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Asian Lineage H5N1 Highly Pathogenic Avian Influenza Virus Replication in Feathers of Waterfowl

(水禽の羽における H5N1 亜型高病原性鳥インフルエンザ
ウイルスの増殖に関する研究)

Yu YAMAMOTO

Contents

Abbreviations	iv
Preface	v
Chapter I	
Mortality with Severe Nonpurulent Encephalitis and Feather Lesions in Domestic Ducks (<i>Anas platyrhyncha</i> var. <i>domestica</i>) Inoculated with Highly Pathogenic Avian Influenza Virus A/chicken/Yamaguchi/7/2004(H5N1)	
Introduction	2
Materials and Methods	3
Results	6
Discussion	20
Summary	23
Chapter II	
Confirmatory Analyses for Asian Lineage H5N1 Highly Pathogenic Avian Influenza Virus Replication in Waterfowl Feathers	
Introduction	25
Materials and Methods	25
Results	29
Discussion	38
Summary	40
Chapter III	
Advantages and Disadvantages of H5N1 Highly Pathogenic Avian Influenza Virus Replication in Feathers of Domestic Ducks	
Introduction	42
Materials and Methods	43
Results	46
Discussion	52
Summary	55

Conclusions	56
Acknowledgments	58
References	59
Summary in Japanese (和文要旨)	69

Abbreviations

AI	: avian influenza
Ck/Yama/7/04	: A/chicken/Yamaguchi/7/2004(H5N1)
Ck/Miya/K11/07	: A/chicken/Miyazaki/K11/2007(H5N1)
EID ₅₀	: 50% egg infectious dose
H, HA	: hemagglutinin
HI	: hemagglutination inhibition
HP	: highly pathogenic
HPAI	: highly pathogenic avian influenza
LP	: low pathogenic
N, NA	: neuraminidase
PI	: postinoculation
RT-PCR	: reverse transcription-polymerase chain reaction
Ws/Akita/1/08	: A/whooper swan/Akita/1/2008(H5N1)

Preface

Avian influenza (AI) is caused by influenza A virus which belongs to the family *Orthomyxoviridae* (78, 84). The virus has 8 RNA segments which encode 10-11 viral proteins (13, 78, 84). The virus is classified into subtypes by its surface antigens, hemagglutinin (HA; H1-16) and neuraminidase (NA; N1-9) (78, 84). In terms of virulence for poultry such as chickens, AI virus is recognized as low pathogenic (LP) virus or highly pathogenic (HP) virus (78, 84). Generally, highly pathogenic avian influenza (HPAI) viruses isolated from birds are H5 and H7 subtypes, while LP viruses have all 16 HA subtypes including H5 and H7 (78, 84). HPAI virus is highly virulent for poultry and causes significant economic losses to the industry (78, 84). Prevention and urgent action after outbreaks are required to control the disease.

Wild waterfowl are considered natural hosts of AI virus (47, 78, 84). The viruses perpetuated among waterfowl are generally LP viruses (78, 84). The virus is transmitted by indirect fecal-oral route through contaminated open water in fields (25, 39). LPAI infection in waterfowl is asymptomatic, and the virus replication is confined to the lower intestinal tracts leading to virus shedding in their feces (30, 72, 77, 78). The isolation of HPAI virus from waterfowl is extremely rare event (78, 84). With experimental infections using HPAI viruses (excluding Asian lineage H5N1 subtype HPAI virus) and domestic ducks, the AI viruses which are highly virulent for chickens have caused asymptomatic infection in inoculated ducks, indicating that waterfowl have the different susceptibility from that of chickens against the HPAI virus (1, 2, 15, 57, 73).

However, the unique relationship among the HPAI virus and waterfowl was found after the emergence of Asian lineage H5N1 HPAI virus in the late 1990's (57, 79). This virus caused significant mortality not only to poultry but also to wild birds including waterfowl (17, 33, 37, 74). Furthermore, humans and some species of mammals have contracted the disease by close contact with infected birds (5, 21, 24, 29, 60). H5N1 HPAI virus continues to cause mortality in poultry and wild bird populations over the Eurasian continent including Japan. Some possible pathways of H5N1 HPAI virus transmission, such as migratory

birds, transport of poultry and poultry products, and live bird markets, have been proposed, although a major cause of the spread of the virus remains unclear (12, 18, 31, 43, 52, 79).

The fact that H5N1 HPAI virus has caused profound mortality to wild birds including waterfowl indicates that infected waterfowl can be associated with the viral transmission (18, 52, 74, 79). Actually, epidemiological studies in Southeast Asia revealed that free-range domestic ducks can play a prominent role in regional spread of the virus (20, 40, 59). In Japan, domestic ducks (*Anas platyrhynchos* var. *domestica*) called *Aigamo* in Japanese are reared as free-ranging ducks in water-soaked rice paddy fields for weed control and meat production. Therefore, these birds may have opportunities for direct contact with migratory wild birds infected with H5N1 HPAI virus, and then contribute to viral transmission.

The present thesis was aimed to investigate the pathogenesis and viral replication in waterfowl infected with Asian lineage H5N1 HPAI virus isolated in Japan. In particular, the author focused on the viral replication in feathers, and discussed the possible role of waterfowl feathers in viral transmission from infected birds. Feathers of birds are uniquely developed outgrowths of the skin (87). Feathers are covering the whole body so that they can be lost to the environment by regular physiological process (molting) or other accidental causes (plucking, stress, disease and others) (23, 87).

The present study comprises 3 chapters. In the first chapter, the author report the susceptibility and pathogenesis of Japanese domestic ducks inoculated with Asian lineage H5N1 HPAI virus. In chapter II, the author reported that the pathological details of viral replication in waterfowl feathers and the viral transmission via infective feathers of domestic ducks. In chapter III, the author present the usefulness of domestic duck feathers in clinical examination and the long-term viral persistence in feathers detached from the bodies of infected domestic ducks.

Chapter I

Mortality with Severe Nonpurulent Encephalitis and Feather Lesions in Domestic Ducks (*Anas platyrhyncha* var. *domestica*) Inoculated with Highly Pathogenic Avian Influenza Virus A/chicken/Yamaguchi/7/2004(H5N1)

Introduction

Wild waterfowl are generally considered natural hosts of AI virus (72, 78). Usually, AI infection in waterfowl is asymptomatic, and the virus replication is confined to the lower intestinal tracts leading to virus shedding in their feces (30, 72, 77, 78). However, since reporting on the mortality of wild ducks infected with Asian lineage H5N1 HPAI virus in Hong Kong in 2002 (17), highly virulent viruses against wild and domestic waterfowl have spread in Asia (26, 34, 35, 37, 65). These facts are epidemiologically important because wild waterfowl may be at risk for shedding HPAI viruses along their migration routes (36, 37, 58).

The HPAI virus strain A/chicken/Yamaguchi/7/2004(H5N1) (Ck/Yama/7/04) was isolated from the outbreaks in Japan in 2004 (42). Ck/Yama/7/04 caused 100% mortality to chickens within 1 day after the intravenous inoculation (42). Genetic analysis revealed that Ck/Yama/7/04 has multiple basic amino acids at HA cleavage site, and is highly close to HPAI virus isolated in South Korea in 2003 (41). Many migratory birds come to Japan from the Eurasian continent through People's Republic of China or Korean peninsula to overwinter (46). Therefore, the series of HPAI outbreaks in Japan in 2004 was suspected to be associated with migratory birds that may carry the virus (46).

Japanese domestic ducks (*Anas platyrhynchos* var. *domestica*) called *Aigamo* in Japanese are a crossbreed of wild mallard and domestic ducks. They are free-ranging ducks in water-soaked rice paddy fields and are used for weed control and meat production. In the rice paddy fields, these ducks have opportunities for direct contact with wild birds infected with AI virus. Therefore, it is necessary to investigate the pathogenicity of HPAI virus for Japanese domestic ducks and to consider possible virus shedding from infected ducks.

The pathogenicity of AI virus is generally determined by the mortality rate of 8- to 4-week-old chickens inoculated intravenously with any AI virus isolates, according to the criteria established by the World Organisation for Animal Health (84). The presence of multiple basic amino acids at HA cleavage site of the virus is also an indicator of the highly virulent nature for chickens (84). However, this genetic analysis is not always applicable to the virus pathogenicity for waterfowl,

because many studies reported that waterfowl have the different susceptibility from that of chickens against the HPAI viruses (1, 2, 15, 49, 50, 57, 73). Therefore, the author hypothesized that the true viral pathogenicity to waterfowl should be determined by intravenous inoculation, the same way it is selected in chickens.

The purpose of this study was to investigate the pathogenicity of Ck/Yama/7/04 for Japanese domestic ducks and to examine the pathological lesions and viral distribution in ducks inoculated with the virus. The results of intranasal inoculation study, which is an effective approach to analyze the pathogenesis in natural infection, were also described.

Materials and Methods

Animals. Domestic ducks (*Anas platyrhyncha* var. *domestica*) were obtained from a breeder at 1 day of age and raised with commercial food in an isolated facility. One-day-old birds randomly selected for the virus inoculation were directly moved into negative-pressure isolators of Biosafety Level 3–approved laboratories (National Institute of Animal Health, Tsukuba, Japan). Other birds for inoculation were moved into negative–pressure isolators for acclimatization 1 week before the experiment. The birds were provided with food and water *ad libitum*. All experimental procedures were approved by the Ethics Committee of the National Institute of Animal Health in Japan (authorization number 572, 653, 737).

Viruses. The HPAI virus Ck/Yama/7/04 was used for this study (42). The stored virus was propagated for 36–48 hours in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C. The infectious allantoic fluid was harvested and stored at –80°C until use.

Study 1. Pathogenicity test. Domestic ducks were divided into 3 groups, 1-day-old, 2-week-old, and 4-week-old, with 8 birds in each group. The birds were inoculated intravenously with $10^{7.3}$ 50% egg infectious doses (EID₅₀) of the virus. All birds were monitored to evaluate clinical symptoms and mortality for 10 days. All surviving birds on day 10 postinoculation (PI) were euthanized

and examined by serological test.

