NOTE *Avian Pathology*

Zoonotic Risk for Influenza A (H5N1) Infection in Wild Swan Feathers

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ABSTRACT. We examined whooper swans naturally infected with avian influenza virus (H5N1) to evaluate the possible zoonotic risk of swan feathers. Viruses were isolated from feather calami. Immunohistochemical testing revealed that virus antigens were present in the feather epidermis and feather follicle wall epidermis of some feathers. RT-PCR and genetic sequencing using paraffin sections of swan feathers confirmed the presence of avian influenza virus (H5N1) in the feather tissue. These results indicate that the feathers could have the risk for zoonotic infection from infected wild swans.

KEY WORDS: feathers, H5N1 subtype, Influenza A virus, swans, zoonosis.

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Among the wild bird species, swans such as whooper swans (*Cygnus cygnus*) and mute swans (*Cygnus olor*) are frequently found dead or emaciated after avian influenza (AI) virus (H5N1) infection [1, 5, 10, 11]. The neurological symptoms have often been reported as characteristic signs in experimentally and naturally infected swans [2, 4, 5, 10], although some swans may exhibit an asymptomatic course during the infection. Combined with migrating behavior, the high susceptibility to the AI virus (H5N1) and the virus shedding from infected swans are of great concern in terms of virus transmission to distant places [2, 4, 5, 11]. Moreover, wild swans have been considered as direct sources for human influenza A (H5N1) infections in the Republic of Azerbaijan, which was the first suspected occurrence of people contracting the disease from wild birds [3].

In 2006, human infections of influenza A (H5N1) of avian origin were found in the Republic of Azerbaijan [3]. Epidemiological investigation revealed seven cases including four fatalities that were possibly linked to wild swans infected with the virus [3]. The most probable source for human infection was defeathering of dead wild swans for making pillows [3]. If this speculation is true, how did people become infected with the virus by defeathering swans? Did people contact tainted blood or body fluids adhering to the swan's body? Did the virus actually exist in the feather tissue?

We previously reported that currently circulating AI virus (H5N1) of Asian lineage can replicate in the feather epidermal cells of domestic ducks and geese in experimental infection [15]. The feather lesion was found even in asymptomatic waterfowl which can play an important role for virus transmission [6, 8, 15]. Similar lesions were reported in swans experimentally inoculated with the virus [4]. These findings indicate that feathers of infected waterfowl may have an epidemiological importance for AI (H5N1) outbreaks. However, to the authors' knowledge, it has not been clear whether the feathers could be affected in those waterfowl species through natural infection. Confirming the viral replication in feathers of naturally infected waterfowl would be beneficial for risk analysis from epidemiological and public health viewpoints.

We examined two whooper swans infected with H5N1 subtype AI virus (clade 2.3.2) found in Aomori prefecture, northern Japan where a series of outbreaks occurred in the wild swan population in 2008 [11, 14]. The adult swan was found emaciated on the shore of Lake Towada in April [14]. The juvenile swan was found dead on the shore of the same lake in May [14]. After AI (H5N1) infections were confirmed with tracheal swab samples, their whole bodies were stored at −80°C. In August, the skin including feathers was collected from both birds after thawing for virological and pathological examination. At the necropsy, the adult swan's body had a small hole at the abdomen and lacked the pancreas and parts of the intestine. In the juvenile swan, only the heart, lung and kidney were left in the body cavity probably because of predation by wild animals.

Virus isolation using 10-day-old embryonated chicken eggs was performed with the basal part of the plucked contour feather, called the calamus, and skin of the head. Viruses were isolated from feather calami of the adult swan with a virus titer of $10^{3.5}$ 50% egg infectious doses per gram.

The skin collected from the head, neck, shoulder, wing, abdomen, back and thigh was fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 3 μm. Sections were stained with hematoxylin and eosin. Immunohistochemistry was performed to detect the viral antigen with a Histofine Simple Stain MAX-PO (M) kit (Nichirei Inc., Tokyo, Japan). A mouse monoclonal antibody specific for the influenza A virus matrix protein (diluted 1:500; clone GA2B, AbD Serotec, Kidlington, UK) was used as the primary antibody. Pathological analysis revealed that, although many feathers were mature feathers with scarce epidermal tissue, virus antigens were present in the feather epidermis and feather follicle wall epidermis of some developing feathers of the adult swan (Fig. 1a). The affected epi-

