducks in poultry markets in Cambodia. Surveillance, biosafety, and biosecurity efforts should be bolstered along the poultry value chain to limit spread and infection risk at the animal–human interface.

Since 2014, highly pathogenic avian influenza viruses (HPAIVs) with H5 hemagglutinin (HA) genes grouped in the genetic clade 2.3.4.4 have spread globally causing severe outbreaks in Africa, Europe, Asia, and most recently, North America (*1*). These viruses cause devastating outbreaks in poultry, rapidly evolve, and continuously reassort with other avian influenza viruses (AIVs), posing a threat to food security in many parts of the world and substantial zoonotic infection risk.

The Study

Since 2017, the Institut Pasteur du Cambodge and National Animal Health and Production Research Institute in Cambodia have partnered with the Food and Agriculture Organization of the United Nations to enhance ongoing longitudinal AIV surveillance in live bird markets and poultry storage facilities throughout Cambodia. This active surveillance reveals high levels of AIV circulation with ≈30%–50% of ducks and ≈20%–40% of chickens testing positive for various influenza A subtypes. Most detected HPAIVs were H5N1 HA clade 1 viruses during 2005–2014 and H5N1 HA clade 2.3.2.1c viruses since 2014; H5N6 clades 2.3.4.4g and 2.3.4.4h were detected sporadically during 2018–2020 (Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/29/1/22-0934-App1.pdf). Other subtypes also circulate, including novel H7Nx low pathogenicity avian influenza viruses (LPAIVs) (*2*,*3*).

During active surveillance of live bird markets (National Ethics Committee for Health Research Approval no. 149/NECHR/2020) in late 2021, domestic ducks (*Anas platyrhynchos*) at Orussey (Phnom Penh, n = 1), Takmao (Kandal, n = 2), Chba Ampov (Phnom Penh, n = 1), and Takeo (Takeo, n = 1) tested positive for HPAIV H5 HA but negative for neuraminidase (NA) subtype N1 by real time reverse transcription PCR (RT-PCR). We determined these samples were the H5N8 subtype after further RT-PCR analysis (Appendix 1). Positive samples originated from Orussey and Chba Ampov markets during week 37, Takmao market during week 41, and Takeo market during week 46 of 2021 (Figure 1). Full genome sequencing on a GridION instrument (Oxford Nanopore Technologies, https://www.nanoporetech.com) confirmed these samples were HPAIV H5N8 and H5 HA clade 2.3.4.4b (*4*).

All H5N8 HA sequences from Cambodia encoded proteins with 2-4 amino acid differences

Author affiliations: University of Hong Kong, Hong Kong, China (K.M. Edwards, X. Wei, V. Dhanasekaran); Institut Pasteur du Cambodge, Phnom Penh, Cambodia (J.Y. Siegers, S. Yann, E.A. Karlsson); Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia (A. Aziz, Y.-M. Deng, I.G. Barr); National Animal Health and Production Research Institute, Phnom Penh (C. Bun, S. Bunnary, S. Tum); Australian Center for Disease Preparedness, Geelong, Victoria, Australia (L. Izzard, F. Wong); Food and Agriculture Organization of the United Nations, Phnom Penh (M. Hak); Johns Hopkins University, Baltimore, Maryland, USA (P. Theilen); Royal Veterinary College, London, UK (N.S. Lewis, J. James); OIE/FAO International Reference Laboratory for Avian Influenza, Swine influenza and Newcastle Disease, Weybridge, UK (N.S. Lewis); Food and Agriculture Organization of the United Nations, Bangkok, Thailand (F. Claes)

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[1]These authors contributed equally to this article.

