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Histopathological Analysis of Surviving Mallards Infected with H5 Subtype Clade 2.3.4.4b High Pathogenicity Avian Influenza Viruses

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Abstract

High pathogenicity avian influenza (HPAI) viruses of clade 2.3.4.4b have caused outbreaks worldwide, affecting both wild and domestic birds, as well as occasionally mammals. Mallards (*Anas platyrhynchos*), the natural hosts of influenza A viruses, are major migratory birds in Japan and strong contributors to the transmission and maintenance of the viruses. To further understand the pathological consequences and potential for viral persistence, histopathological examinations were conducted on mallards that survived 14 days post-inoculation with H5N1 and H5N8 clade 2.3.4.4b HPAI isolates. Five of the ten mallards that underwent autopsy exhibited multifocal encephalitis of variable severity. Two showed severe encephalomalacia and calcifications, one of which also had persistent viral antigens within the necrotic foci. Furthermore, marked atrophy of the lymphoid tissues, particularly the thymus, was observed and appeared to correlate with the severity of encephalitis. These findings indicate that residual encephalitis and lymphoid depletion can persist despite resolution of the infection, suggesting potentially lasting health effects in mallards following HPAI viral infection. Notably, some mallards showed no histological lesions, indicating their potential as viral carriers.

Discipline: Animal Science
Additional key words: calcification, encephalitis, experimental infection, necrosis, thymic atrophy

Introduction

Avian influenza viruses (AIVs) are classified into subtypes based on the antigenicity of their two surface proteins. Prevalent across a wide range of avian species worldwide, 16 of the 18 known hemagglutinin (HA) subtypes and 9 of the 11 neuraminidase subtypes have been identified in birds. Among these, wild waterfowl, primarily species of the orders *Anseriformes* (ducks, geese, and swans) and certain *Charadriiformes* (shorebirds, gulls, terns, and auks), are recognized as primary natural reservoirs of AIVs (Webster et al. 1992). Within these groups, mallards (*Anas platyrhynchos*) and other dabbling ducks play a crucial role in maintaining the viruses. AIVs can be transmitted from wild birds to terrestrial poultry, such as chickens and turkeys, typically in the form of low pathogenicity avian influenza (LPAI) viruses, which may cause subclinical infections, mild respiratory disease, or reduced egg production. In poultry, LPAI viruses can evolve into high pathogenicity avian influenza (HPAI) viruses through the acquisition of insertions at the HA cleavage site, which facilitates systemic infection and results in flock mortality rates of up to 100% (Lee et al. 2021). Initially, outbreaks of H5 and H7 HPAI viruses were primarily observed in poultry; therefore, these viruses were thought to be adapted to poultry with limited concern regarding their potential to infect or spread among wild birds, including waterfowl. However, since 2002, viruses of the A/goose/Guangdong/1/96 (Gs/GD) lineage (Xu et al. 1999) have been shown to cause systemic infection and mortality in a wide range of avian species, including domestic, zoo, and wild birds, such as waterfowl (Chen et al. 2005, Ellis et al. 2004, Usui et al. 2020). The Gs/GD lineage has

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diversified into multiple clades (0-9), with some clades further classified into subclades (Smith et al. 2015). Clade 2.3.4.4, which is predominant worldwide, has been subdivided into subclades a through h (World Health Organization 2020). Based on a consensus among researchers in Japan conducting definitive HPAI diagnoses, clade 2.3.4.4b has been further categorized into genetic groups (G1, G2a, G2b, G2c, & G2d) according to phylogenetic analysis of the HA gene (Nishiura et al. 2025).

Many studies have investigated the pathogenicity of Gs/GD lineage HPAI viruses in domestic duck species and captive mallards, revealing a wide range of clinical presentations, from subclinical infections to severe diseases with high mortality rates (Bingham et al. 2009, Pantin-Jackwood et al. 2016, Spackman et al. 2023, Tang et al. 2009, Tanikawa et al. 2022). These studies demonstrated that both symptomatic and subclinically infected ducks can shed large quantities of HPAI virus, thereby contributing to increased environmental viral loads and facilitating the long-distance spread of infection. Although previous studies using mallards inoculated with the Gs/GD lineage clade 2.3.4.4 HPAI virus have focused on histopathological lesions in the peak phase of the infection (DeJesus et al. 2016, Leyson et al. 2021, Pantin-Jackwood et al. 2016), the histopathological characteristics in mallards that survived the viral infection have not been thoroughly described. This study performed histopathological analysis on mallards that survived infection with the clade 2.3.4.4b HPAI virus. The objective of this analysis was to evaluate the potential risks associated with viral persistence, its effects on the host, and the possibility of mallards subsequently acting as carriers.