Study 2. *Histopathological study.* Twelve 2-week-old birds were inoculated intravenously with Ck/Yama/7/04 in the same manner as in experiment 1. Three birds were euthanatized on each of days 1, 3, 5, and 7 PI and examined by clinical observation, pathological analysis and virus isolation. In addition, 2- and 4-week-old birds in study 1 were added to study 2 since the same inoculum dose was used in both studies.

Study 3. *Intranasal infection study.* Study 3 was performed to examine the pathological changes in domestic ducks in intranasal infection adopted as a natural infection route. Thirty-three domestic ducks were divided into 3 groups: 1-day-old (n = 15), 2-week-old (n = 9), and 4-week-old (n = 9) birds. The birds were inoculated intranasally with $10^{7.3}$ EID₅₀ of virus. All birds were monitored to evaluate clinical signs and mortality. Because of the mortality in the 1-day-old group, euthanized or dead birds were examined on days 1, 3, 4, 5, 6, and 7 PI. In the 2- and 4-week-old groups, 3 birds were euthanized on each of days 3, 6, and 14 PI. Birds were examined by histopathology and serological test.

Histopathology. Following postmortem examination in studies 2 and 3, the brain, eye, conjunctiva, beak, tongue, trachea, esophagus, thymus, lung, heart, proventriculus, gizzard, liver, spleen, pancreas, duodenum, small intestine at Meckel's diverticulum, cecum, large intestine (rectum), bursa of Fabricius, adrenal gland, kidney, genital organs, spinal cord (on day 10 PI), skeletal muscle, thigh bone, and skin were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 3 μ m, and stained with hematoxylin and eosin.

Immunohistochemistry. The immunohistochemistry was performed to detect the influenza virus antigen. A Histofine Simple Stain MAX-PO (M) kit (Nichirei Inc., Tokyo, Japan) was used according to the manufacturer's protocols. The sections were pretreated with 10 mmol of citrate buffer at pH 6.0 in a microwave oven at 500 W for 10 minutes for antigen retrieval. A mouse monoclonal antibody, specific for the influenza A virus matrix protein (diluted 1:500; clone GA2B, Oxford Biotechnology Ltd., Kidlington, U.K.), was used as the primary antibody. Negative control sections were used with the phosphate-buffered saline

instead of the primary antibody.

Severity index of histopathologic lesions and viral antigens. The severity of histopathologic lesions and viral antigen distribution in study 2 was scored according to the following scale: 0 = none, 1 = mild, 2 = moderate, 3 = severe. The severity index of each tissue in each test period was calculated by adding lesion scores, then dividing by the sum of the total number of birds used for histopathologic evaluation.

Virus isolation. Virus isolation using 10-day-old embryonated chicken eggs was performed with the supernatant of 10% (wt/vol) homogenate of collected organs in study 2. The cerebrum, liver, rectum, and skin of the head, which includes many down feathers, were collected from the 2-week-old group. In the 4-week-old group, the cerebrum, lung, liver, pancreas, rectum, and skeletal muscle were collected and stored at -80°C . Recovery of the infectious virus was determined by the positivity of allantoic fluids for HA activity. The virus titer was expressed as EID_{50}/g .

Serological test. The immune response against HPAI virus was measured by hemagglutination inhibition (HI) tests using 4 HA units of Ck/Yama/7/04 as antigen (84). The serum samples were extracted from 2- and 4-week-old birds on day 10 PI in study 1, and from 2- and 4-week-old birds on day 14 PI in study 3. An HI antibody titer $<1:16$ was considered negative for antibody production. Preinoculation sera from birds were also examined and were negative for antibodies against the virus.

Results

Study 1

Mortality and clinical signs. Mortality and mild-to-severe neurologic symptoms that started on day 3 PI or later were observed (Table 1). Mild-to-severe depression was common to all inoculated birds from days 1 to 5 PI.

All 8 birds in the 1-day-old group died within 4 days after the virus inoculation. Three birds were found dead on days 1 and 2 PI. Three out of 5 birds that died on days 3 and 4 PI exhibited severe neurologic signs of drowsiness, ataxia, and intermittent generalized seizure.

In the 2-week-old group, 2 of 8 inoculated birds exhibited mild neurologic signs of persistent torticollis, circling, and ataxia from days 4 or 8 to 10 PI. In the 4-week-old group, 1 bird suffered severe depression and a generalized seizure, and died on day 4 PI.

Serological test. The antibody was detected in all surviving birds on day 10 PI in study 1. The HI antibody titer ranged from 1:40 to 1:320 in 2-week-old birds and from 1:20 to 1:160 in 4-week-old birds.

Table 1. Mortality and neurologic signs of domestic ducks inoculated intravenously with Ck/Yama/7/04.

Group	No. of total birds	% Mortality	% Neurologic signs
1-day-old	8	100	37.5
2-week-old	8	0	25
4-week-old	8	12.5	12.5

Study 2

Mortality and clinical signs. In study 2, 4 of 12 inoculated birds exhibited neurologic signs. Severe neurologic signs started on day 3 PI in 1 bird and on day 4 PI in 2 birds. The signs were drowsiness, ataxia, intermittent generalized seizure, and leg paralysis. The other bird showed mild neurologic signs, including persistent torticollis and circling movement from days 4 to 7 PI.

Gross findings. Hyperemic meninges were commonly observed in birds with neurologic symptoms. Bilateral or unilateral corneal opacity was observed in birds on day 3 PI or later. Focal-to-diffuse, white, necrotic lesions were found in the pancreas of the 2-week-old birds on day 3 PI.

Histopathology. Major histopathologic lesions were common to all inoculated groups, depending on the course of infection. They were characterized by the nonpurulent encephalitis and necrosis of the feather epidermal cells.

Two-week-old group. Histologic changes of the 2-week-old group are summarized in Table 2. In the cerebrum, histopathologic lesions were common to inoculated birds regardless of the manifestation of neurologic signs. The lesions were mainly observed in the submeningeal and periventricular areas (Fig. 1). In severely affected cases, lesions were symmetrically distributed and expanded to the parenchyma of the nidopallium and mesopallium.

On day 3 PI, small-to-large foci of neuronal necrosis, characterized by uniformly eosinophilic, spheroidized neurons with spongy changes of the adjacent neuropils, were observed (Figs. 2A and 2B). Mild perivascular cuffing of mononuclear cells and a few heterophils was infrequently found around the foci of neuronal necrosis, accompanied by occasional meningeal inflammation. Some glial and inflammatory cells of perivascular cuffing exhibited fragmented nuclei.

On day 5 PI or later, gliosis and foci of mild-to-severe perivascular cuffing composed of lymphocytes and plasma cells predominated (Figs. 2C and 2D). These lesions were occasionally accompanied by lymphoplasmacytic infiltrations at the meninges. A few lymphocytes and plasma cells also infiltrated the parenchyma. Mitotic figures of inflammatory cells and glial cells were often present. Neuronal necrosis was rarely scattered in these lesions.

In the cerebellum, focal necrosis of Purkinje cells was randomly distributed on

day 3 PI. Granule cells beneath the necrotic foci of Purkinje cells became necrotic and often possessed pyknotic or fragmented nuclei. On day 5 PI or later, there were focal lesions composed of the loss of Purkinje cells, mild-to-severe gliosis in the molecular and Purkinje cell layers, and mild lymphoplasmacytic perivascular cuffing in the molecular layer, granular layer, and white matter.

Lesions in the brainstem were much less common than in the cerebrum and cerebellum, and if they existed, they were mild lesions. The spinal cord on day 10 PI lacked the histopathologic change.

In many feathers, especially in developing feathers, focal-to-diffuse necrosis of the feather epidermal cells with a few heterophils was observed on days 3 to 7 PI (Figs. 2E and 2F). These feather lesions were distributed from the epidermal collar to the pulp cap and, less frequently, to the developing rachis and barb ridge. The feather follicular epithelium lacked the histopathologic changes. Similar focal necrosis with heterophil infiltrations was observed in the epithelium of the beak and was prominent in the palatine epithelium of the upper beak (Fig. 2G and 2H). Focal necrosis was often observed in the epithelium of the scaled skin of the legs and the epithelium of the tongue.

Focal necrosis of myocardial cells with a few heterophils was observed on day 3 PI (Fig. 2I). The necrotic myocardial cells were often mineralized. There were lymphoplasmacytic infiltrations and foci of the fibroblast proliferation with mild fibroplasia in myocardium on days 5 to 10 PI. These were associated with the loss of myocardial cells.

In the skeletal muscle, multifocal-to-diffuse necrosis with infiltrations of heterophils and macrophages was observed on day 3 PI (Fig. 2J). On day 5 PI or later, these lesions were replaced by lymphoplasmacytic infiltrations and regeneration of skeletal myocytes, which were characterized by the proliferation of immature myocytes with basophilic cytoplasm and one or more nuclei centrally located.

In the pancreas, multifocal-to-massive necrosis of the acinar cells was observed on day 3 PI (Fig. 2K). Focal necrosis with a few heterophils in the liver was mild and confined to day 1 PI.

Focal necrosis of the corneal epithelium was observed on day 3 PI (Fig. 2L).

There was edema with a few heterophils and mild capillary formation in the substantia propria of the cornea from days 3 to 10 PI. Mild necrosis of the bone marrow and loss of osteoblasts was found on day 3 PI. Other organs lacked significant histologic lesions.

Four-week-old group. The histologic lesions of the bird that died on day 4 PI in study 1 were consistent with those of the 2-week-old birds on days 3 and 5 PI. Lesions in the brain of the dead bird were more severe than those in the visceral organs. Other birds euthanatized on day 10 PI in the 4-week-old group lacked notable histologic lesions.

Immunohistochemistry. Viral antigens detected were strongly associated with necrotic lesions in systemic organs on day 3 PI. (Table 2 and Fig. 2). Viral antigens were also detected in the cornea (epithelial cells), tongue (epithelial cells), intestine (smooth muscle cells and myenteric nerve plexus), adrenal gland (cortical cells and medullary cells), ganglion cells, bone marrow (osteoblasts and osteoclasts), arteries (smooth muscle cells), and a few endothelial cells.

