



## Communication

# A Lethal Case of Natural Infection with the H5N8 Highly Pathogenic Avian Influenza Virus of Clade 2.3.4.4 in a Mandarin Duck

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**Abstract:** Recent global outbreaks of highly pathogenic avian influenza viruses (HPAIVs) of the H5N8 subtype in poultry and wild birds have raised concerns about animal and human health, particularly after its first evidence of zoonotic transmission from birds to humans. Here, we report a lethal infection with the H5N8 HPAIV in a mandarin duck that had previously demonstrated resistance to the H5N8 HPAIV infection. In addition, we revealed that the isolated virus was a genetic reassortant between the existing H5N8 HPAIV and LPAIV(s). Although further studies are warranted to assess the impact of the genetic reassortment on virus pathogenicity, the potential role of mandarin ducks in HPAIV dissemination should be re-evaluated.

**Keywords:** highly pathogenic avian influenza virus; H5N8; mandarin duck



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Since their initial detection in China in 2010, highly pathogenic H5Nx avian influenza viruses (HPAIV) of clade 2.3.4.4 have been circulating in wild and domestic birds [1]. During the 2020/2021 winter season, outbreaks of H5N8 HPAIVs (clade 2.3.4.4) in wild and domestic birds have been reported in several European [2–5] and Asian [6–8] countries, most likely due to viral dissemination by migratory waterfowl. In addition, the first zoonotic evidence of an H5N8 HPAIV was reported from poultry farm workers in the South of Russia on 20 February 2021 (<https://www.ecdc.europa.eu/en/publications-data/threat-assessment-first-human-cases-avian-influenza-h5n8>, accessed on 10 February 2021). Therefore, investigating the evolution and ecology of H5N8 HPAIVs is important not only for poultry production, but also for public health.

Mandarin ducks (*Aix galericulata*) are perching ducks that belong to the family Anatidae, of the order Anseriformes. They are widely found in eastern Asian countries along the East Asia/Australia flyway, including Japan, Taiwan, China, and South Korea. Under experimental conditions, H5N6 and H5N8 HPAIVs of clade 2.3.4.4 did not cause symptomatic infection in mandarin ducks [9,10], whereas H5N1 HPAIVs of clade 2.3.2.1 infections produced high mortality [11]. In fact, an H5N8 HPAIV of clade 2.3.4.4, A/Mandarin duck/Korea/K20-551-4/2020 (H5N8), has recently been isolated from a fecal sample, but not a carcass, of a mandarin duck [6], indicating limited viral pathogenicity in mandarin ducks. Here, we report a lethal case of natural infection with a H5N8 HPAIV of clade 2.3.4.4 in a mandarin duck.

On 22 December 2020, a mandarin duck showing neurological clinical signs, including ataxia with torticollis (Video S1), was captured at the Kogawa Dam in Izumi, Kagoshima Prefecture, Japan, by a local governmental staff member. When brought to Kagoshima University for diagnostic purposes the following day, the sick mandarin duck was lethargic (Video S2). Tracheal and cloacal swabs tested negative for the influenza A viral antigen using the influenza rapid diagnostic test ESPLINE A Influenza (Fujirebio Inc., Tokyo, Japan). However, an influenza A viral M gene was detected in the RNA from the tracheal swab by reverse transcription-PCR as previously described [12]. By inoculating the tracheal swab into embryonated chicken eggs, followed by rapid diagnostic testing, we isolated an AIV. Subsequent genetic analyses revealed that the isolated AIV was an H5N8 HPAIV of clade 2.3.4.4, named A/mandarin duck/Kagoshima/KU-d57/2020 (H5N8).

To genetically characterize the HPAIV, we determined the full-length nucleotide sequences of the coding regions of all eight gene segments using MinION (Oxford Nanopore Technologies, Oxford, UK) with Flongle flow cells. Briefly, PCR amplicons specific to each individual gene segment were processed with a Direct cDNA Sequencing Kit (Oxford Nanopore Technologies [13]) and sequenced with Flongle flow cells using MinION control software. The MinION-generated sequencing readings (Q score > 7) were mapped onto a contemporary H5N8 HPAIV isolate A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8) as a reference strain (mapping set at 5 iterations and a 60% consensus threshold). The resultant sequence coverages for each gene segment were over 100. The consensus sequences for each gene segment were generated using Geneious Prime v.2020.2.4 (Biomatters Ltd., Auckland, New Zealand). We had confirmed in advance that the nucleotide sequences from A/environment/KU-ngr-G/2018 (H3N8) [14] determined by this methodology were almost identical to those determined by Sanger sequencing (data not shown) as reported previously [15,16].