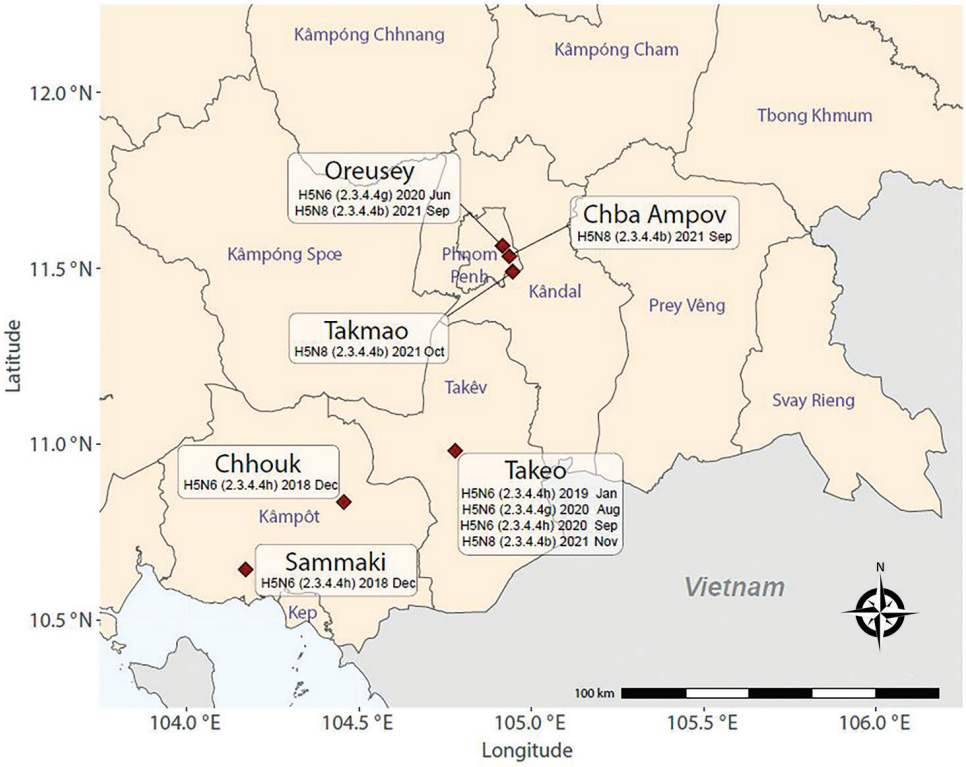

Figure 1. Location of live bird markets where highly pathogenic clade 2.3.4.4b avian influenza A(H5N8) viruses were detected in Cambodia during 2018–2021. The map shows where both H5N6 and H5N8 subtypes of avian influenza A were detected.

relative to the clade 2.3.4.4b candidate vaccine strain A/Astrakhan/3212/2020(H5N8) (Table 1). HA mutations T192I and H276N (according to the H3 numbering system) were shared across all H5N8 HA proteins, whereas A188V occurred in 2 sequences, and E273K, T312S, and I339K each occurred only once. Those mutations did not correlate with previously reported phenotypic traits. Consistent with other clade 2.3.4.4b HA proteins, H5N8 viruses from Cambodia retained the HPAIV cleavage site motif, REKRRKR|GLF. The NA sequences did not contain stalk deletions or markers of antiviral drug resistance. However, other genes encoded amino acid residues associated with increased replication capacity and mammalian pathogenicity, including V89, V292, D309, R389, and T598 in PB2; G622 in PB1; D30, M43, and A215 in M1; and S42 and M106 in NS proteins (*5*).

We performed hemagglutination inhibition assays to assess potential cross-reactivity among the 2.3.4.4b viruses isolated from ducks in Cambodia by using 2 key reference viruses: A/Astrakhan/3212/2020, the recommended candidate vaccine virus for clade 2.3.4.4.b; and A/domestic_duck/England/074477/2021, a recently identified clade 2.3.4.4b virus from poultry associated with human infection in the United Kingdom (*6*). H5N8 viruses from Cambodia with V188, I192 and N276 in HA showed good recognition by antiserum raised against A/Astrakhan/3212/2020. However, A/duck/Cambodia/f1PPOreu241D3/2021 (with I192 and N276, and K339 in HA) and A/duck/Cambodia/f6T241D4/2021 (with I192, K273, N276, S312 in HA) showed reduced recognition by the antiserum (Table 2).

Table 1. Amino acid mutations in hemagglutinin relative to the reference strain A/Astrakhan/3212/2020 in clade 2.3.4.4b avian influenza A(H5N8) viruses detected in Cambodia, 2021*

	HA amino acid position†					
H5 clade 2.3.4.4b strain	188	192	273	276	312	339
A/Astrakhan/3212/2020 (CVV)‡	A	T	E	H	N	I
A/duck/Cambodia/f6T241D4/2021		I	K	N	S	
A/duck/Cambodia/f4K241D3_C/2021	V	I		N		
A/duck/Cambodia/f4K241D4/2021	V	I		N		
A/duck/Cambodia/f1PPOreu241D3_C/2021		I		N		K
A/duck/Cambodia/f1PPChba241D6/2021		I		N		

*Blank cells indicate no mutation. HA, hemagglutinin.
†Amino acids were numbered according to the hemagglutinin H3 numbering system.
‡Candidate vaccine virus reference strain (GISAID accession no. EPI_ISL_1038924; https://www.gisaid.org).

Table 2. Hemagglutination inhibition titers of isolated virus strains in study of clade 2.3.4.4b avian influenza A(H5N8) virus detected in Cambodia, 2021*

Strain	Titer
A/duck/Cambodia/f1PPOreu241D3/2021	40
A/duck/Cambodia/f4K241D4/2021	80
A/duck/Cambodia/f4K241D3/2021	80
A/duck/Cambodia/f6T241D4/2021	40
A/domestic_duck/England/074477/2021	80
A/Astrakhan/3212/2020/2020 (CVV)†	160

*Hemagglutination inhibition titer using ferret antiserum against the reference virus strain A/Astrakhan/3212/2020.
†Candidate vaccine virus reference strain (GISAID accession no. EPI_ISL_1038924; https://www.gisaid.org).