Virus isolation. The viruses were isolated from all organs examined, except on day 10 PI (Table 3). The highest virus titer was detected in the brain. In the skin, virus titers of 10^3 EID₅₀/g or more were isolated from days 1 to 7 PI.

Table 2. Mean scores of the severity of histopathological lesions and viral antigens in 2-week-old domestic ducks inoculated intravenously with Ck/Yama/7/04.^a

Organ		Days postinoculation				
		1	3	5	7	10
Cerebrum	Necrosis	0 ^b	2.7	1.3	1.3	0.4
	Perivascular	0	1.3	1.3	2	2.3
	Gliosis	0	0.7	1	2	2.1
	Viral antigens	0	2.3	0.7	0.3	0
Cerebellum	Necrosis	0	2.3	1.3	0.7	0.3
	Perivascular	0	0.7	1.7	1.3	1.8
	Gliosis	0	0	2.3	2	0.8
	Viral antigens	0	2.3	0.3	0.3	0
Brain stem	Necrosis	0	0.7	0.3	0	0.1
	Perivascular	0	0.7	0	0.7	0.9
	Gliosis	0	1	0.7	0	0.6
	Viral antigens	0	0.7	0	0	0
Epidermis of feathers	Necrosis	0	1.7	1.3	0.3	0
	Viral antigens	0	1.3	1	0.3	0
Epithelium of the beak	Necrosis	0	2.3	1	0.7	0.1
	Viral antigens	0.3	2.3	1	0.7	0.5
Pancreas	Necrosis	0.4	2.7	0.3	0	0
	Viral antigens	0	1.7	0	0	0
Liver	Necrosis	1.4	0	0	0	0
	Viral antigens	0.7	0	0	0	0
Heart	Necrosis	0.4	1	0.7	0	0
	Inflammation	0	0.3	1.7	0.7	1.5
	Viral antigens	0.3	0.7	0	0	0
Skeletal muscle	Necrosis	0	2.7	1	0.3	0
	Inflammation	0	2.7	1.3	2	0.9
	Regeneration	0	0	1.7	1.7	0.6
	Viral antigens	0	2.3	0.3	0	0

^a The results of birds on days 1-7 PI (study 2) and on day 10 PI (study 1).

^b Mean scores of histopathological lesions and viral antigens in 3 birds (0 = none, 1 = mild, 2 = moderate, 3 = severe).

Table 3. The virus isolation from domestic ducks inoculated intravenously with Ck/Yama/7/04 in study 2, expressed as log₁₀ EID₅₀/g.^a

Organ	2-week-old group (Duck A-O)												4-week-old group (Duck P-W)		
	1 dpi ^b			3 dpi			5 dpi			7 dpi			10 dpi	4 dpi ^c	10 dpi
	A	B	C	D	E	F	G	H	I	J	K	L	M-O	P	Q-W
Cerebrum	2.5	3	2.5	7	5.5	7	4.5	– ^d	–	–	–	–	–	8	–
Skin	4	5.5	5.5	5	5	4	5.5	5	3	3.5	5	–	–		
Rectum	6	6	4.5	5	5	2.5	–	4.5	–	–	–	–	–	6.5	–
Liver	3.5	5.5	4	3	3	2	–	–	–	–	–	–	–	2	–
Lung														6.5	–
Pancreas														5.5	–
Skeletal muscle														7	–

^a The results of birds on days 1-7 PI (study 2) and on day 10 PI (study 1).

^b dpi, days postinoculation.

^c Duck P died on day 4 PI.

^d – = negative (<10² EID₅₀/g).

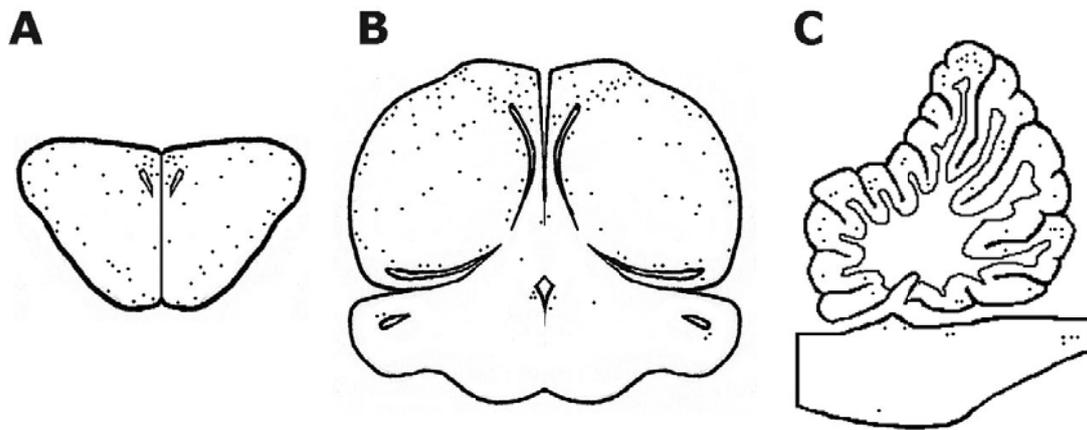


Figure 1. Distribution of histologic lesions in the brain of 2-week-old domestic ducks in study 2. Dots indicate histopathologic lesions in 2 coronal sections of the (A) anterior part and (B) posterior part of the cerebrum and the (C) sagittal section of the cerebellum.

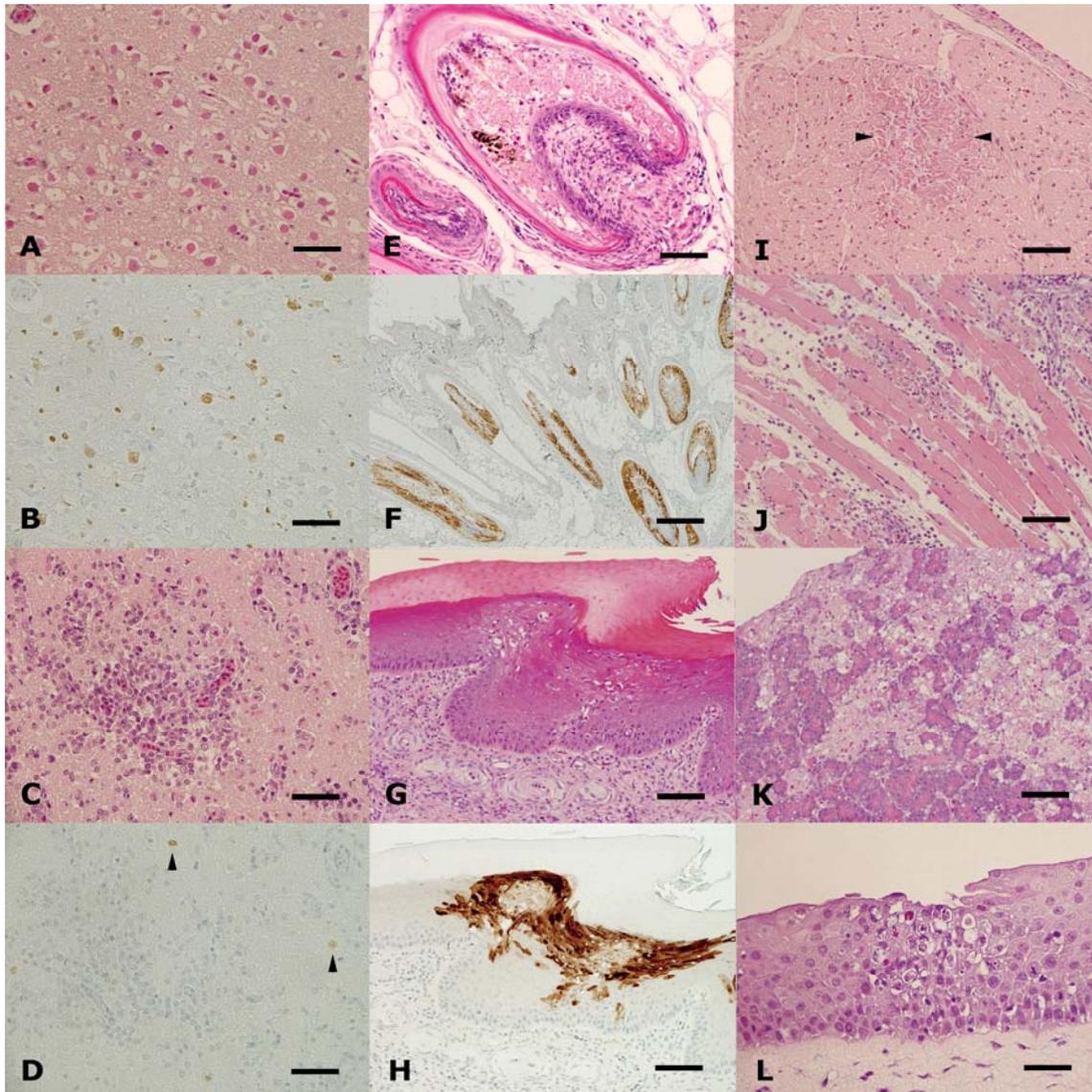


Figure 2. Histopathological findings in domestic ducks intravenously inoculated with Ck/Yama/7/04.

A-B: Cerebrum on day 3 PI. (A) Neuronal necrosis with spongy changes of the adjacent neuropils. Hematoxylin and eosin stain. Bar = 50 μ m. (B) Viral antigens were detected in neurons and glial cells. Immunohistochemistry. Hematoxylin counterstain. Bar = 50 μ m.

C-D: Cerebrum on day 7 PI. (C) Severe nonpurulent encephalitis composed of gliosis and perivascular cuffing of lymphocytes and plasma cells. Hematoxylin and eosin stain. Bar = 50 μ m. (D) Viral antigens were scarcely distributed (arrowheads). Immunohistochemistry. Hematoxylin counterstain. Bar = 50 μ m.

