Article DOI: http://doi.org/10.3201/eid3006.240194

EID cannot ensure accessibility for supplementary materials supplied by authors. Readers who have difficulty accessing supplementary content should contact the authors for assistance.

Concurrent Infection with Clade 2.3.4.4b Highly Pathogenic Avian Influenza H5N6 and H5N1 Viruses, South Korea, 2023

Appendix

Methods

Surveillance and Sample Collection

On 3 December 2023, a broiler duck farm owner in Goheung, Jeolla-do, reported an increase in mortality of 39 day-old broiler ducks, to the municipal administration. Staff members of the veterinary service in Jeolla-do visited this farm and collected oropharyngeal and cloacal swab samples from 20 birds in each flock. The swabs were pooled by flock in sample collection phase. All of these pooled swabs were H5 positive, and the samples were transferred to the Animal and Plant Quarantine Agency (APQA) for confirmatory diagnosis. For AI active surveillance program on wild bird, on 4 December 2023, staff members at the Livestock Health Control Association, the organization supporting government for sampling, disease control and sanitation, captured 29 live wild mandarin ducks in Jeongeup in Jeolla-do by mist net and collected oropharyngeal and cloacal swab samples from each, then released the wild birds at the site of capture. The samples were delivered to the APQA the next day, with H5N6 HPAI viruses subsequently isolated from oropharyngeal swabs of two mandarin ducks (WA875 and WA859). Active periodic virological surveillance by the veterinary service of Jeolla-do on 5 and 20 December, 2023, found that birds at two broiler duck farms in Muan and Jangheung, respectively, were infected with H5 viruses. Following transfer to the APQA, these positive samples were confirmed to be H5N6 and H5N1 HPAIs, respectively.

Subtyping and Pathotyping

All the samples delivered to APQA were analyzed by qRT-PCR using VDx® AIV qRT-PCR Ver 2.1 (NP-AIV-37, NP-AIV-38, NP-AIV-39, Median Diagnostics, Gangwon, Korea), targeting the AIV matrix and the H5 and H7 genes according to the manufacturer's protocol. H5 positive samples were subjected to in-house HA/NA subtyping by endpoint RT-PCR. Briefly, nucleic acids were extracted from the samples using NX-48 Viral NA kits with Nextractor ® NX-48 (Genolution, Seoul, Korea). Amplicons of eight gene segments of the viruses in these samples and virus isolates were generated by one-step RT-PCR using a previously described protocol (*1*). Gene sequences were amplified using a SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (12574–026, Invitrogen, Carlsbad, CA, USA), two forward primers; CommonA-Uni12G (5'-GCCGGAGCTCTGCAGATATCAGCGAAAGCAGG-3') and CommonA-Uni12 (5'-GCCAGAGCTCTGCAGATATCAGCAAAAGCAGG-3'), and one reverse primer; CommonA-Uni13G (5'- GCCGGAGCTCTGCAGATATCAGTAGAAACAAGG-3'). Each reaction mixture contained 25 µL 2X RT-PCR buffer, 2 µL commercial reverse transcription and PCR polymerase, 0.5 µL of each forward primer (10 µM), 1 µL of reverse primer (10 µM), 10 µL of RNA extracted from a target sample, and 11 µL nuclease free water. The amplification conditions consisted of heating at 42°C for 15 minutes, 55°C for 15 minutes, 60°C for 5 minutes, and 94°C for 2 minutes (ramp rate: 2.5°C/s); 5 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds (ramp rate: 2.5°C/s) and extension at 68°C for 5 minutes (ramp rate: 0.5°C/s); 37 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension 68°C for 5 minutes; and a final extension at 68°C for 5 minutes (ramp rate: 2.5°C/s). For PCR purification, Each RT-PCR product was mixed with 90 µL of Ampure XP SPRI Reagent (A63882, Beckman Coulter, La Brea, CA, USA) and quantified using Qubit dsDNA BR assay kits (Q32853, Invitrogen) with a Qubit 4 fluorometer (Q33226, Invitrogen). Sequencing libraries were generated from each quantified RT-PCR product using a Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technology, Oxford, UK), according to the manufacturer's instructions. All libraries were sequenced on a MinION Mk1C (MIN-101C, Oxford Nanopore Technology) using a Flow Cell (R10.4.1) (FLO-MIN114, Oxford Nanopore Technology) according to the manufacturer's instructions.