The H5N8 viruses from Cambodia shared >95.7% nucleotide sequence homology across their genomes and formed distinct monophyletic lineages in maximum-likelihood phylogenies of several genes (bootstrap support was 100%, except for the matrix protein gene, which was 89%; Appendix Figure 1), implying circulation of a single virus strain ≥10 weeks from September to November 2021. The H5 HA gene was likely derived from H5N8 viruses that have caused widespread outbreaks in poultry and wild birds across Eurasia since early 2020 (*7*) (Figure 2). N8 NA gene segments were closest to that of HPAIV H5N8 detected in wild and domestic waterfowl in China and Korea during 2020–21, sharing most recent ancestry with NA of A/*Cygnus_columbianus*/Hubei/116/2020(H5N8) that was collected in November 2020 (Appendix Figure 1). Both the HA and MP gene segments were most closely related to

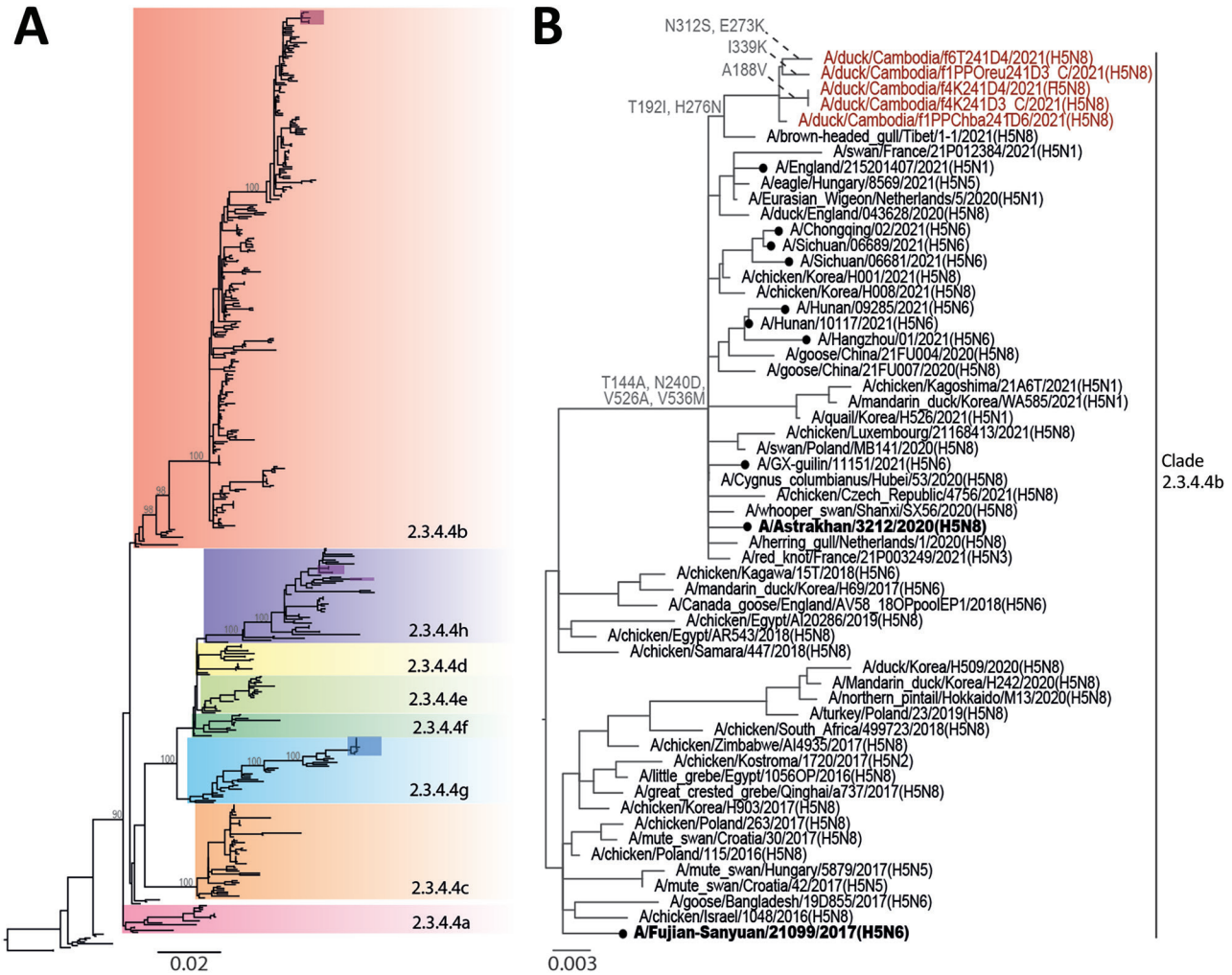

Figure 2. Phylogenetic analysis of the hemagglutinin genes of clade 2.3.4.4 avian influenza A(H5N8) viruses detected in Cambodia. Whole-genome sequencing of isolated viruses was performed and phylogenies were constructed using the maximum-likelihood method. A) Subclades of H5Nx clade 2.3.4.4. Recent isolates from Cambodia are shown in red, purple, and blue shaded boxes. B) Phylogeny of avian influenza A(H5N8) clade 2.3.4.4b isolates. Recent isolates from Cambodia are in red font and amino acid mutations are indicated at select nodes. Candidate vaccine viruses used as reference viruses are in bold font. Closed circles indicate cases of human infection with avian H5Nx viruses. Scale bars indicate nucleotide substitutions per site.

A/brown-headed gull/Tibet/1–1/2021(H5N8), collected in May 2021 (Figure 2, panel B; Appendix Figure 1). In contrast, the other gene segments encoding internal virus proteins were derived from LPAIV (Appendix Figure 1). PB2 and PB1 genes shared common ancestry with LPAIV detected in ducks in Vietnam in 2020. PA and NP genes shared recent common ancestry with LPAIV isolated in 2019 from wild ducks in Korea (PA gene) and China (NP gene). The NS protein gene was most similar to that of LPAIV from ducks in China in 2018. Overall, HPAIV H5Nx clade 2.3.4.4b showed evidence of extensive genetic reassortment with LPAIV found in wild waterfowl, which frequently spillover to and from domestic poultry.