E-F: Skin. (E) Diffuse necrosis of the feather epidermal cells. Feather lesions

were distributed from the epidermal collar to the pulp cap. HE. Bar = 110 μm .

(F) Abundant viral antigens were detected in association with the necrosis. Immunohistochemistry. Hematoxylin counterstain. Bar = 300 μm .

G-H: Beak. (G) Focal necrosis of the epithelium of the beak. Hematoxylin and eosin stain. Bar = 80 μm . (H) Viral antigens were detected in association with the necrosis. Immunohistochemistry. Hematoxylin counterstain. Bar = 80 μm .

I: Heart. Focal necrosis of myocardium (arrowheads). Hematoxylin and eosin stain. Bar = 60 μm .

J: Skeletal muscle. The necrosis of the skeletal myocytes and infiltrations of lymphocytes and macrophages. Hematoxylin and eosin stain. Bar = 80 μm .

K: Pancreas. The necrosis of acinar cells with a few heterophils. Hematoxylin and eosin stain. Bar = 80 μm .

L: Cornea. Focal necrosis of the corneal epithelium. Hematoxylin and eosin stain. Bar = 40 μm .

Study 3

Clinical signs and mortality. In the 1-day-old group, except for 3 birds on day 1 PI and 2 birds on day 3 PI, all 10 remaining birds exhibited neurological signs followed by mortality between days 3 and 7 PI. Neurologic symptoms were composed of drowsiness, ataxia, and intermittent generalized seizure (Fig. 3A). Corneal opacity was observed in 6 birds on day 3 PI or later. In the 2- and 4-week-old groups, no birds exhibited neurologic signs or mortality. Four birds in the 2-week-old group and 1 bird in the 4-week-old group exhibited persistent corneal opacity.

Gross lesions. In the pancreas of 1-day-old birds on day 3 or later, focal-to-diffuse white lesion consistent with necrosis was observed (Fig. 3B). Severe atrophy of the bursa of Fabricius was common in dead birds. In 2-week-old birds, similar focal lesions of the pancreas were found on day 3 PI. Birds in the 4-week-old group lacked significant findings.

Histopathology and immunohistochemistry. Nonpurulent encephalitis was commonly observed in all groups. In 1-day-old birds, severe, focal-to-diffuse lesions of neuronal necrosis with viral antigens were found on day 3 PI. Subsequently, lymphoplasmacytic perivascular cuffing with gliosis was observed throughout the olfactory bulb, cerebrum, cerebellum, and brain stem. In the cervical to lumbar spinal cord, there was necrosis of ependymal cells with viral antigens and mild myelitis of the gray matter. In the 2- and 4-week-old groups, mild-to-moderate focal perivascular cuffing by lymphocytes and plasma cells was observed in the brain and cervical spinal cord.

In some birds in all age groups, necrosis and/or viral antigens were observed in the feather epidermis (Table 4). In the 1-day-old group, necrosis of the feather epidermis with a few heterophils was found in 3 birds (Fig. 3C), although a small number of feathers were affected in each bird. Viral antigens were detected in association with necrotic changes, and were rarely seen in the adjacent follicular wall epithelium (Fig. 3D). In 2-week-old birds, although no apparent necrosis of the feather epidermis was present, thickened feather sheaths with irregular melanin pigments were often observed in 1 bird. This finding was considered a postnecrotic change of the feather epidermis. Viral antigens were detected in the

feather epidermal cells beneath the affected sheath. Three 4-week-old birds had necrosis of the feather epithelium with viral antigens.

The other lesion in dead birds in the 1-day-old group was focal-to-diffuse necrosis with viral antigens of the heart, pancreas, liver, skeletal muscle, adrenal gland, and ganglion. The epithelial necrosis of the beak, tongue, and scaled skin of the legs also was common and accompanied by abundant viral antigens. Two dead birds had local, small necrosis of the epidermis with heterophilic infiltration, and viral antigens were detected in 1 bird. Occasionally, heterophilic keratitis, heterophilic arteritis, and lymphocytic depletion in the bursa of Fabricius were observed. Two- and 4-week-old birds on days 3 and 6 PI had similar lesions in the pancreas, heart, artery, skeletal muscle, and cornea, but they were mild and restricted to small areas.

Serological test. A serum HI antibody titer ranged from 1:40 to 1:80 in all 2- and 4-week-old birds on day 14 PI.

Table 4. Histologic feather lesions and viral antigens in domestic ducks inoculated intranasally with Ck/Yama/7/04.

Group	Days postinoculation						
	1	3	4	5	6	7	14
1-day-old	0/3 ^a (-) ^b	0/3(-)	1/2(+)	1/3(+)	1/2(+)	0/2(-)	
2-week-old		0/3(-)			0/3(+)		0/3(-)
4-week-old		1/3(+)			2/3(++)		0/3(-)

^a Number of birds with feather lesions / No. examined birds.

^b (virus antigens); ++ = positive in 2 birds, + = positive in 1 bird, - = negative.

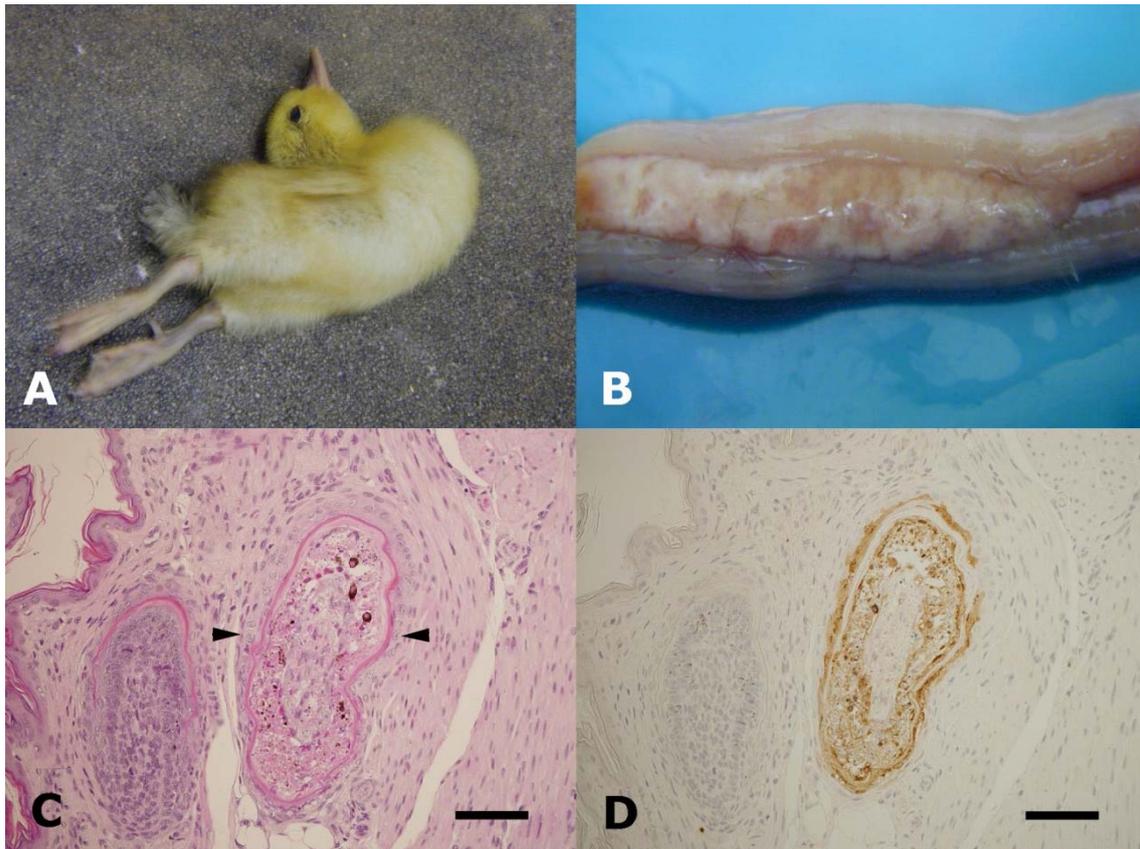


Figure 3. Clinical and pathological findings in domestic ducks inoculated intranasally with Ck/Yama/7/04.

A: Opisthotonos observed in the 1-day-old group on day 5 PI.

B: Pancreas. White necrotic lesions in the 1-day-old group on day 6 PI.

C-D: Skin. (C) Diffuse necrosis of the feather epidermal cells (arrowheads).

Hematoxylin and eosin stain. Bar = 60 μ m. (D) Abundant viral antigens were

detected in the necrotic feather epidermis and adjacent follicular wall epithelium.

Immunohistochemistry. Hematoxylin counterstain. Bar = 60 μ m.

Discusson

It was found that HPAI virus isolate Ck/Yama/7/04 has a pathogenicity that could cause mortality and severe neurologic symptoms for Japanese domestic ducks. Especially, the high mortality of the 1-day-old group in intravenous and intranasal infection indicates that domestic ducks have an age-related susceptibility against Ck/Yama/7/04 infection. Similar data on high mortality in young ducks has been reported in experimental infection using Ck/Yama/7/04 (27, 32) and other Asian lineage H5N1 HPAI virus strains (48). In HPAI field outbreaks in South Korea caused by the virus genetically close to Ck/Yama/7/04 (41), the increased mortality was recorded in 2-week-old commercial ducks, but not in adult breeder ducks (34).

Clinical symptoms and systemic histopathologic lesions with viral antigens were observed in inoculated birds. Regardless of the mortality, some inoculated ducks exhibited neurologic symptoms and histological nonpurulent encephalitis, suggesting that Ck/Yama/7/04 is neurovirulent for domestic ducks. Nonpurulent encephalitis observed in study 1 was characterized by severe neuronal necrosis from day 3 PI and subsequent inflammatory reaction such as perivascular cuffing and gliosis. Considering the severity of histopathologic lesions with viral antigens and the high level of virus titer in the brain on day 3 PI, it is likely that the onset of severe neurologic symptoms and mortality is associated with neuronal necrosis caused by virus replication.