Materials and methods

1. Animal experiments and virus isolates

Twelve 12-week-old mallards were randomly assigned to three groups of four mallards and experimentally infected via intranasal inoculation with 10^6 EID_{50} of three different clade 2.3.4.4b HPAI virus isolates: A/chicken/Akita/7C/2021 (Akita7C) (H5N8, G2a), A/chicken/Kagoshima/21A6T/2021 (Kagoshima6T) (H5N1, G2b), and A/chicken/Iwate/21A7T/2022 (Iwate7T) (H5N1, G2d). Three 14-week-old mallards that were not inoculated with viruses served as negative controls. All procedures were conducted in biosafety level 3 facilities in accordance with the procedures approved by the Institutional Committee for Ethics of Animal Experiments (approval no. R4-I002-NIAH, R4-I002-NIAH-2, R4-I002-NIAH-3, and R4-C01-NIAH-2).

2. Histopathology and immunohistochemistry

Surviving mallards were euthanized at 14 days post-inoculation (dpi) by an intraperitoneal injection of sodium pentobarbital, followed by cardiac exsanguination for postmortem examination. Tissue samples from the liver, spleen, kidney, heart, lung, intestines, pancreas, brain, thymus, bursa of Fabricius, and eyes were collected and fixed in 10% neutral-buffered formalin. These tissues were then processed routinely, embedded in paraffin wax, sectioned at a thickness of 3 µm-5 µm, and stained with hematoxylin and eosin. Based on the initial microscopic findings, additional sections from selected cases were stained using the Von Kossa technique. Encephalitis severity was evaluated by assessing the extent of perivascular mononuclear cell infiltration (perivascular cuffs) and gliosis: mild: few foci with fewer than five layers of mononuclear cell aggregates; moderate: multifocal to coalescing distribution and more than five layers of mononuclear cell aggregates with gliosis; severe: locally extensive distribution with marked gliosis and necrosis.

Immunostaining was performed using an antibody-labeled polymer method. After dewaxing the tissue sections, endogenous peroxidase activity was blocked using 3% hydrogen peroxide in ultrapure water. Antigen retrieval was achieved by digesting the sections with 1 mg/mL of actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) in phosphate-buffered saline (PBS) for 10 min at 37°C. Sections were blocked with 5% skim milk in PBS for 20 min at room temperature. Following blocking, the sections were incubated overnight at 4°C with a mouse monoclonal antibody specific for the nucleoprotein of the influenza A virus (1:1,000; HYB 340-05; Statens Serum Institut, Copenhagen, Denmark) and a rabbit polyclonal antibody specific for glial fibrillary acidic protein (GFAP) (ready-to-use IR524; Agilent Dako, Santa Clara, CA, US). A Histofine Simple Stain MAX-PO kit (Nichirei Bioscience, Tokyo, Japan) was used as the secondary antibody. Immunoreactivity was detected using 3,3′-diaminobenzidine (Histofine DAB substrate kit; Nichirei Bioscience).

Results

1. Clinical signs

All mallards infected with Akita7C and Kagoshima6T survived, but one mallard in each group showed neurological signs and depression (Table 1). In addition, all mallards infected with Iwate7T exhibited neurological signs, including wrynecks, circling, and loss of balance, and two of these died (Sakuma et al. 2025).

Table 1. Summary of findings

Virus			Mallard no.	Clinical signs[a]	Gross lesion	Microscopic lesions	IHC[b]
Isolate	Subtype	Group					
Akita7C	H5N8	G2a	C32	—	Thymic atrophy	Encephalitis (++)[e]	—
			C33	—	NSL[c]	—	—
			C34	—	NSL	—	—
			C35	Neurological signs	Thymic atrophy	Encephalitis (+++)	—
Kagoshima6T	H5N1	G2b	C36	—	NSL	—	—
			C37	—	Thymic atrophy	—	—
			C38	Depression	Encephalomalacia, thymic atrophy	Encephalitis (+++)	+ (Brain)
			C39	—	NSL	—	—
Iwate7T	H5N1	G2d	C41	(Death)	ND[d]	ND	ND
			C42	Wryneck	Thymic atrophy	Encephalitis (+)	—
			C43	(Death)	ND	ND	ND
			C44	Wryneck	Thymic atrophy	Encephalitis (++)	—