In addition to various LPAIVs, multiple H5 subtypes and clades circulate in Cambodia (Appendix Table 1). H5N1 clade 2.3.2.1c viruses are detected regularly. H5N6 clade 2.3.4.4g viruses were found in Takeo and Orussey markets in chickens in 2019 and ducks in 2020, and H5N6 clade 2.3.4.4h viruses were detected sporadically in Kampot province in late 2018, Takeo province during 2019–2020, and Phnom Penh in 2020 (Figures 1, 2; Appendix Figures 2–5). Therefore, detection of H5N8 clade 2.3.4.4b viruses in these same markets is a major concern because further reassortment might occur. Since 2018, outbreaks of reassorted HPAIV H5Nx clade 2.3.4.4b with NA subtypes N8, N6, N1, N3, and N5 have increased in frequency (*8*). These viruses have disseminated intercontinentally across migratory flyways and regionally via poultry trade, often causing considerable economic losses. In 2021, H5Nx clade 2.3.4.4b viruses caused severe outbreaks in Europe, Africa, and Asia, particularly in wild birds in western China and in domestic poultry in Vietnam (*9*). Since January 2022, HPAIV H5N1 clade 2.3.4.4b has been detected in waterfowl, birds of prey, and poultry across North America (*10*).

H5Nx clade 2.3.4.4b viruses also pose a zoonotic risk to humans and other species. In February 2021, a total of 7 cases of asymptomatic human infections with HPAIV H5N8 clade 2.3.4.4b were reported in poultry farm workers in Russia following a poultry outbreak (*11*). H5N6 clade 2.3.4.4 viruses have caused 79 human infection cases (including 33 cases in 2021) in China with ≈32 deaths since 2014 and 1 case in Laos (*12*), and 3 cases of H5Nx were reported in Nigeria (*9*). HPAIV H5Nx clade 2.3.4.4 viruses have also been detected in domestic cats in China and Korea (*1*) and red foxes in The Netherlands (*1*), and serologic evidence exists for infection in swine (*13*). More recently, HPAIV H5N1 clade 2.3.4.4b containing HA genes closely related to A/Astrakhan/3212/2020 have caused human infections in the United Kingdom (*14*) and United States (*15*). HPAIV H5N1 clade 2.3.3.4b has not been detected in Cambodia.

Conclusions

Because of the global spread, economic impact, and zoonotic potential of HPAIV clade 2.3.4.4b viruses, active, longitudinal surveillance in live bird markets must be maintained in Cambodia, the Greater Mekong Subregion, and globally to monitor further introduction and reassortment events. In addition, surveillance of influenza-like illness needs to be maintained among persons in close contact with infected or deceased poultry. To combat the spread of HPAIV in Cambodia and other countries, viral monitoring, biosafety, and biosecurity efforts should be bolstered along the poultry value chain. Early warning and rapid control will limit infections at the animal–human interface to reduce potential pandemic risk.

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About the Author

Mrs. Edwards serves as project manager for the Pathogen Evolution Laboratory in the School of Public Health at the University of Hong Kong. Her research focuses on the ecology, epidemiology, and evolution of infectious disease at the human–animal interface.