In contrast, the chickens naturally (45) or experimentally (K. Nakamura, pers. comm.) infected with Ck/Yama/7/04 did not exhibit any neurologic signs, although Ck/Yama/7/04 caused 100% mortality to chickens in experimental infection. The pathologic lesions of the brain in chickens were characterized by a few, minimal necrosis of neurons with viral antigens, and mild focal gliosis (45 and K. Nakamura, pers. comm.). These results suggest that clinical and pathologic differences between ducks and chickens result from a different pathogenesis depending on the species of the bird infected with the virus.

Interestingly, the feather epidermis of inoculated ducks exhibited necrotic changes with viral antigens in intravenous and intranasal infection. The feather

lesion have not been reported in waterfowl or chickens infected with Asian lineage H5N1 HPAI virus until the present study was conducted (17, 32, 34, 35, 37, 45, 50, 51, 65). Similar lesions of the epithelium in the beak, scaled skin of the legs, tongue and feather follicle wall suggest Ck/Yama/7/04 has the tropism for a certain type of the stratified squamous epithelium in ducks. In contrast, pathological changes of the stratified squamous epithelium of the esophagus, cloaca, and skin epidermis were highly exceptional in the present study, although 1 dead bird in the 1-day-old group of study 3 had small necrosis with viral antigens in the skin epidermis. It is not clear whether the virus is unlikely to replicate in the skin epidermis, or whether lesions will disappear due to rapid epidermal turnover even if formed. However, the former is probable because no postnecrotic changes associated with skin epidermis, such as inflammation or crust formation, were observed in the course of the disease.

In addition, viruses were isolated from skin that included many feathers in study 2. The possibility of the contamination to the skin from feces cannot be eliminated. However, the author used clean, dry skin from the head, which was less likely to be contaminated. On day 7 PI, viruses were isolated from the skin but not from the rectum. Therefore, virus titers of 10^3 EID₅₀/g or greater detected from the skin may be associated with necrotic lesions of the feathers. These results raise the possibility that the viruses may be released from feathers of domestic ducks infected with Ck/Yama/7/04, leading to possible viral transmission to other birds or mammals. In the viral diseases that affect the feather epidermis of birds, such as psittacine beak and feather disease or papovavirus infection, infective feathers or feather dusts are potential sources of the infection (54, 55). Marek's disease virus replicates actively in the feather follicle wall epithelial cells which are the main route for virus shedding from infected chickens (10).

Histologic examination revealed that the corneal opacity was attributable to the keratitis caused by Ck/Yama/7/04. Lesions in the substantia propria of the cornea might be a secondary change to the necrotic lesion of the corneal epithelium. Similar clinical findings referred to cloudy eyes reported in ducks infected with Asian lineage H5N1 HPAI virus, although histologic examination was not performed (26, 65). It is likely that the corneal opacity is a characteristic finding

in waterfowl infected with Asian lineage H5N1 HPAI virus.

In conclusion, the author found that Ck/Yama/7/04 could induce mortality, severe nonpurulent encephalitis, and feather lesions in domestic ducks. The feather lesions with viral antigens and the virus isolation from the skin containing feathers suggest that Ck/Yama/7/04 has a predilection for feathers. Further careful study is needed to investigate the possible viral release from waterfowl feathers.

Summary

Japanese domestic ducks were inoculated with Asian lineage H5N1 subtype HPAI virus isolate Ck/Yama/7/04 to investigate the pathogenicity of the virus, pathological lesions and viral distribution in inoculated ducks. One-day-old, 2-week-old, and 4-week-old domestic ducks (*Anas platyrhyncha* var. *domestica*) were inoculated intravenously with the virus. Clinically, the birds exhibited mild-to-severe neurologic symptoms and corneal opacity. All birds in the 1-day-old group and 1 bird in the 4-week-old group died within 4 days after the virus inoculation. Histologic changes were characterized by severe nonpurulent encephalitis, necrotic lesions of the feather epidermis, focal necrosis of myocardial cells, pancreatic acinar cells, skeletal myocytes, and corneal epithelial cells. Viral antigens were detected in association with necrotic changes. Viruses were isolated from all examined organs including the skin with many feathers. Serum antibody against the virus was detected in all surviving birds on day 10 PI by HI tests. In intranasal infection study using 1-day-old, 2-week-old, and 4-week-old domestic ducks, the mortality was observed only in the 1-day-old group. Histopathological changes were similar to those in birds inoculated intravenously. These results suggest that Ck/Yama/7/04 has a pathogenicity that causes mortality with neurologic symptoms, nonpurulent encephalitis, and feather lesions for domestic ducks. Feather lesions with viral antigens and the virus isolation from the skin suggest that Ck/Yama/7/04 has a predilection for feathers in domestic ducks.

Chapter II

Confirmatory Analyses for Asian Lineage H5N1 Highly Pathogenic Avian Influenza Virus Replication in Waterfowl Feathers

Introduction

Since the late 1990's, an epidemic of Asian lineage H5N1 HPAI virus has spread in Eurasia and Africa, causing fatal infections in poultry, wild birds, mammals, and humans (79). The epidemiological studies revealed that the outbreaks of HPAI in Southeast Asia had a relationship with the distribution of free-range domestic ducks reared for rice farming and meat production (20, 40, 58). Even asymptotically infected domestic ducks can shed the virus from the cloaca and oral cavity (26, 66) and contribute to viral maintenance and spread (20, 40). Therefore, focusing on the epidemiologic role of domestic waterfowl in H5N1 HPAI virus epidemics is important.

The author found that, in experimental infection with Japanese domestic ducks, HPAI virus strain Ck/Yama/7/04 caused mortality, nonpurulent encephalitis, and necrosis of the feather epidermis with viral antigens (chapter I). Infectious virus was isolated from the skin of infected ducks (chapter I). Viral replication in feathers of domestic ducks by Ck/Yama/7/04 raises the possibility that feathers containing the virus can be released to the environment from the body of infected waterfowl, and then may play a role in virus transmission in fields.

However, except for studies in chapter I, this feather lesion has not been reported in H5N1 HPAI virus-infected waterfowl (17, 32, 34, 37, 49, 50, 65). Different susceptibilities to H5N1 HPAI virus have been reported among several duck species (7). Therefore, confirming the replication of other Asian lineage H5N1 HPAI viruses in feathers of other waterfowl species would be necessary to discuss the possible involvement of contaminated feathers in H5N1 HPAI virus transmission. The infectivity of contaminated feathers to unaffected birds also needs to be investigated.

In chapter II, the pathological details of the feather lesion caused by H5N1 HPAI viruses in 3 waterfowl species were investigated. The author also reports virus transmission by oral inoculation of feathers of infected domestic ducks.

Materials and Methods

Animals. Two species of domestic waterfowl, ducks and geese, were used for experimental infection in studies 3 and 5. Geese were selected because wild geese (*A. indicus*) accounted for a large proportion of the deaths in HPAI outbreaks at Qinghai Lake in People's Republic of China in 2005 (11). All experimental procedures were approved by the Ethics Committee of the National Institute of Animal Health in Japan (authorization number 572, 653, 737 and 07-118).

In study 4, the author examined 2 whooper swans naturally infected with H5N1 HPAI virus (H5 clade 2.3.2). These swans were found in Aomori prefecture, northern Japan where a series of outbreaks occurred in the wild swan population in 2008 (74, 85). The adult swan was found emaciated on the shore of Lake Towada in April (85). The juvenile swan was found dead on the shore of the same lake in May (85).

Viruses. Two different clades of H5N1 HPAI virus were used in experimental infection. Ck/Yama/7/04 is classified as H5 clade 2.5 (74) and genotype V (42). A/chicken/Miyazaki/K11/2007(H5N1) (Ck/Miya/K11/07) belongs to H5 clade 2.2 (M. Mase, unpub. data) and genotype Z, which has spread from China to Japan, Europe, and Africa (6, 11). The stored virus was propagated for 36–48 hours in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C. The infectious allantoic fluid was harvested and stored at –80°C until use.

Study 4. *H5N1 HPAI viruses in feathers of domestic ducks and geese.* Experimental infection was performed to see whether the feather lesion, which was found in chapter I, is common to other waterfowl species and Asian lineage H5N1 HPAI viruses. 4-week-old ducks (n = 2) and geese (n = 2) were inoculated intranasally with 10⁷ EID₅₀ of Ck/Yama/7/04. Similarly, 4-week-old ducks (n = 2) and geese (n = 2) were inoculated intranasally with 10⁷ EID₅₀ of Ck/Miya/K11/07. Inoculated birds were euthanized with an overdose injection of sodium pentobarbital (i.v.) on days 3 and 5 PI. Skin samples containing feathers were examined by pathological, ultrastructural and virological analysis.

Study 5. *Examination of naturally infected swans.* Two wild swans infected with H5N1 HPAI virus were examined to confirm the viral replication in feathers of wild waterfowl naturally infected with the virus. After H5N1 HPAI

virus infections were confirmed with tracheal swab samples in May 2008 (85), their whole bodies were stored at -80°C . In August, the skin with feathers was collected from both birds after thawing for pathological and virological examination.

Study 6. *Virus transmission study.* The author performed virus transmission study to investigate the infectivity of feathers taken from an infected domestic duck. To prepare feathers infected with Ck/Yama/7/04, a 2-week-old duck was inoculated intravenously with $10^{7.3}$ EID₅₀ of virus in 0.2 ml, as performed in chapter I. This bird died on day 4 PI, and its skin of the head and neck was collected and stored at -80°C . This skin sample was proven to have the necrosis of the feather epidermis by histology. The virus titer of the skin including feathers after homogenization was 10^6 EID₅₀/g. Then, a 0.2 g sample of down feathers was plucked from the skin.

After evenly dividing down feathers into 10 parts in a small amount of the phosphate-buffered saline without homogenization or chopping into small pieces, 9 2-week-old ducks (A-I) were orally inoculated with approximately 20 mg of down feathers per bird. Three birds were euthanized on each of days 3, 6, and 14 PI. The infection in inoculated birds was confirmed by immunohistochemistry, virus isolation from cloacal swabs, and serological test.