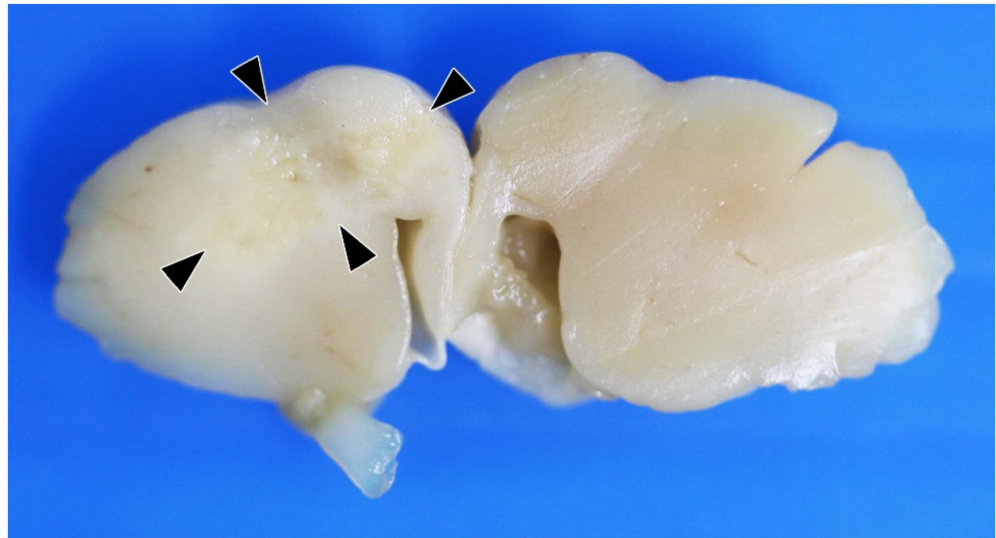
BLAST analysis of the determined sequences against the Global Initiative on Sharing Avian Influenza Data (GISAID) and NCBI databases revealed that the PA, HA, NA, M, and NS gene segments from the isolate showed a high similarity (99.53–99.81%) to their counterparts from H5N8 HPAIVs recently isolated in Japan and South Korea (Table 1). By contrast, the closest relatives to the PB2, PB1, and NP gene segments were those from different low pathogenic avian influenza viruses (LPAIVs), isolated from Asian countries (Table 1). Phylogenetic analyses also revealed that the PB2, PB1, and NP gene segments from A/mandarin duck/Kagoshima/KU-d57/2020 (H5N8) are derived from different LPAIVs (Supplementary Figure S1), indicating that the isolate is a genetic reassortant generated by the intermixing of gene segments between H5N8 HPAIV and various LPAIV(s). The same genetic constellation of contemporary H5N8 HPAIVs isolated in South Korea was reported as genotype E3 [17]. Molecular marker analyses based on the deduced amino acid sequences revealed that this H5N8 HPAIV isolate did not harbor typical mammalian adaptive mutations (e.g., lysine at amino acid position 627 of the PB2 protein) [18].

**Table 1.** Closest relatives of genes from A/mandarin duck/Kagoshima/KU-d57/2020 (H5N8).

Gene	Accession No. <sup>a</sup>	Closest Relative <sup>b</sup>	Identity (%)
PB2	MZ620715	A/environment/Korea/CSM3/2002 (H3N6)	97.49
PB1	MZ620716	A/duck/Hokkaido/56/2017 (H12N2)	99.38
PA	MZ620717	A/northern pintail/Hokkaido/M13/2020 (H5N8)	99.81
HA	MZ620718	A/Madarin duck/Korea/K20-551-4/2020 (H5N8)	99.53
NP	MZ620719	A/common teal/Shanghai/NH110923/2019 (H1N1)	98.05
NA	MZ620720	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.57
M	MZ620721	A/environment/Kagoshima/KU-ngr-J2/2020 (H5N8)	99.79
NS	MZ620722	A/Mandarin duck/Korea/H242/2020 (H5N8)	99.64

<sup>a</sup> Accession numbers in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>, accessed on 10 February 2021) are listed. <sup>b</sup> Representative viruses with the highest nucleotide sequence identity found in the GISAID database on 10 February 2021 are listed.