[a] From Sakuma et al. (2025). [b] IHC, immunohistochemistry. [c] NSL, no significant lesions. [d] ND, no data (no samples available for examination). [e] mild, +; moderate, ++; severe, +++

2. Gross lesions and histopathology

Autopsies were performed on the 10 surviving mallards, excluding the two that died following inoculation with Iwate7T (Table 1). Thymic atrophy was observed in 6 of the 10 mallards, with varying degrees of severity. In severe cases, the thymus was thin, translucent, and barely discernible. One Kagoshima6T-inoculated mallard (C38) presented an asymmetrical, well-demarcated, and locally extensive area of encephalomalacia in the dorsal aspect of the telencephalon (Fig. 1a). No other significant macroscopic findings were observed.

Histological examination revealed multifocal encephalitis of varying severity in five mallards. Common findings included mononuclear cell infiltration forming cuffs around the capillaries, significant gliosis with an increased number of astrocytes and microglial cells, and dilated, swollen axons (spheroids). Two mallards (C35 and C38) showed extensive necrosis of the subpial grey matter in the hyperpallium (Fig. 1b). The neuroparenchyma was disrupted by occasional cavitation, infiltrated by numerous foamy macrophages, lymphocytes, and plasma cells. Within the affected area, many plump reactive astrocytes aggregated, often forming clusters (Fig. 1c). Multifocal accumulation of minerals, interpreted as dystrophic calcifications, was often present within multinucleated giant cell macrophages (Fig. 1d). The von Kossa histochemical technique highlighted the presence of mineralized inclusions (Fig. 1e). Intracapillary thrombi with admixed karyorrhectic debris were infrequently observed adjacent to the necrotic foci. Mallard C38 also exhibited notable lymphoplasmacytic infiltration in the choroid of the eyes, primarily in the pecten (Fig. 1g), extending to the optic nerve. All examined mallards showed hyperplasia of the lymphoid follicles and lymphocytolysis in the spleen (Fig. 1h). Significant cortical atrophy in the thymus, which correlated with the severity of encephalitis, was also observed (Fig. 1i). Reductions in both cortical and medullary lymphocytes were observed in the bursa of Fabricius. Multifocal lymphoplasmacytic aggregates and stromal proliferations were observed in the liver and pancreas. No lesions were observed in the negative control group.

3. Immunohistochemistry

Influenza A viral antigens were observed in the necrotic foci of encephalitis in Mallard C38, which was inoculated with Kagoshima6T (Fig. 1f). In contrast, no viral antigens were detected in other organs or individuals. GFAP immunoreactivity revealed extensive astroglial scarring adjacent to the necrotic areas in Mallards C35 and C38.

Discussion

This study assessed the histopathological observations of mallards infected with three H5 clade 2.3.4.4b HPAI viruses. The findings revealed marked histopathological lesions in some infected mallards along with the persistence of viral antigens. Mallards typically show no or only mild disease, even when infected with the HPAI virus, despite shedding large amounts of the