Histopathology and immunohistochemistry. The skin and plucked counter feathers in studies 4 and 5, and major organs of inoculated ducks in study 5 were 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.

Transmission electron microscopy. For ultrastructural examination in study 4, the skin and plucked contour feathers were fixed in 3% glutaraldehyde in 0.1M phosphate buffer, postfixed in 1% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H-7500 transmission electron microscope (Hitachi Corp., Tokyo,

Japan).

Virus isolation. Virus isolation using 10-day-old embryonated chicken eggs was performed with the skin of the head in studies 4 and 5, and plucked counter feathers in study 5. The supernatant of 10% (wt/vol) homogenate of the skin and 3–4 sticks of feather calamus, the basal part of the feather shaft, was made and titrated. In study 6, cloacal swabs were collected in 1 ml of the phosphate-buffered saline with antibiotics (10000 unit/ml penicillin and 10 mg/ml streptomycin) for virus isolation. Samples were centrifuged at $6000 \times g$ at 4°C , and supernatant fluid of each sample was stored at -80°C . The virus titer was expressed as EID_{50}/g in studies 4 and 5 and as $\text{EID}_{50}/\text{ml}$ in study 6.

Reverse transcription-polymerase chain reaction (RT-PCR). In study 5, total RNA was extracted from paraffin sections of the 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 HA gene (H5-248-270F and H5-671-647R) (83). Positive PCR products were further analyzed by direct sequencing method with 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.).

Serological test. In study 6, the immune response against H5N1 HPAI virus was measured by HI tests using 4 HA units of Ck/Yama/7/04 as antigen. An HI antibody titer $<1:16$ was considered negative for antibody production. Preinoculation sera from birds were negative for antibodies against the virus.

Results

Study 4

Clinical signs. Inoculated birds did not exhibit apparent clinical signs, except for unilateral corneal opacity in a goose inoculated with Ck/Yama/7/04 euthanized on day 5 PI.

Histopathology and immunohistochemistry. Results of histopathologic and virologic examinations are summarized in the Table 5. Histologically, viral antigens were occasionally detected in the feather epidermal cells with or without epidermal necrosis (Fig. 4). Some affected feathers were accompanied by heterophilic and lymphocytic infiltration in the inner feather pulp. Other tissues in the skin were negative for influenza virus by immunohistochemical analysis with the exception of very rare positive reaction in stromal cells in the feather pulp.

Transmission electron microscopy. Ultrastructurally, round, enveloped virions 80 to 100 nm in diameter were observed between feather epidermal cells in both domestic ducks and geese (Fig. 5). Virions budding from cell surface were occasionally observed.

Virus isolation. Virus isolation from the skin was positive in 1 duck and 1 goose inoculated with Ck/Yama/7/04; the viral titers were $10^{3.5}$ and $10^{4.5}$ EID₅₀/g, respectively. All ducks and geese inoculated with Ck/Miya/K11/07 tested positive for the isolation; the viral titers were $10^{2.5}$ – $10^{4.5}$ EID₅₀/g.

Table 5. Histopathology of feathers and virus isolation from the skin in domestic ducks and geese inoculated with 2 H5N1 HPAI viruses.

dpi ^a	Ck/Yama/7/04		Ck/Miya/K11/07	
	Duck	Goose	Duck	Goose
3	+/+ ^b (3.5) ^c	-/- (-)	+/+ (4.5)	+/+ (4.4)
5	+/+ (-)	+/+ (4.5)	-/+ (3.8)	+/+ (2.5)

^a dpi, days postinoculation.

^b Epidermal necrosis of feathers/detected viral antigens; + = positive, - = negative.

^c (Viral titer of the skin expressed as log₁₀EID₅₀/g); - = negative (< 10^{1.5} EID₅₀/g).

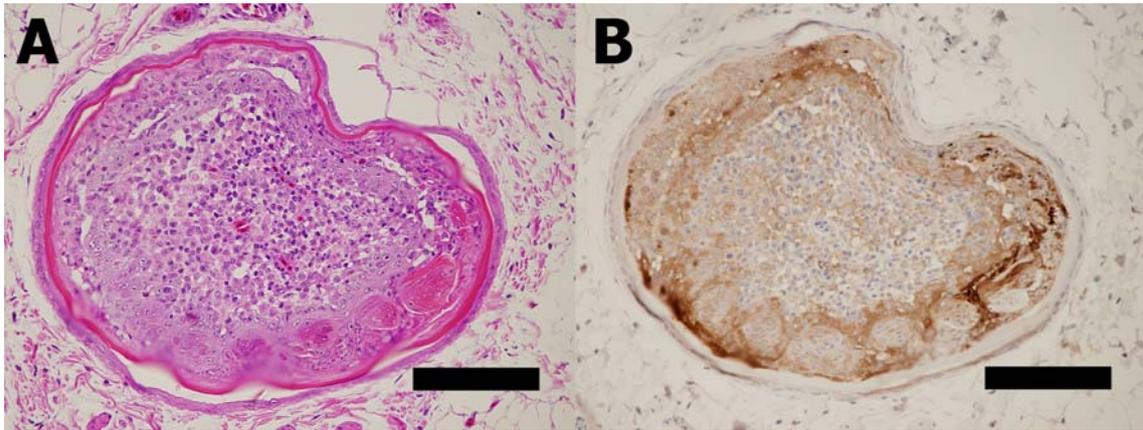


Figure 4. Histopathologic changes in a goose infected with Ck/Miya/K11/07.

A: Outer epidermal necrosis of the feather with inflammation in the inner feather pulp. Hematoxylin and eosin stain. Bar = 120 μ m.

B: Influenza viral antigens detected in feather epidermal cells. Immunohistochemistry. Hematoxylin counterstain. Bar = 120 μ m.

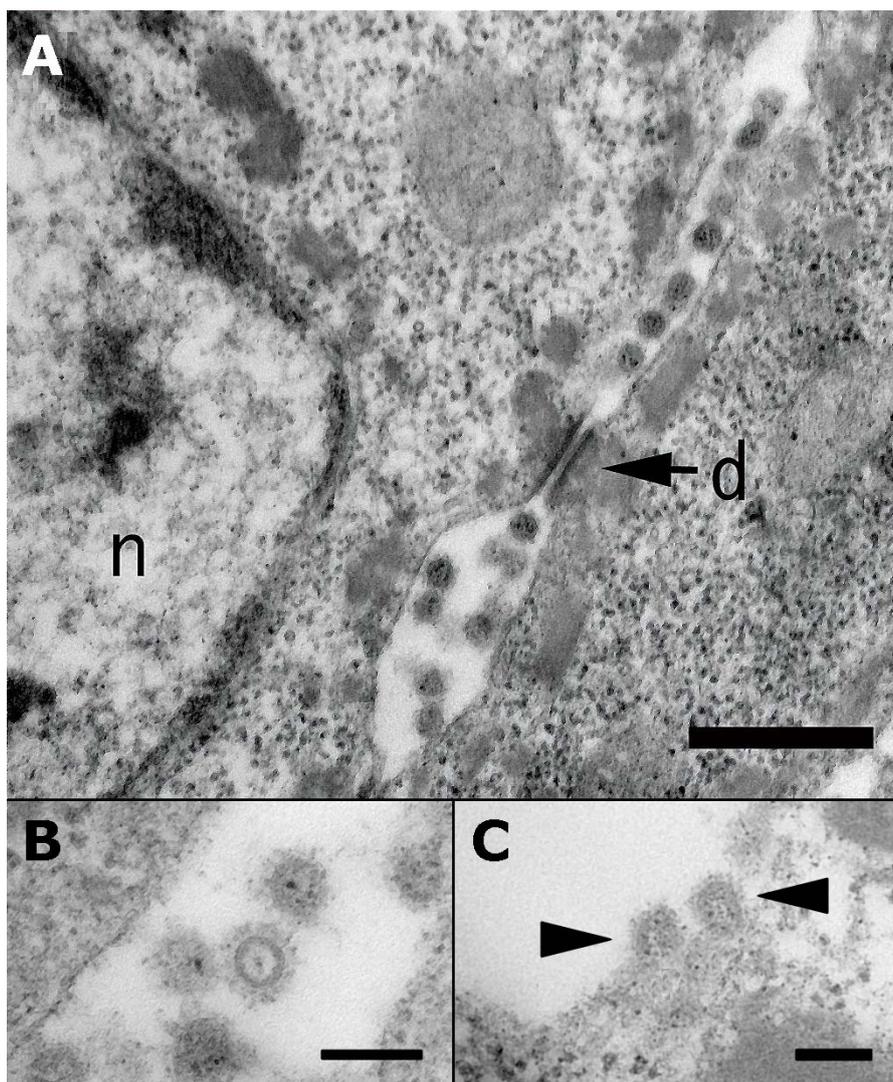


Figure 5. Ultrastructure of feather epidermal cells of a duck infected with Ck/Yama/7/04.

A: Electron microscopy of the feather epidermis showing virions observed between epidermal cells with the desmosome (d) and nucleus (n). Uranyl and lead citrate. Bar = 500 nm.

B: Spherical virion with envelope spikes. Uranyl and lead citrate. Bar = 100 nm.

C: Budding process of virion (arrowheads). Uranyl and lead citrate. Bar = 100 nm.

Study 5

Gross finding. 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.

Histopathology and immunohistochemistry. 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 epithelium of some developing feathers of the adult swan (Fig. 6A). The affected epidermal tissue positive for viral antigens often exhibited necrotic changes. The feathers of the juvenile swan were negative for immunohistochemistry.

RT-PCR. Total RNA from paraffin sections of adult swan's feathers was positive for viral matrix and H5 AI viral genes (Fig. 6B). Direct sequencing 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 RT-PCR.

Virus isolation. Viruses were isolated from feather calami of the adult swan with a virus titer of $10^{3.5}$ EID₅₀/g. The feathers and skin of the juvenile swan were negative for virus isolation.