Generation of Consensus Genome Sequences

Raw reads were collected for basecalling using MinKNOW V. 23.11.3, with the options, 'Enable read splitting: on', 'Override read splitting min score: on', 'Minimum read splitting score: 58', 'Flow cell product code: FLO-MIN114', 'Chemistry: DNA-400bps-5kHz', 'Model: Super-accurate basecalling', 'Modified basecalling: None', 'Barcoding kits: SQK-NBD114–96', 'Trim barcodes: on', 'Barcode both ends: on', 'Mid-read barcode filtering: off', 'Override minimum barcoding score: off', and 'Alignment reference: off'. Only passed reads were retained for further analysis.

Sequences were assembled using CLC Genomics workbench 23.0.4. Initial import settings followed the options 'Paired reads: off', 'Discard read names: on', 'Discard quality score: off', and 'Illumina options: off'. Reads were mapped to the reference sequences using the default parameters of CLC Genomics workbench 23.0.4, including 'Match score: 1', 'Mismatch cost: 2', 'Cost of insertions and deletions: Linear gap cost', 'Insertion cost: 3', 'Deletion cost: 3', 'Insertion open cost: 6', 'Insertion extend cost: 1', 'Deletion open cost: 6', 'Deletion extend cost: 1', 'Length fraction: 0.5', 'Similarity fraction: 0.8', 'Global alignment: off', 'Auto-detect paired distances: on', 'Non-specific match handling: Map randomly' and 'Masking mode: No masking'. Local realignment was performed using the options 'Realign unaligned ends: on', 'Multi-pass realignment: 2', and 'Guidance-variant settings: off'. Consensus sequences were extracted using the options 'Low coverage definition threshold: 0', 'Low coverage handling: Remove regions with low coverage and Join after removal', and 'Conflict resolution: Vote'. All the sequence data determined in this study were registered in Global Initiative on Sharing Avian Influenza Data (GISAID) under the sequences EPI_ISL_18819959 for A/duck/Korea/D448-N6/2023(H5N6), EPI ISL 18819960 for A/duck/Korea/D448-N1/2023(H5N1), EPI_ISL_18819961 for A/duck/Korea/D449/2023 (H5N6), EPI_ISL_18819826 for A/mandarin

duck/Korea/WA875/2023(H5N6), and EPI_ISL_18819797 for A/duck/Korea/D502/2023(H5N1).

Pairwise Comparisons

Nucleotide identity between viral gene segments of A/duck/Korea/D448-N6/2023(H5N6) and A/duck/Korea/D448-N1/2023(H5N1) were determined by comparing coding region sequence. The percent nucleotide identifies for segments 1–5 and 7–8 were found to be 91.1%, 94.8%, 95.5%, 97.9%, 92.6%, 99.2%, and 95.7%, respectively. Segment 6 in the two viruses were found to be of different subtypes.

Phylogenomic Analysis

Reference nucleotide sequences in the GISAID databases, specifically the isolates in the 100 top BLAST hits for the eight genes of the isolated HPAI viruses, were downloaded by BLAST searches on 30 December 2023. These sequences were aligned with MAFFT using the default parameters for FASTA alignment. All untranslated regions (UTRs) were removed, with only protein-coding sequences of each segment being retained. Phylogenetic analyses of the HA gene and the N1 and N6 subtypes of the NA gene were performed using 518, 362, and 141 sequences, respectively; whereas phylogenetic analyses of the PB2, PB1, PA, NP, MP, and NS genes were performed using 802, 795, 797, 809, 804, and 775 sequences, respectively. Maximum likelihood trees based on the aligned sequences were constructed using RAxML on XSEDE version 8.2.12 (2) and CIPRES Science Gateway (https://www.phylo.org/) (3) Trees were displayed with interactive iTOL (https://itol.embl.de/login.cgi), with bootstrap values over than 75% noted on the nodes (4)

References

- Van Poelvoorde LAE, Bogaerts B, Fu Q, De Keersmaecker SCJ, Thomas I, Van Goethem N, et al. Whole-genome-based phylogenomic analysis of the Belgian 2016–2017 influenza A(H3N2) outbreak season allows improved surveillance. Microb Genom. 2021;7:000643. <u>PubMed</u> <u>https://doi.org/10.1099/mgen.0.000643</u>
- 2. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3. <u>PubMed</u> <u>https://doi.org/10.1093/bioinformatics/btu033</u>
- Miller MA, Schwartz T, Pickett BE, He S, Klem EB, Scheuermann RH, et al. A RESTful API for access to phylogenetic tools via the CIPRES science gateway. Evol Bioinform Online. 2015;11:43–8. <u>PubMed https://doi.org/10.4137/EBO.S21501</u>
- 4. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 2021;49(W1):W293–6. <u>PubMed</u> <u>https://doi.org/10.1093/nar/gkab301</u>

Appendix Figures (following pages). Sequences of H5Nx viruses. Red and black squares indicate H5N6 and H5N1 viruses isolated in Korea in December 2023. Black circles indicate H5Nx viruses isolated in Korea from 2020 to July 2023. Sequences determined in this study are shown in bold.