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Address for correspondence: Erik A. Karlsson, Institut Pasteur du Cambodge Virology Unit, 5 Monivong Blvd, PO Box 983, Phnom Penh, Cambodia; email: ekarlsson@pasteur-kh.org

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Detection of Clade 2.3.4.4b Avian Influenza A(H5N8) Virus in Cambodia, 2021

Appendix 1

Materials and Methods

Sample Collection and Screening

We collected tracheal, cloacal, and environmental samples (August 2017–December 2021) from ducks and chickens as described previously (*1*). We extracted viral RNA by using the QIAamp Viral RNA Mini Kit (QIAGEN, https://www.qiagen.com) in accordance with the manufacturer's protocol. We screened all samples by real-time reverse transcription PCR (RT-PCR) of the matrix protein gene and then by using H5 HA-specific primers, as previously described (*2*). We propagated matrix gene-positive samples, if there was sufficient sample and high virus concentration (RT-PCR Ct<30), in 10-day-old embryonated chicken eggs for virus isolation. We tested original isolates for all known subtypes of avian influenza virus by using influenza multiplex RT-PCR.

Genome Sequencing

We amplified whole genomes of influenza A viruses from Cambodia by using custom Uni12/Inf-1 and Uni13/Inf-1 barcoded primers (*3*), kindly provided by Peter Thielen, and SSIII One-step RT-PCR with Platinum Taq High Fidelity kit (Thermo Fisher Scientific, https://www.thermofisher.com). We prepared sequencing libraries by using a ligation sequencing kit (Oxford Nanopore Technologies, https://www.nanoporetech.com). Samples were sequenced by using the GridION platform (Oxford Nanopore Technologies). We then demultiplexed, quality trimmed, and filtered sequencing reads by using Porechop software (https://www.github.com/rrwick/Porechop). We deposited a total of 159 gene segment sequences obtained from 20 influenza A viruses in GenBank under accession numbers ON716288–446.

To generate the consensus sequence, IRMA (Integrated Resource Management Applications, https://irma.nps.gov/Portal) was run with default settings. We manually inspected

consensus sequences for errors, such as insertion-deletion mutations and mixed bases, and corrected if required. We used a minimum 100-bp cutoff for depth coverage for all gene segments. The average per sample depth was 46,800 reads (minimum, 6,000; maximum, 150,000; median, 30,000) and the average per gene depth was 38,000 reads (minimum, 13,000; maximum, 86,000; median, 39,000). Several (4 out of 20) randomly selected viruses underwent resequencing by the World Health Organization Collaboration Center in Melbourne, Australia, for confirmation and quality control. Sequences that were problematic at the multibasic cleavage site were confirmed by using Sanger sequencing at Macrogen (https://www.macrogen.com). A positive control (A/H3N2) and several negative controls were included at each step of the sequencing protocol.

Phylogenetic Analysis

We used metadata from Nextstrain (*4*) (accessed on 2022 Jan 19) to prepare subsets of HPAIV H5 HA sequences, which were downloaded from the GISAID database (*5*) (https://www.gisaid.org, accessed on 2022 Jan 19). We downloaded all available N6 and N8 subtype neuraminidase sequences and genes encoding internal proteins of all H5Nx subtype viruses from GISAID and GenBank. For each of the internal gene datasets, we added the top 10 BLAST matches from GISAID and GenBank that were closest to the H5Nx sequences from Cambodia. We excluded duplicate (according to strain name), laboratory-derived, mixed subtype, and low coverage (<90% of full length) sequences from downstream analysis. We aligned sequences with MAFFT v.7.490 (*6*) and constructed phylogenetic trees by using the best-fit nucleotide substitution model in IQ-TREE v.2.1.4 (*7*). We visualized and annotated the trees by using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

Hemagglutinin Inhibition Assays

We conducted hemagglutinin inhibition (HI) assays according to standard methods (*1*). We treated serum samples with a receptor-destroying enzyme and conducted erythrocyte adsorption before HI testing. In brief, 1 volume of heat-treated serum sample was added to 4 volumes of receptor-destroying enzyme (APHA Scientific, http://apha.defra.gov.uk/apha-scientific/index.htm). We incubated the resulting solution for 16 h at 37°C before heat-inactivation at 56°C for 1 h. We added packed erythrocytes from specific pathogen-free chickens at a ratio of 1:5 (erythrocyte:serum), incubated at room temperature for 30 min, centrifuged at 1000 ×*g*, and transferred the supernatant to a clean tube. We performed the HI assay at 4°C by

using 4 hemagglutinating units of the indicated H5 HPAIV strains as antigens, ferret antiserum against the reference virus, and chicken erythrocytes. We serially diluted serum samples 2-fold for HI assays starting at a dilution of 1:4 and tested the samples in duplicate. We included control wells for each sample to test for nonspecific hemagglutination, and positive antiserum and negative control serum (taken from specific pathogen-free chickens) were also tested for each assay.

Data Availability

Code and accession numbers are available at https://github.com/vjlab/cambodia-H5N8.

