

1 **Title:**

2 **Highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b in domestic**
3 **ducks, Indonesia, 2022**

4

5 Keywords: HPAI H5N1, clade 2.3.4.4b, ducks, whole genome sequencing, Indonesia

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23 **Abstract**

24 Highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b viruses were detected and
25 isolated from domestic ducks in South Kalimantan, Indonesia during April 2022. The viruses
26 were genetically similar to clade 2.3.4.4b H5N1 viruses recently detected in East Asia from
27 2021-2022. Further investigation is necessary to determine the source of virus incursion.

28 H5N1 subtype of avian influenza (AI) virus A/goose/Guangdong/1/96 (Gs/GD/96) lineages
29 have caused highly pathogenic AI (HPAI) outbreaks in poultry since 1996. In 2008, various
30 novel reassortant viruses bearing the genetic backbone of the hemagglutinin (HA) of
31 Gs/GSD/96 from clade 2.3.4 with different combination of neuraminidase (NA) were
32 identified in domestic duck and live poultry markets in China including H5N2, H5N5, H5N6,
33 H5N8 (Lee et al., 2017). Clade 2.3.4 continued to evolve resulting several fourth order
34 lineages, including clade 2.3.4.4, which have undergone genetic reassortment with other
35 H5N1 clades and low pathogenic AI (LPAI) viruses resulting eight genetic groups (2.3.4.4a-
36 h) and multiple genotypes (Lee et al., 2017). Clade 2.3.4.4 of H5N8 subtype viruses have
37 spread across many countries from Asia to Europe, Africa, and North America (Lee et al,
38 2017; Kwon, et al., 2023) and repeated outbreaks due to infection of H5N8 clade 2.3.4.4
39 group B (2.3.4.4b) viruses have been reported between 2016 and mid-2020 (Yehia et al,
40 2018; Engelsma et al, 2022). However, a new strain of HPAI H5N1 clade 2.3.4.4b virus
41 emerged in late 2020 which led to an increase the scale of wild bird outbreaks and poultry
42 worldwide and this virus has almost entirely replaced H5N8 clade 2.3.4.4b globally since late
43 2021 (Venkatesan, 2023; Wiley and Barr, 2022)

44

45 In April 2022, several poultry cases were reported from small-holder duck farms located next
46 to marshes in Hulu Sungai Utara District, South Kalimantan Province, Indonesia (Appendix,
47 Figure 1). We collected 18 oropharyngeal swabs which pooled in four viral transport media
48 and two tissue samples from dead birds from these farms. All samples were detected AI
49 positive by PCR, but only 3 pooled swabs could be isolated from embryonic chicken eggs.
50 They were then characterized using Illumina sequencing platform followed by phylogenetic
51 and sequence analysis based on complete genome sequencing. We conducted the
52 hemagglutination inhibition (HI) assay for the virus isolates and performed necropsy and

53 hematoxylin and eosin staining for gross- and histo-pathology of collected tissues from dead
54 ducks.

55

56 Whole genome sequence of three virus isolates, A/duck/Hulu Sungai Utara/A0522064-
57 06/2022, A/duck/Hulu Sungai Utara/A0522064-03-04/2022 and A/duck/Hulu Sungai
58 Utara/A0522067-06-07/2022, have been deposited to GISAID under ID isolate numbers
59 EPI_ISL_17371282, EPI_ISL_17371283, and EPI_ISL_17371284, respectively.

60 Phylogenetic analysis of the HA gene showed that all three viruses clustered with recent H5
61 clade 2.3.3.4b viruses from Asia and Europe. However, they appeared to be more genetically
62 close related to H5N1 clade 2.3.4.4b viruses from wild birds and poultry in China, South
63 Korea, and Japan between October 2021 and February 2022 (Figure). The tree topology of
64 the other gene segments (PB2, PB1, PA, NP, NA, MP, and NS) showed that all three viruses
65 were also situated close to H5N1 clade 2.3.4.4b from China, South Korea, and Japan
66 (Appendix Figures 2-5). These virus isolates shared 99.8-100% nucleotide sequences
67 similarity in each viral segment and all were identified as HPAI on the basis of multiple basic
68 amino acid sequences detected in the HA cleavage site (REKRRKR|G). None of these have
69 molecular determinants related with increase binding affinity or efficient replication in
70 mammals including humans (Chutinimitkul et al., 2010; Suttie et al., 2019) (Appendix Table
71 1). The BLAST tool search (<https://www.ncbi.nlm.nih.gov/blast>) and pairwise distance
72 analysis showed that all eight gene segments had 98.4-99.8% nucleic acid sequence identities
73 to those of the H5N1 clade 2.3.4.4b viruses from China, South Korea, and Japan (Table)
74 indicating that they had a close common ancestor.