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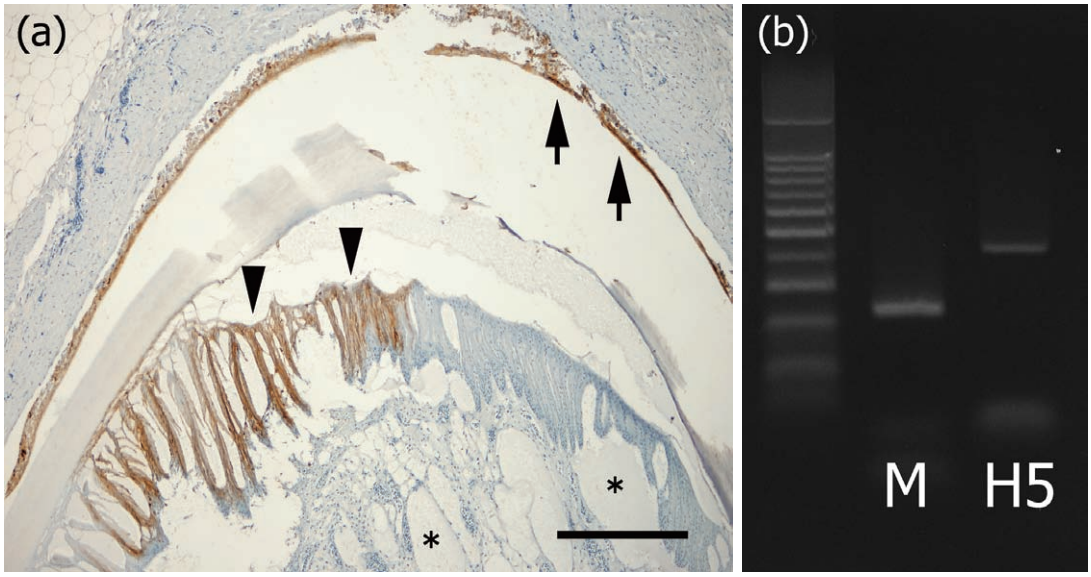

Fig. 1. (a) Immunohistochemical staining of the skin of the whooper swan. Influenza A virus antigens were detected in the feather epidermis (arrowheads) and necrotic feather follicle wall epidermis (arrows). Clear spaces (asterisks) in the feather tissue are histopathological artifacts caused by freezing preservation (bar=200 μm). (b) Positive results of RT-PCR for matrix (M) gene (232 bp) and H5 hemagglutinin gene (424 bp).

dermal tissue positive for viral antigens often exhibited necrotic changes.

Total RNA was extracted from paraffin sections of the adult swan feathers with an RNeasy FFPE Kit (QIAGEN, Hilden, Germany). One-step RT-PCR (SuperScript III One-Step RT-PCR System; Invitrogen, Carlsbad, CA, U.S.A.) was performed with the primers for influenza A virus matrix gene (M30F and M264R2) and H5 hemagglutinin gene (H5-248–270F and H5–671–647R) [13]. Samples were positive for both viral genes (Fig. 1b). Direct sequencing by 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.) revealed that the nucleotide sequences of positive RT-PCR products were identical to those of the virus strain A/whooper swan/Aomori/1/2008(H5N1) (GenBank accession nos. AB458242 and AB458239), the previously recorded sequences obtained from the virus in the swan's tracheal swab. The feathers of the juvenile swan were negative for virus isolation, immunohistochemistry and RT-PCR.

We found that AI virus (H5N1) can exist in the feather tissue of wild swans naturally infected with the virus. From an epidemiological viewpoint, the basal portion of the feather is more important, because the base has many more active epidermal cells that allow the virus to replicate than any other parts of the feather. In addition, the necrotic feather follicle wall epidermis that may contain the virus can be attached to the surface of the feather sheath. Therefore, the result indicates that the feathers, especially when plucked by people, could have the risk for zoonotic infection from infected wild swans.

Our finding is different from the result of a pathological study where naturally infected swans in Germany lacked any pathological changes in the skin [9]. German virus isolate belonged to clade 2.2 [7, 9], which is a different viral lineage from clade 2.3.2 in the present study [11]. However, the same German isolate replicated in swan feathers in experimental infection [4], indicating that the development of the feather lesion in natural infection does not depend on the phylogenetic factor of AI virus (H5N1). The severity of the disease in each case or the careful examination focusing only on feathers in the present study may have attributed to our finding of swan feathers.

The feather finding in wild swans indicates that similar feather lesions could occur in other waterfowl species through natural infection. In the previous experimental infection study using domestic ducks, AI virus (H5N1) was isolated from feathers for a longer period than from swabs [16]. Besides, affected feathers had the infectivity to healthy domestic ducks when birds were orally inoculated with feathers [17]. The data in the present study emphasize the possible epidemiological importance of waterfowl feathers and call for further risk analysis of feathers in the field where AI (H5N1) occurs.

Wild swans are natural hosts of AI virus of all hemagglutinin subtypes [12]. Therefore, combined with the result of immunohistochemistry, the detection of H5 specific gene from paraffin sections of feathers by RT-PCR and subsequent genetic sequencing were useful to confirm the presence of AI virus (H5N1) in the feather tissue. When only fixed samples are available for examination of wild birds, genetic analysis using formalin fixed paraffin embedded tissues could be used as supplemental information for AI

(H5N1) diagnosis.

Wild swan cases in the present study highlight the epidemiological importance of swan feathers to veterinarians and the general public who may handle diseased wild birds in the field. To reduce the risk of zoonotic infection, when wild swans are found and suspected of AI (H5N1) infection, people should avoid handling the birds without appropriate protective equipment.

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