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Appendix Table 1. Influenza A virus H5Nx subtypes isolated from live bird markets and sequenced in study of clade 2.3.4.4b avian influenza A(H5N8) virus detected in Cambodia, 2017–2021*

Subtype	H5Nx viruses detected					
(H5 HA clade)	2017	2018	2019	2020	2021	Total
H5N1 (2.3.2.1c)	12	19	33	9	12	85
H5N2 (2.3.2.1c)	0	1	1	0	0	2
H5N6 (2.3.4.4h)	0	3	1	1	0	5
H5N6 (2.3.4.4g)	0	0	0	10	0	10
H5N8 (2.3.4.4b)	0	0	0	0	5	5
Totals	12	23	35	20	17	110

*H, hemagglutinin; HA, hemagglutinin; N, neuraminidase.

Appendix Table 2. Amino acid mutations in HA from avian influenza A(H5N6) clade 2.3.4.4g viruses detected in Cambodia, 2021*

	HA amino acid position†												
H5 clade 2.3.4.4g strain name	5	94	140	151	165	167	192	199	242	276	371	379	507
A/chicken/Vietnam/Raho4-Cd-20–421/2020‡	V	P	Q	V	E	S	T	T	A	R	K	D	S
A/duck/Cambodia/e5PPOreu241D3/2020	I			L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e5PPOreu241D4/2020	I	A		L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e5PPOreu241D8/2020	I			L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D6/2020	I		L	L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D5/2020	I		L	L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D9/2020	I		L	L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D10/2020	I		L	L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D15/2020	I		L	L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D16/2020	I		L	L	K	N	E	A	E	N	R	N	T
A/duck/Cambodia/e8T241D11/2020	I		L	L	K	N	E	A	E	N	R	N	T

*Blank cells indicate no mutation. HA, hemagglutinin.
†H5N6 HA segments were compared to the reference strain A/chicken/Vietnam/Raho4-Cd-20–421/2020. Amino acids were numbered according to the hemagglutinin H3 numbering system.
‡Candidate vaccine virus (GISAID accession: EPI_ISL_1379443).

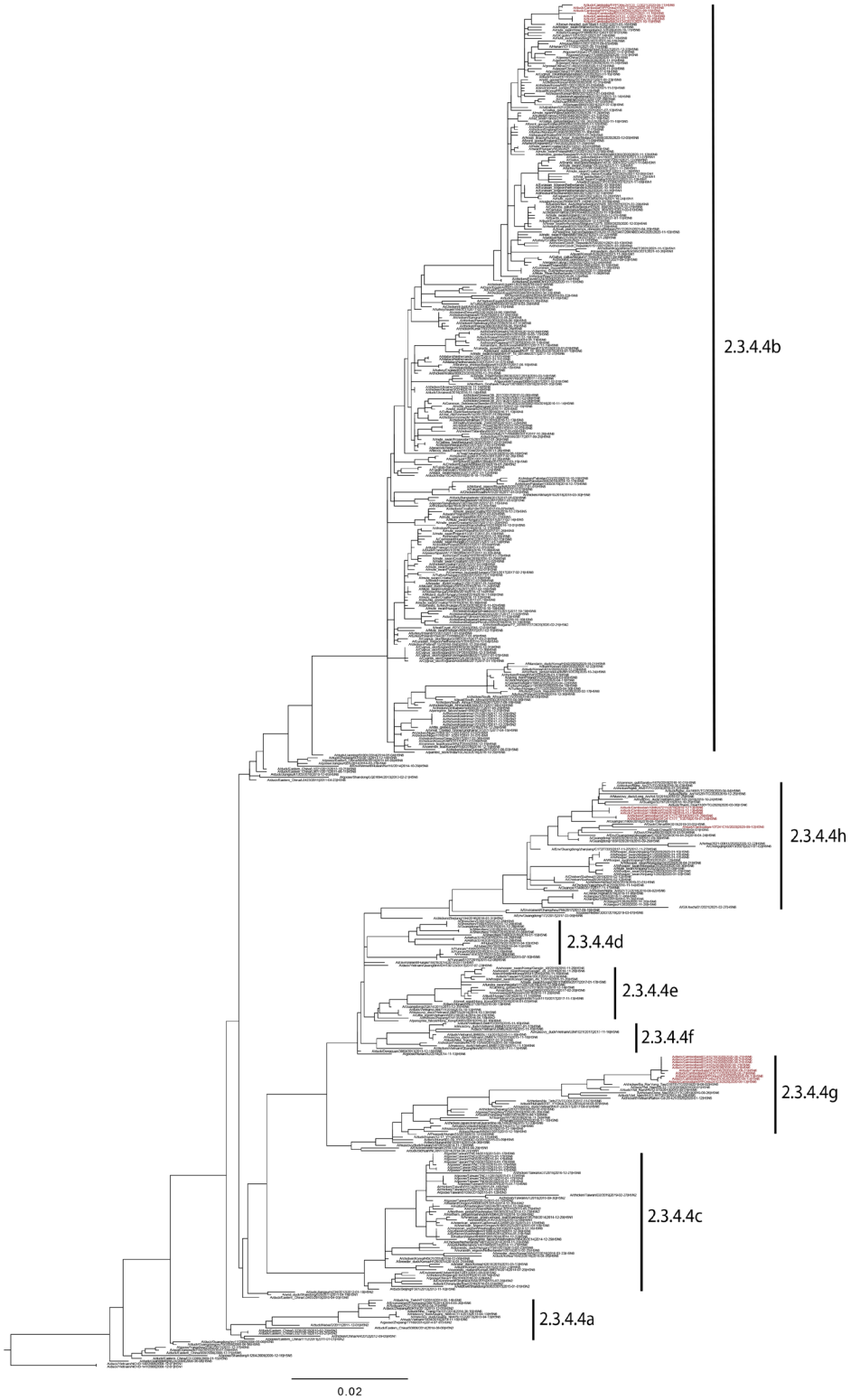
Appendix Table 3. Amino acid mutations in HA from avian influenza A(H5N6) clade 2.3.4.4h viruses detected in Cambodia, 2021*