75

76 The HI assay revealed low reactivities of the virus isolates against representative antisera
77 from HPAI viruses that have been circulating in poultry in Indonesia, including the H5N1

78 vaccines that are currently used (Appendix, Table 2). The gross- and histo-pathology in
79 naturally infected ducks showed multiorgan haemorrhages with prominent lesion in tissues
80 were congestion and necrosis in parenchymal cells often accompanied with inflammatory cell
81 infiltrates (Appendix, Figure 6)

82

83 Wild migratory birds have been considered play a role in the intercontinental spread of HPAI
84 H5Nx clade 2.3.4.4 (Lee et al, 2017; Caliendo et al, 2022, Zhang et al, 2023). This study
85 reports the identification of HPAI H5N1 clade 2.3.4.4b viruses isolated from domestic ducks
86 in South Kalimantan, Indonesia. Although we could not yet determine the source of virus
87 incursion to this area, the role of wild aquatic birds on virus transmission cannot be rule out
88 since the infected farms were connected to water course from the marshes which provide
89 opportunity for intermingle contacts between naturally infected wild birds to naïve ducks or
90 these ducks could be infected through contaminated water.

91

92 Limitation of data on AI virus genome sequence and wild bird surveillance hampering us to
93 determine the exact role of wild birds in the spread of HPAI H5N1 clade 2.3.4.4b into
94 Indonesia. This highlights the importance of both epidemiology and molecular surveillance
95 on these species to support better preparedness for such pandemic threats due to continued AI
96 virus evolution.

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148 **Figure Legend**

149 Phylogenetic tree of the HA gene of H5N1 clade 2.3.4.4b viruses isolated from domestic
 150 ducks (indicated in red taxa) during poultry outbreaks in South Kalimantan, Indonesia in
 151 April 2022. The evolutionary history was inferred by using Maximum Likelihood method
 152 using the best fit substitution model (GTR+G) involving 67 HA-H5 sequences from GISAID
 153 (<http://www.gisaid.org>) with a total of 1656 positions in the final dataset. The tree is drawn
 154 to scale, with branch lengths measured in the number of substitutions per site (0.01) shown in
 155 bottom left.

156

157 **Table Title**

158 Sequence homology of each gene segment of the virus isolates to other H5N1 clade 2.3.4.4b
 159 viruses from China, Korea, and Japan, October 2021 to February 2022.

Virus Name (GISAID ID No. Collection Date)	Nucleic acid similarity (%)							
	PB2	PB1	PA	HA	NP	NA	MP	NS
Between the virus isolates (EPI_ISL_17371282, EPI_ISL_17371283, EPI_ISL_17371284 2022-04-05)	100	99.8- 99.9	99.8- 99.9	99.9- 100	100	100	100	99.8- 100
A/mandarin duck/Korea/WA585/2021 (EPI_ISL_6959592 2021-10-26)	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 2021-11-08)	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 2021-12-08)	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 2022-01-10)	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 2022-02-22)	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 2021-11-12)	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 2021-12-07)	99.6	99.3- 99.4	99.1- 99.2	98.4- 98.6	99.1	99.6	99.3	99.0
A/teal/Miyazaki/211109-32/2021 (EPI_ISL_15613494 2021-11-09)	99.4	99.3	99.3	98.7- 98.9	99.1	99.6	99.2	99.2
A/chicken/Ehime/TU11-2-24 25/2022 (EPI_ISL_15063431 2022-01-04)	99.8	92.5	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 2022-01-27)	99.6	99.3	99.3- 99.4	98.6- 98.7	99.3	99.6	99.3	99.2

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