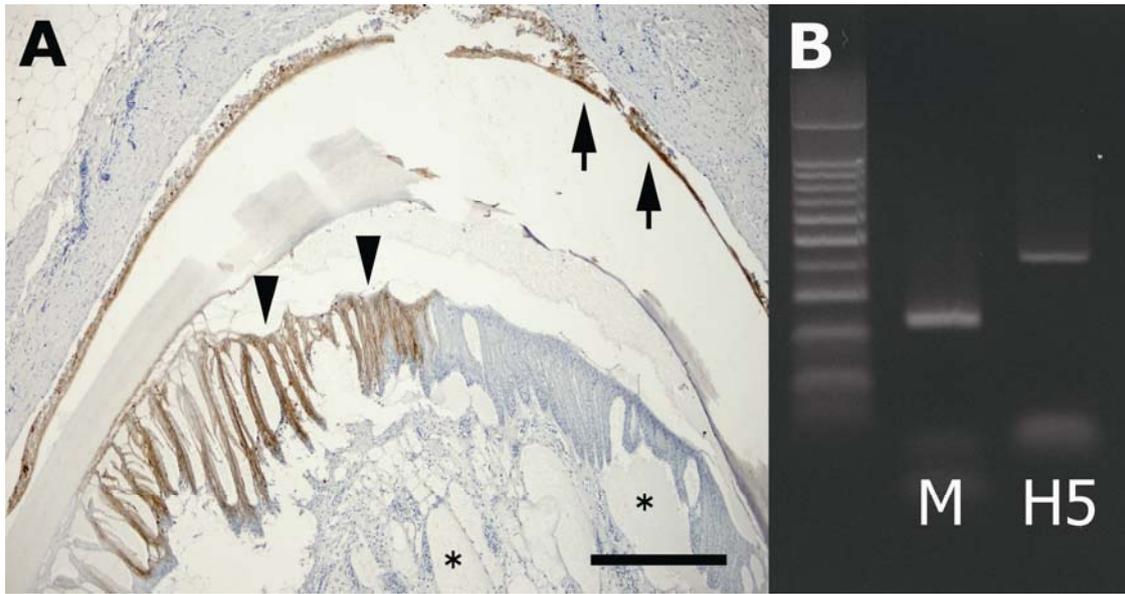


Figure 6. Histopathology and RT-PCR results of feathers of a whooper swan naturally infected with H5N1 HPAI virus.

A: Influenza A virus matrix antigens were detected in the feather epidermis (arrowheads) and necrotic feather follicle wall epithelium (arrows). Clear spaces (asterisks) in the feather tissue are histopathological artifacts caused by freezing preservation. Immunohistochemistry. Hematoxylin counterstain. Bar = 200 μ m.

B: Positive results of RT-PCR for matrix (M) gene (232 bp) and H5 HA gene (424 bp).

Study 6

The infection was confirmed by the immunohistochemistry, virus isolation from cloacal swabs, or HI antibody production in 7 of 9 ducks that were orally inoculated with feathers (Table 6). Clinical signs, gross lesions, and histological changes were similar to those reported in 2- and 4-week-old birds in chapter I .

One bird exhibited persistent corneal opacity from day 4 to 14 PI. Grossly, focal necrosis of the pancreas was observed in the birds on day 3 PI. Histologic lesions with viral antigens were mostly observed on days 3 and 6 PI. These lesions were composed of nonpurulent encephalitis (Fig. 7A), myelitis in the cervical to thoracic spinal cord, and epidermal necrosis of the feather (Figs. 7B and 7C), beak, feather follicle wall, scaled skin of the legs, and tongue. Focal necrosis of the pancreas (Fig. 7D) and heart was mild-to-moderate and accompanied by lymphocytic infiltration. Mild heterophilic keratitis and myositis were rarely observed. The viral antigens were detected in many surface epithelial cells of the bursa of Fabricius in 2 birds that were positive for the virus isolation on day 3 PI. One bird on day 6 PI was also positive for the virus isolation. The virus titers of cloacal swabs ranged from $10^{1.5}$ to $10^{4.5}$ EID₅₀/ml.

According to the serological test, 1 bird on day 6 PI and 2 birds on day 14 PI were positive for HI antibodies.

Table 6. Histopathology, virus isolation, and serology in 2-week-old domestic ducks (A-I) orally inoculated with feathers of a duck infected with Ck/Yama/7/04.

	3 dpi ^a			6 dpi			14 dpi		
	Duck A	Duck B	Duck C	Duck D	Duck E	Duck F	Duck G	Duck H	Duck I
Major histological lesions									
Encephalomyelitis	+ ^b (+) ^c	- ^d	-	-	+(+)	-	+(-)	-	-
Necrosis of the feather epidermis	+(+)	-	-	-	+(+)	-	-	-	-
Necrosis of the beak or legs	-(+)	-	-	-	+(+)	+(+)	-	-	-
Necrosis of the heart	+(+)	-	-	-(+)	+(-)	+(-)	-	-	-
Necrosis of the pancreas	+(+)	-	-	+(+)	-	+(-)	-	-	-
Keratitis	-	-	-	-	+(-)	-	-	-	-
Epithelial hyperplasia of the bursa of Fabricius	+(+)	+(+)	-	-	-	-	-	-	-
Virus isolation from cloacal swab	4.5 ^e	2.5	-	1.5	-	-	-	-	-
Titer of HI antibodies	-	-	-	-	1:20	-	1:40	1:40	-

^a dpi, days postinoculation.

^b + = positive.

^c (virus antigens); + = positive; - = negative.

^d - = negative for histological lesions and virus antigens.

^e Virus titer expressed as log₁₀ EID₅₀/ml; - = negative (<10^{1.5} EID₅₀/ml).

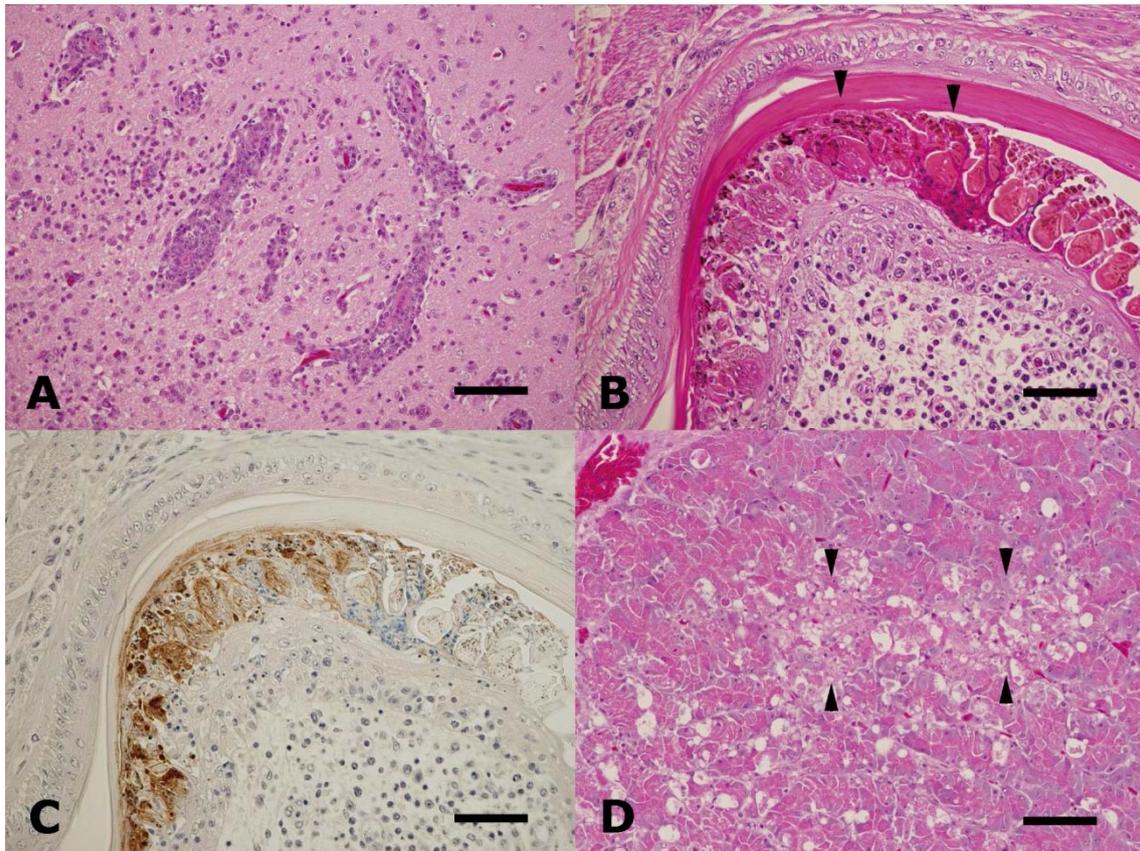


Figure 7. Histopathological findings in domestic ducks orally inoculated with feathers of a duck infected with Ck/Yama/7/04.

A: Cerebrum. Perivascular cuffing with gliosis. Hematoxylin and eosin stain. Bar = 80 μ m.

B-C: Contour feather. (B) Epidermal necrosis (arrowheads) with inflammatory cells in the feather pulp. Hematoxylin and eosin stain. Bar = 50 μ m. (C)

Detected viral antigens. Immunohistochemistry. Hematoxylin counterstain. Bar = 50 μ m.

D: Pancreas. Focal necrosis (arrowheads) and vacuolization of acinar cells. Hematoxylin and eosin stain. Bar = 70 μ m.

Discussion

It was found that 2 different clades of H5N1 HPAI virus isolated in 2004 and 2007 could replicate in the feather epidermal cells of domestic ducks and geese in experimental infection. Ultrastructural observation of virions showing the budding process from the cell surface provided a direct evidence of its replication. The important finding is that histologic feather lesions and virus isolation from the skin were found in inoculated birds that did not exhibit apparent clinical signs. These results indicate that Asian lineage H5N1 HPAI viruses have strong tropism to the feather epidermal tissue of domestic waterfowl. Although 1 goose inoculated with Ck/Yama/7/04 was negative for all examinations, this might have resulted from individual differences in susceptibility or the limited area of the skin used for the examination. In H5N1 HPAI virus epidemics in Southeast Asia, asymptomatic domestic ducks played a key role in regional spread of the virus (20, 40, 58). Given that the feathers can easily drop off, blow away, or be reduced to dust, the result of chapter II indicates that feathers of domestic waterfowl infected with H5N1 HPAI virus can be potential sources of virus shedding, along with their feces and respiratory secretions (26, 66).