	HA amino acid position†																
H5 clade 2.3.4.4h strain	4	5	10	41	60	92	125	142	166	187	192	202	214	222	242	450	536
A/Guangdong/18SF020/2018‡	V	V	S	D	V	N	R	Q	M	A	T	V	V	Q	A	K	M
A/duck/Cambodia/c18MKAP189/2018	M				I			T	K		A		M			R	V
A/duck/Cambodia/c18MKAP211/2018	M				I			T	K		A		M			R	V
A/duck/Cambodia/c18MKAP214/2018	M				I			T	K		A		M			R	V
A/chicken/Cambodia/c9T241C17T/2019	M				I	S		T	K		A	I					V
A/duck/Cambodia/e10T241C18/2020		F	N	N			S			V				R	T		V

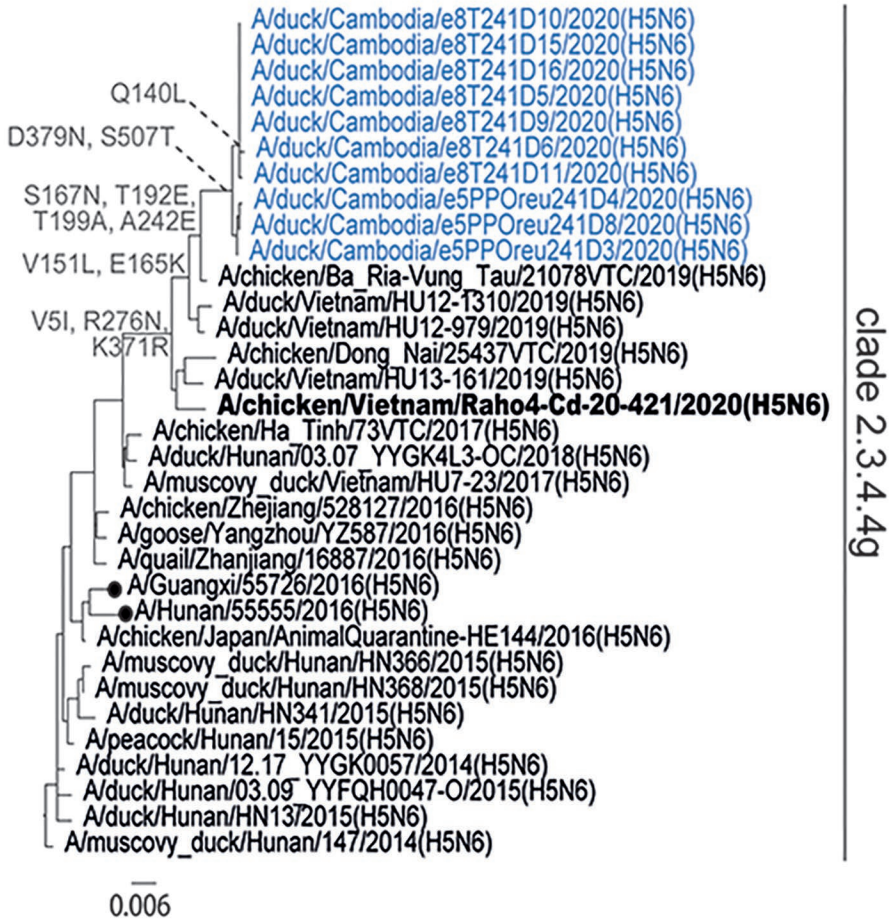
*Blank cells indicate no mutation. HA, hemagglutinin.
†H5N6 HA segments were compared to the reference strain A/Guangdong/18SF020/2018. Amino acids were numbered according to the hemagglutinin H3 numbering system.
‡Candidate vaccine virus (GISAID accession no. EPI_ISL_337274).