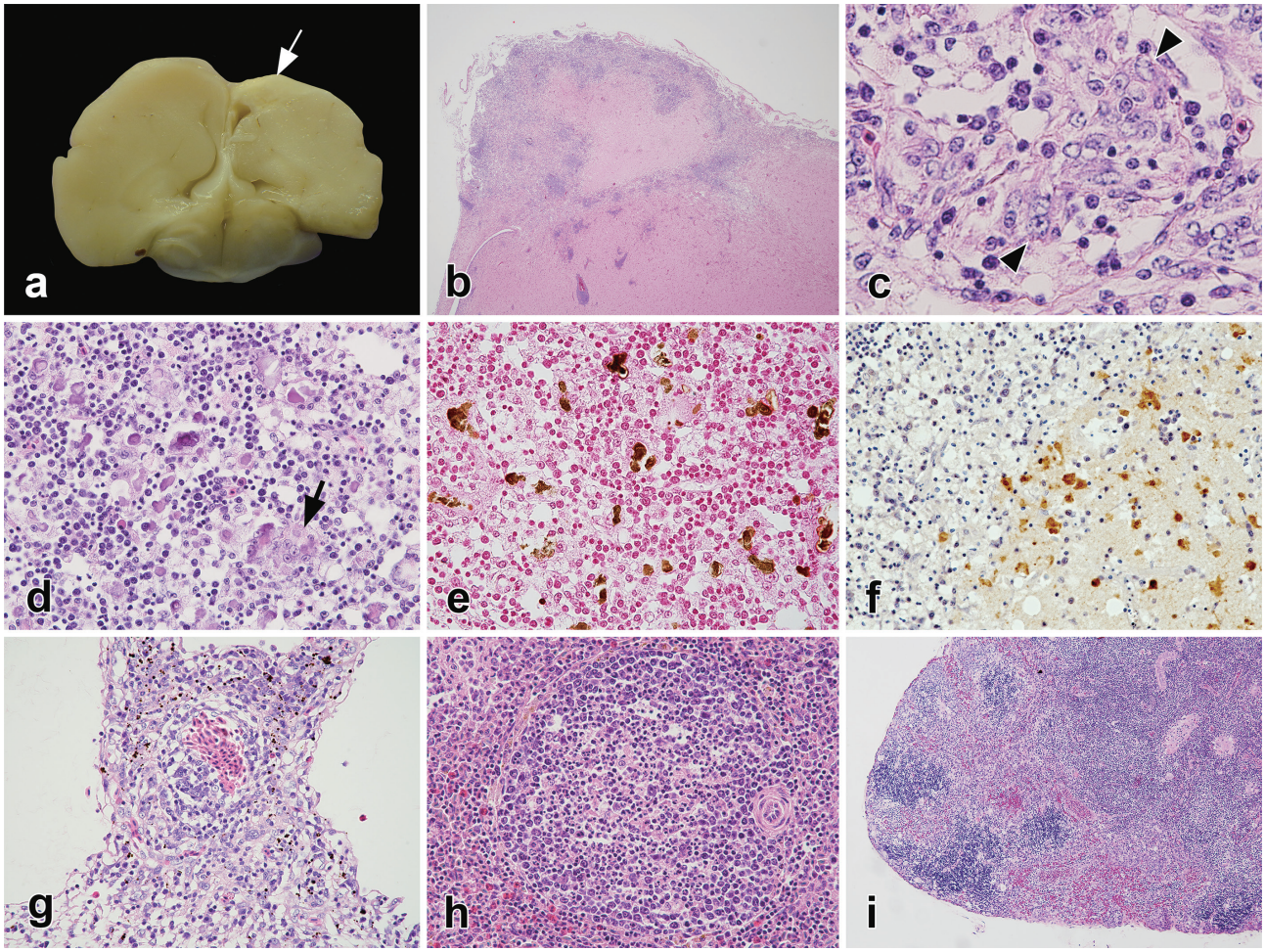

Fig. 1. Gross and histological lesions in mallards inoculated with H5 clade 2.3.4.4b high pathogenicity avian influenza viruses
(a) Brain, C38. Well-demarcated, locally extensive area of encephalomalacia in the dorsal telencephalon (arrow). (b) Brain, C38. Extensive necrosis in the subpial grey matter of the hyperpallium. (c) Brain, C38. Aggregates of plump and reactive astrocytes (arrowhead) adjacent to necrotic foci in the brain. (d) Brain, C38. Multifocal calcifications often phagocytosed by multinucleated giant cells (arrow) within the necrotic foci of the brain. (e) Brain, C38, the Von Kossa histochemical technique. Highlighted fine-granular mineral deposits, corresponding to the region shown in (d). (f) Brain, C38. Immunohistochemistry for nucleoprotein of Influenza A virus. Area of necrosis are immunopositive. (g) Eye, C38. Lymphoplasmacytic infiltration of *Pecten oculi*. (h) Spleen, C38. Lymphocytolysis of reactive lymphoid follicles. (i) Thymus, C42. Severe cortical atrophy of the thymus.

virus (Keawcharoen et al. 2008, Kwon et al. 2010). This resistance contrasts with the high susceptibility of chickens. In fact, inoculation with 10^6 EID_{50} of either Akita7C or Kagoshima6T resulted in 100% mortality in chickens (Takadate et al. 2023), whereas the same dose of either strain caused no mortality in mallards (Sakuma et al. 2025). However, contrasting results were observed with Iwate7T, which caused mortality in mallards at 10^6 EID_{50} and even at lower doses, such as 10^4 EID_{50} (Sakuma et al. 2025). These results suggest that Iwate7T may possess relatively higher pathogenicity in mallards. Recent studies have reported that the H5 Gs/GD lineage clade 2.3.4.4b HPAI viruses occasionally cause severe neurological signs (e.g., ataxia and torticollis) via viral replication in the brain, resulting in mortality among experimentally infected mallards (Leyson et al. 2021, Spackman et al. 2023, Tarasiuk et al. 2023). Leyson et al. (2021) demonstrated that reassortant viruses with substituted polymerase basic protein 2, nucleoprotein, and matrix protein gene segments exhibited significantly reduced mortality in mallards. The differences in pathogenicity observed in the present study may be attributed to variations in these gene segments. Future studies will be required to elucidate the mechanisms underlying the increased pathogenicity of Iwate7T.