Appendix Figure 1. Phylogenetic tree of PB2 gene.



Appendix Figure 2. Phylogenetic tree of PB1 gene.



Appendix Figure 3. Phylogenetic tree of PA gene.

AP AL LOCATION AN INVESTIGATION AND IN A AND A AN
the statements apple is advanced using the compared on the statements the statements apple is advanced using the statements and the statements the statements apple is advanced using the statements the statements and the statements and the statements
• The Additional production from the Control and Product 2014 (Control and Control and
Vet B., NUME (Assess to June 111) Vetta (Assess to Disc a Helicola Collar) and the set of the other and the International Collar Assessment (Assessment Collar) and the set of the other and the International Collar Collar Collar assessment (Assessment Collar) (Assessment Collar) (Assessment Collar) and the set of the International Collar Col
Contract, including and only University of a the second seco
Yer Visit 2. A URCT (Secondary of a Direct Sill 2019) studied to 1.6 (s) Part Visit 2. A URCT (Secondary of an URL NO 2. (Secondary 2.0 (s) (Secondary 2.0
Exit is provide process shows for the end of the e
 A. S. A. B. S. S.
EVEN IN THE OWNER OF A STATE
19 Construction of the state
W Statistics of the statist
A set of the set of
1 Chi Su, diversityi and constraints and constraint straints interpreter traints and all Phile and an annual straint straints and an annual straints and an annual straints and provide and an annual straints and an annual straints and an annual straints and an annual straints. A 2012 July Interpreter and annual straints and an annual straints and an annual straints. In Provide A 2012 July Interpreter and annual straints and annual straints and an annual straints.
INT IS, LOCIENTIA da Lue vivolenza 120001 (LOCIE-1-10) INT IS, LOCIENTIA da Lue vivolenza 12000000000000000000000000000000000000
The TRUE AND ENDING AND
in Prive, an Utility is a strange and an a field in Standard and a strange with the strate and a strategy of the strategy o
VPI M., UP WEND AND AND WE AND WE AND IN THE ADDRESS OF ADDRESS AND ADDRESS
E. D. H. S. Marcelland P. and Marcelland T. B. Marcelland Methylation Methy
FPT M., REEDE AND WAVE TO SUPERVISE AND ADDRESS ADD
Vin ID, BUTCH-LINE Wank ID TRACKS OF TRACKS OF ORDER VIOLENCE AND INTERVIOLENCE AND INTERVIEWED AND ADDRESS OF ADDRESS
C) In it is all instanting investments on the control of the co
The Yes, Strattering's - United in Signal and Analytics and particular strategy of the Signal and Analytics and Signal and Analytics and Signal and Analytics and Signal and
a) An ellis internet sub constraints of AN ellis and A
Previous Assessing the previous dimension in the control of the previous and previous dimension of a control of the control of the previous dimension of the control of the control of the control of the previous dimension of the control of the control of the control of the control of the end of the control of the end of the control of the end of the control of the end of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the end of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the control of the control of the control of the end of the control of the end of the control of the control of the control of the control of the end of the control of
All M. (2007) A review of the characterized and an advance of the characterized and advance of t
(a) In the control of the control
On the case of the sense of the
Province in the second se
to the contract of the co
The Annual
Proc. 2010;201:2010;Accionent Research Control Co
The start is a start of the start is a start of the start
PF PL, All Child (La service) and an equation to (an example of the example of
1 of the second relation of the second relation of the relation of the second relation o
(c) A set of a set
Personal Science of the second science of science of the second
term in the term of term of the term of t
 In the state of th
We have a determine the second
Control A, Milling Control A, Service S,
The second secon
Wirtlei, A. Bill, William and A. S.
Instrumenteringueschertretargenzungen sind zur einen juriergebenannt ihr eine Ausschertretargenzungen sind zur einen juriergebenannt ihr eine Ausschertretargenzungenzenz
 Prova Late Late Design and mero same and white Late Protocol 2018 as 100 Prova Late Late Design and mero same and white Late Protocol 2018 as 100 Prote Late Design and the Protocol 2018 (PA) (2018) and 2018 as 100 Prote Late Design and the Protocol 2018 (PA) (2018) and 2018 as 100 Prote Late Design and the Protocol 2018 (PA) (2018) and 2018 as 100 Prote Late Design and the Protocol 2018 (PA) (2018) and 2018 as 100 Protocol 2018 (PA) (PA) (PA) (PA) (PA) (PA) (PA) (PA)
Event Control (Control (Contro) (Control (Control (Contro) (Control (Contro) (Control (Contro) (C
Colin C., 21 Anny Park Annowani California (2014) and annual calify differentiation (4-1) and annual calify a set of the set of t
8.7 No. 2 VARIATE (Constraints and Antonia De VARIATE Series an
 An the presenter a council wave wave wave and an account and account and account account
Line to interventing in a proceeding of the process and the process of the pro
(IPPLIX, LOBIC VPT) unclosed rowshill LAME 2015(BPR) 2015(CH):
 Versit, Webber auf volume 1, warvest 4, etc. (2012) and (2012) a
Christian La constructar, annual neuro esta esta constructaria con esta provincia a constructaria a constructaria con esta constructiva con esta constructiva con esta constructiva con esta constructiva constructaria constructaria constructaria con esta constructiva constructiva constru
8.4 Tr. S., JATTORIS, Vol. 44, Aux Manuel (2014) 151 01 00000101 000000 00 00 0000000000
CP 40, STORENT ANA ANALYSIS SINCE YARANGA SINCE ANALYSIS SINCE ANALYSIS CP 40, STORENT ANALYSIS SINCE YARANGA SINCE ANALYSIS SINCE YARANGA SINCE ANALYSIS CP 40, STORENT ANALYSIS SINCE YARANGA SINCE ANALYSIS SINCE YARANGA SINCE
(2) Als under Diright and wave even on the start of products as a Al of all also under products and an and also products as a Al of all also under products and also products as a Al of all also products and also products and all also products and products and also products and also products and products and products and also products and also products and products and products and also products and products and products and products and products and products and products and products and products and also products and products an
Pert B. Littlerform visual mean even of UE 20 perturbation 2 of the Pert B. Littlerform visual mean even of UE 20 perturbation 2 of the Pert B. Littlerform visual mean even of UE 20 perturbation 2 of the Perturbation
⁴⁴ A los res is the cost digate single-contrast transportant process and a loss of loss of loss of loss of loss of loss of loss of loss of loss of loss o
 Provide State Sta
In Proc. 19, 2019 Proc. A Constrainty of Constra