Appendix Figure 1. Phylogenetic analysis of different genes of avian influenza A(H5N8) viruses detected in Cambodia. Phylogenies were constructed using the maximum-likelihood method. Recent clade 2.3.4.4b isolates from Cambodia are in red font. Scale bars indicate nucleotide substitutions per site. MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.

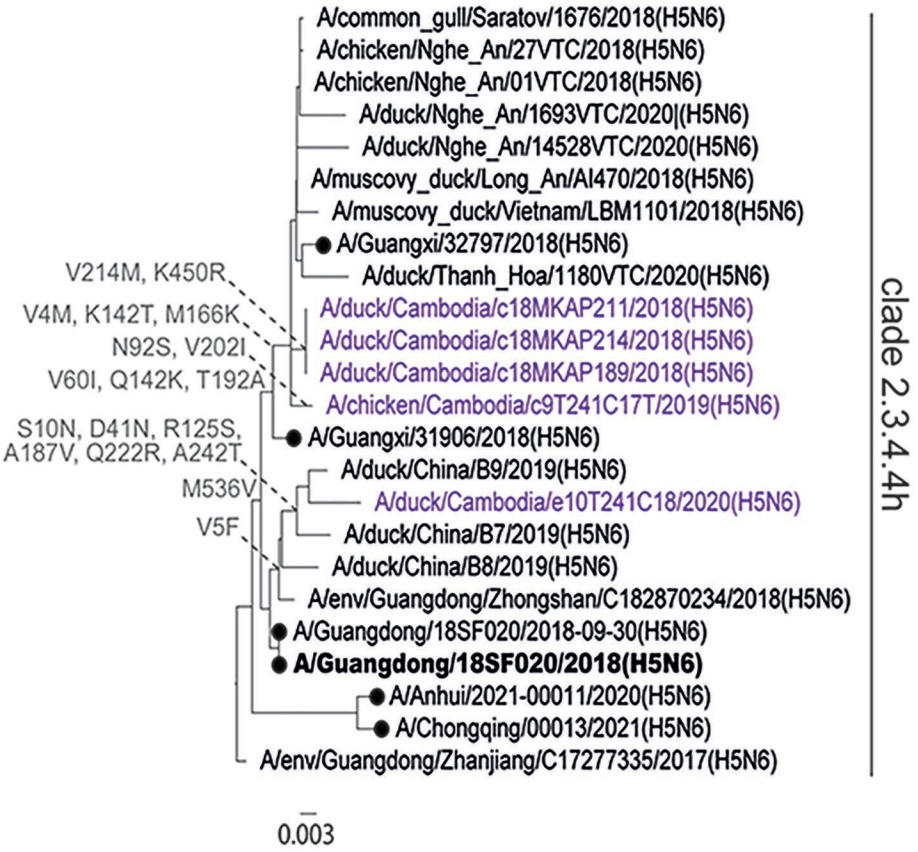
Appendix Figure 2. Phylogenetic analysis of different genes of avian influenza A(H5N6) viruses detected in Cambodia. Phylogenies were constructed using the maximum-likelihood method. Recent clade 2.3.4.4g (purple font) and 2.3.4.4h (blue font) isolates from Cambodia are shown. Scale bars indicate nucleotide substitutions per site. MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.



Appendix Figure 3. Phylogenetic analysis of the hemagglutinin gene of clade 2.3.4.4 viruses generated using the maximum-likelihood method. Recent isolates from Cambodia are in red font. Scale bar indicates nucleotide substitutions per site.



Appendix Figure 4. Phylogenetic analysis of hemagglutinin genes from avian influenza subtype H5N6 in study of clade 2.3.4.4 avian influenza viruses detected in Cambodia. Dendograms were constructed using maximum-likelihood phylogeny and show phylogeny of avian influenza A(H5N6) clade 2.3.4.4g isolates from Cambodia. Recent isolates from Cambodia are in blue font and amino acid mutations are indicated at select nodes. Candidate vaccine viruses used as reference viruses are in bold font. Closed circles indicate cases of human infection with avian H5N6 clade 2.3.4.4g viruses. Scale bar indicates nucleotide substitutions per site.



Appendix Figure 5. Phylogenetic analysis of hemagglutinin genes from avian influenza subtype H5N6 in study of clade 2.3.4.4 avian influenza viruses detected in Cambodia. Dendograms were constructed using maximum-likelihood phylogeny and show phylogeny of avian influenza A(H5N6) clade 2.3.4.4h isolates from Cambodia. Recent isolates from Cambodia are in purple font and amino acid mutations are indicated at select nodes. Candidate vaccine viruses used as reference viruses are in bold font. Closed circles indicate cases of human infection with avian H5N6 clade 2.3.4.4h viruses. Scale bar indicates nucleotide substitutions per site.