Lymphohistiocytic perivascular encephalitis is one of the most common lesions observed in ducks infected with HPAI viruses (Brown et al. 2006, Sturm-Ramirez

et al. 2004, Yamamoto et al. 2007, Yamamoto et al. 2016). Experimental infections in call ducks revealed the progression of brain lesions; necrosis was most severe at three days post-infection (dpi) and subsequently decreased, whereas perivascular cuffing increased in severity and peaked at 10 dpi in the cerebrum, persisting as residual lesions (Yamamoto et al. 2007). This study showed locally extensive necrosis, marginal gliosis, and prominent calcification in the areas with severe encephalitis. The distribution and histopathological features of the brain resembled those reported in previous studies on the clade 2.3.4.4b HPAI virus in waterfowl species (Anis et al. 2018, Khalil et al. 2022); however, to our knowledge, locally extensive necrosis and multifocal calcification are uncommon findings that have not been previously reported in these contexts. Morphologically, this calcification is presumed to be dystrophic, resulting from neuronal necrosis. Similar calcifications have been observed in bald eagles (*Haliaeetus leucocephalus*) and golden eagles (*Aquila chrysaetos*) naturally infected with the West Nile virus and are regarded as non-specific findings associated with chronic necrotizing viral encephalitis (Wunschmann et al. 2014). Mallards with marked calcification exhibit only transient clinical signs. This calcification may be a sequela of a viral infection. The etiology of the malacia observed in Mallard C38 remains uncertain, as it may have resulted from direct viral invasion of neuronal structures, leading to necrosis, or alternatively, from ischemic damage secondary to endothelial injury and subsequent thrombotic infarction.

Immunohistochemistry demonstrated that no viral antigens were detected in almost all organs of the affected mallards at 14 dpi. In Mallard C38, which exhibited severe encephalitis, viral antigens were detected within the necrotic lesions. Similar findings have been reported in pigeons experimentally infected with the HPAI virus, where viral antigens and lesions persisted beyond 19 dpi (Klopfleisch et al. 2006). A recent study by Foret-Lucas et al. (2023) suggested that the neurological disorders observed in ducks infected with clade 2.3.4.4b H5N8 HPAI viruses were attributed not to direct viral neurotropism, but rather to early viral colonization of the brain combined with prolonged survival after the onset of virus replication. Ducks may effectively suppress viral proliferation in the lungs, thereby avoiding acute respiratory distress and allowing sufficient time for viral replication in the brain (Foret-Lucas et al. 2023).

Severe atrophy of the lymphoid tissue, particularly in the thymic cortex, was also observed in this study and correlated with the severity of encephalitis. Lymphoid atrophy and apoptosis have been documented in avian species infected with the HPAI virus (Kwon & Swayne 2010, Nooruzzaman et al. 2019, Perkins & Swayne 2001, Perkins & Swayne 2002). In addition, severe encephalomalacia and atrophy of the thymus and bursa of Fabricius have been observed in waterfowl species, such as geese (Perkins & Swayne 2002), similar to the lesions observed in Mallard C38 in the present study. Comparisons with negative controls suggest that the lymphoid tissue atrophy was likely caused by viral infection. However, it remains unclear whether lymphoid depletion results from direct damage by the virus or indirect effects, such as cytokine-mediated responses. The relationship between lymphocyte depletion and encephalitis in HPAI viral infections remains poorly understood. Notably, a study on Newcastle disease virus infection in ducks also showed that the severity of encephalitis correlated with lymphocyte depletion in immune organs (Hishikawa et al. 2024), consistent with the current findings. Further studies with larger sample sizes are required to confirm this relationship. Considering that long-distance migration is one of the most demanding physiological activities in wild birds, this can lead to immunosuppression (Weber & Stilianakis 2007). Clarifying the relationship between the immune status and disease progression is crucial for understanding HPAI viral infections in migratory birds.

In conclusion, mallards infected with the HPAI virus exhibited varying degrees of encephalitis, with some showing severe lesions while others showed none. The observed correlation between the degree of lymphoid atrophy and the severity of encephalitis highlights the importance of host immune status in disease progression. Notably, mallards without histological sequelae retained their mobility, highlighting their potential role as carriers of the virus.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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