 A defaulting to equip the set of the set o
How the interpretation of the section of the s
10 The second registration construction of a second registration of
Print and an analysis of the set of the
 Control (1) (See 10.00) A second control (1) (See 10.
(a) Privat, assessible and the Auto Cano Weith ScaleWeith Scale
4 44 5. Unitability of a constrainty of co
 Control, 2021 Annual Advances (2021) (2021) (2021) (2021) (2021) Control (2021) (2021) (2021) (2021) (2021) (2021) (2021) (2021) (2021) Control (2021) (
Print Transitioner and Transition State Sta
the starts is used provide provide starts and star
[1] Contra Standard, Barton Markan, Barton Marka
 A set of a section product in the contrast of a set of the contrast of the contra
Transmission and a second product of the second and a second product of the second pr
per construct and BERCANDE for large 2000-000-000 and 2000-000 and 2000-000-000-000-000-000-000-000-000-00
a chi
 In contrast and ordingly matters in the contrast contrest contrest contrest contrast contrast contrast contrast cont
 A set of the set of
B) In the control of the set of the product control of the set of the product of the product of the set of the product of the p
1 1
1 4 4 7 TO 1000 (2) A Los Nacional 2010 (2) 2010 (2) 4000 (2) 10 4 411 (2) 1000 (2) 10 (2)
In other a strateging particular light method and strateging the strateging of the s
 P. A. L. HARTAN, Hartowski K. K.

the second and t
 The standard Billion and an advance of the standard and advance of the standard advance o

Appendix Figure 4. Phylogenetic tree of HA gene.



Appendix Figure 5. Phylogenetic tree of NP gene.



Appendix Figure 6. Phylogenetic tree of NA gene of N6 subtype.



Appendix Figure 7. Phylogenetic tree of NA gene of N1 subtype.



Appendix Figure 8. Phylogenetic tree of MP gene.



Appendix Figure 9. Phylogenetic tree of NS gene.