Because of its severe neuropathic symptoms, the infected mandarin duck was humanely euthanized by CO<sub>2</sub> inhalation, in accordance with the standards relating to the Methods of Destruction of Animals by the Ministry of the Environment on 24 December 2020 and subjected to pathological examination and virus titration. On autopsy, no significant lesions other than emaciation were identified. However, after the fixation of the major organs with 10% neutralized formalin, a yellowish white, irregularly shaped focus was identified on the cut surface of the right cerebrum (Figure 1). Histopathologically, the focus consisted of necrosis and rarefaction with infiltration by numerous foamy macrophages (Supplementary Figure S2A). Surrounding the focus, neuronal necrosis, degeneration of the neuropile, and minimal perivascular cuffing were observed (Supplementary Figure S2B). Virus presence was confirmed in neurons in the vicinity of the focus by immunohistochemistry, using antibodies specific for influenza A viral M1 protein (clone GA2B; Bio-Rad Laboratories, Hercules, CA, USA) (Supplementary Figure S2B, insert). These findings suggest that the duck's head tilt was attributable to focal necrosis in the right cerebrum. Intriguingly, degeneration and necrosis of the retina and lymphoplasmacytic infiltration in the choroid were also observed in the left eye (Supplementary Figure S3A). The necrotic and degenerative photoreceptor cells were positive for influenza A viral antigens (Supplementary Figure S3B). No significant lesions were observed in the remaining organs, macroscopically or microscopically. These results imply that the H5N8 HPAIV infection in the mandarin duck induced clinical symptoms due to central nervous system damage and impaired eyesight.



**Figure 1.** The cut surface of the cerebrum (frontal view). A yellowish white focus is observed in the right cerebrum (arrowheads).

Viral titers in the major organs were determined by the 50% egg infectious dose (EID<sub>50</sub>) assays in embryonated chicken eggs, as described previously [19]. Intriguingly, viruses were detected in most of the organs tested (Table 2), although significant lesions were pathologically observed only in the right cerebrum and left eye. These results indicate that A/mandarin duck/Kagoshima/KU-d57/2020 (H5N8) caused systemic infection, including in the central nervous system.

In conclusion, we have confirmed a lethal infection with the H5N8 HPAIV of clade 2.3.4.4 in a mandarin duck. While previous studies have described the limited pathogenicity of H5Nx HPAIVs of clade 2.3.4.4 in mandarin ducks, our results indicate that A/mandarin duck/Kagoshima/KU-d57/2020 (H5N8) caused a lethal infection in a mandarin duck, likely due to the central nervous system damage. Although further studies are warranted to assess the impact of the genetic reassortment on virus pathogenicity and transmissibility as previously described [20], the potential role of mandarin ducks in HPAIV dissemination should be re-evaluated.

**Table 2.** Viral titers in organs of the infected mandarin duck.

Organ	Virus Titer (log <sub>10</sub> EID <sub>50</sub> /g) <sup>a</sup>
Brain	4.24
Lung	2.5
Heart	2.24
Liver	2.5
Pancreas	2.24
Kidney	6.24
Colon	ND <sup>b</sup>

<sup>a</sup> Virus recovered from the indicated organs of the infected mandarin duck was titrated by the EID<sub>50</sub> assays in embryonated chicken eggs. <sup>b</sup> ND: virus not detected (detection limit: 1.5 log<sub>10</sub> EID<sub>50</sub>/g).

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/zoonoticdis2010004/s1>. Figure S1: Phylogenetic trees of the PB2 (A), PB1 (B), PA (C), H5 HA (D), NP (E), N8 NA (F), M (G), and NS (H) gene segments of A/Mandarin duck/Kagoshima/KU-d57/2020 (H5N8) and reference strains. Nucleotide sequences were analyzed by the maximum-likelihood method along with the corresponding genes of reference strains using the GTR+G model that was predetermined by a model-test in MEGA-X software (<http://www.megasoftware.net/>, accessed on 10 February 2021). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1000 replications. The virus isolated in this study is indicated by a black circle. Figure S2: Histopathological findings from the right cerebrum. (A) Necrosis with numerous infiltrating foamy macrophages (right side, hematoxylin and eosin [HE] staining, 100× magnification). (B) Several necrotic neurons (arrowheads; HE staining 200×). Viral antigen in a neuron near the necrotic focus (insert, immunohistochemistry). Figure S3: Histopathological findings in the left eye (A) Retinal degeneration and necrosis (arrowheads) and lymphoplasmacytic infiltration in the choroid (hematoxylin and eosin staining, 400× magnification). (B) Viral antigen in photoreceptor cells (immunohistochemistry, 400×). Video S1: A wild mandarin duck showing neurological clinical signs in the field. Video S2: A wild mandarin duck showing lethargy in a safety cabinet in a laboratory.

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**Data Availability Statement:** The data that support the findings of this study are openly available in the GenBank at <https://www.ncbi.nlm.nih.gov/genbank/> (accessed on 10 February 2021), reference numbers MZ620715- MZ620722 and in the GISAID database at <https://www.gisaid.org/> (accessed on 10 February 2021), reference numbers EPI1847769- EPI1847776.

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**Conflicts of Interest:** The authors declare there are no conflicts of interest.

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