In addition, the author found that Asian lineage H5N1 HPAI virus replicated in the feather tissue of whooper swans naturally infected with the virus. Wild swans such as whooper swans (*Cygnus cygnus*) and mute swans (*Cygnus olor*) are frequently found dead or emaciated after H5N1 HPAI virus infection (3, 44, 68, 74). The feather finding in wild swans suggests that H5N1 HPAI virus can be present in the feather tissue of swans and other waterfowl species in natural infection.

The observation of the feather lesion in wild swan is different from the result of a pathological study where naturally infected swans in Germany lacked any pathological changes in the skin (67). German virus isolate belonged to clade 2.2 (64, 67), which is a different viral cluster from clade 2.3.2 in the present study (74). However, the same German isolate replicated in swan feathers in experimental infection (28), indicating that the development of the feather lesion in natural infection does not depend on the phylogenetic factor of H5N1 HPAI

virus. The severity of the disease in each case or the careful examination focusing only on feathers may have attributed to the virus detection in feather of wild swans in the present study.

In the virus transmission study, the HPAI infection was found in 7 of 9 birds that were fed feathers taken from an infected duck. The histologic lesions with viral antigens, virus isolation from cloacal swabs, and HI antibody production confirmed the infection. These findings indicate that infectious viruses released from digested feathers have caused the infection, and that feathers of domestic ducks infected with H5N1 HPAI virus could have infectivity to unaffected birds. In addition, combined with the results of naturally infected swans, the results of chapter II indicate that waterfowl feathers contaminated with the virus may become the source of direct infection for other animals and humans. Human infection of influenza A virus (H5N1) of avian origin in the Republic of Azerbaijan is suspected to have been caused by defeathering of infected wild swans (21). The presence of the virus in wild swan feathers indicates that the feathers, especially when plucked by people, could have the risk for zoonotic infection from infected wild swans. 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. The necrotic feather follicle wall epithelium that may contain the virus can be attached to the surface of the feather sheath.

In conclusion, the studies in chapter II revealed that feathers of waterfowl infected with Asian lineage H5N1 HPAI virus may play a role in virus transmission. At this time, it is unclear to what extent affected feathers contribute to the epidemiology of H5N1 HPAI virus. However, more attention needs to be paid to the possible association of feathers in HPAI field outbreaks caused by Asian lineage H5N1 HPAI virus.

Summary

Experimental infection study was performed to confirm whether or not H5N1 HPAI virus replication in feathers of domestic ducks, which was reported in chapter I, is a common finding to other H5N1 HPAI virus strains or waterfowl species. Domestic ducks and geese were inoculated intranasally with 2 different clades of H5N1 HPAI virus, Ck/Yama/7/04 (clade 2.5) and Ck/Miya/K11/07 (clade 2.2). Together with virus isolation from the skin, virus antigens were detected in the feather epidermal cells of domestic ducks and geese, indicating that virus replication in feathers could occur in those bird species infected with Asian lineage H5N1 HPAI viruses. Spherical virions between the feather epidermal cells were observed by electron microscopic examination. Budding process of virions from the cell surface provided a direct evidence of viral replication in feather epidermal cells. Viral replication in the feather tissue was also found in a dead whooper swan naturally infected with the virus (clade 2.3.2). Furthermore, the author confirmed the viral transmission in domestic ducks by oral inoculation of down feathers taken from a duck infected with the virus. These results suggest that feathers of domestic ducks infected with Asian lineage H5N1 HPAI viruses could have infectivity to birds, animals, and possibly to humans.

Chapter III

Advantages and Disadvantages of H5N1 Highly Pathogenic Avian Influenza Virus Replication in Feathers of Domestic Ducks

Introduction

Free-range domestic ducks can be a key factor in regional spreading of H5N1 HPAI virus (20, 40, 59). Even asymptotically infected domestic ducks can shed the virus from the oral cavity and cloaca for a certain period (26, 59, 66). Therefore, controlling the virus transmission from infected domestic ducks would be important from an epidemiological viewpoint.

The fact that infectious H5N1 HPAI virus can be present in feathers of waterfowl during the infection (chapter II) may have advantages and disadvantages. Since feathers are living tissues that are easily collectible from live birds with minimal damage, feathers may be used as clinical samples for viral detection. Early detection of infected ducks that are shedding the virus would reduce the risk of spreading H5N1 HPAI virus in a region where the virus has been endemic in domestic duck population.

On the other hand, when feathers of infected domestic ducks dropped off the body, feathers containing the virus may serve as fomites in the environment. Some reports investigated the persistence of influenza viruses in environmental media such as bird feces and river water as well as on various environmental surfaces (4, 16, 22, 38, 57, 70, 71, 77, 82). However, despite the profound mortality caused by H5N1 HPAI virus in a number of field chickens and wild birds, its persistence in virus-contaminated organs or tissues, such as carcasses of infected birds, remain to be elucidated (63). Tissue derived from infected birds can become a source of environmental contamination (63). In addition, contaminated tissue can be a source of direct infection for other animals or humans because it can become a target for scavenging wildlife (80), food for domestic animals and humans (5, 29, 60), or because its disposal requires human handling (75).

In chapter III , the author evaluated the usefulness of feathers as clinical samples for early detection of infected domestic ducks. In addition, viral persistence in feathers detached from the body was investigated to discuss the possible role of infective feathers in virus transmission in the environment.

Materials and Methods

Animals. Domestic ducks (*Anas platyrhyncha* var. *domestica*) were obtained from a breeder farm at one day of age and raised using commercial food in an isolated facility. Birds were moved into negative-pressure isolators at Biosafety Level 3–approved laboratories for 1 week of acclimation before virus inoculation. All experimental procedures were approved by the Ethics Committee of the National Institute of Animal Health, Japan (authorization number 07-118).

Viruses. Two H5N1 HPAI viruses, Ck/Miya/K11/07 and A/whooper swan/Akita/1/2008(H5N1) (Ws/Akita/1/08), were used. Ck/Miya/K11/07 is genetically classified as H5 clade 2.2 (6, 74), which belongs to the virus lineage spreading from Asia to Europe, the Middle East, and Africa. Ws/Akita/1/08 was isolated from dead wild swans in 2008 and belongs to H5 clade 2.3.2 (74). The stock virus was propagated for 2 days in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C. The fresh infectious allantoic fluid was harvested and stored at –80°C until use.

Study 7. Duck feathers as clinical samples for virus detection. Three 4-week-old domestic ducks (A–C) were inoculated intranasally with 10^7 EID₅₀ of Ck/Miya/K11/07. Similarly, 3 ducks (D–F) were inoculated intranasally with 10^7 EID₅₀ of Ws/Akita/1/08. I collected 3–5 contour feathers, plucked from the body, and 2 sets of oropharyngeal and cloacal swabs from each duck at 24-hour intervals from days 2 through 10 PI. Samples were examined by commercial rapid tests, virus isolation, and RT-PCR. Feathers were also examined by immunohistochemical testing.

Study 8. Viral persistence in feathers plucked from infected domestic ducks. Two inoculation groups for viruses Ck/Miya/K11/07 and Ws/Akita/1/08 were established. Each group was kept in a single negative-pressure isolator at Biosafety Level 3-approved laboratories during experimental infection. Commercial mineral water (pH 6.7; sodium 6.5mg/L; hardness 30mg/L) was used as drinking water, because the chlorine content in tap water could inactivate the virus (53) and also affect viral persistence in drinking water. Food and drinking water (500 ml) were replenished twice a day. Preinoculation sera from birds

were assessed by HI tests, and were negative for antibodies against the viruses.

Four-week-old domestic ducks ($n = 5$) in each group were inoculated intranasally with 10^7 EID₅₀ of each virus. The birds were monitored and euthanized 3 days after inoculation as active viral replication was observed in the feathers at this timepoint in study 7 performed under the same experimental conditions. Feathers, drinking water, and fecal samples from each group were collected separately in 50-ml polypropylene centrifuge tubes or 125-ml polystyrene storage bottles. At least 100 contour feathers were plucked from the carcass of each bird. Only the feather calamus, which is the basal part of the feather shaft (Fig. 8A), was cut and collected. These were then pooled into 1 sample for each group. A total of 40 ml drinking water was sampled, and the supernatant was recovered after centrifugation at $3,000 \times g$ for 15 min; 5 g fresh feces was collected from the isolator pan and from the intestines of the birds after necropsy. Titration was performed to determine the initial amount of virus present, and viral RNA was determined with RT-PCR on the day of sampling (day 0). Each sample was then divided into 2 parts and placed in incubators set at 4°C or 20°C. Isolation of the infectious virus and viral RNA detection were performed at different time periods from day 3 through day 360. The investigation was deemed complete when each sample produced repeated negative results in virus isolation and viral RNA detection.

Rapid tests. In study 7, on-site rapid tests were performed with a commercial kit, QuickVue Influenza A+B (Quidel Corp., San Diego, CA, USA), which can detect influenza virus nucleoprotein. The first set of swabs was used for rapid tests according to the manufacturer's instructions. For feathers, the author tested 1–2 sticks of the feather calamus (15–30 mg per stick) for rapid tests. Briefly, the author put the calami into the test tube containing attached reagent solution (340 μ l) and chopped them into small pieces with an iris scissor and then placed the test strip in the tube.

Virus isolation. In study 7, a second set of swabs placed in 1 ml of phosphate-buffered saline containing antimicrobial drugs and feather calami were used for virus isolation. In study 8, feather calami, drinking water and feces were examined.

For feathers, the supernatant of 10% (wt/vol) homogenate of 2–4 feather calami was titrated starting with an initial 10-fold dilution in phosphate-buffered saline supplemented with antibiotics. Recovery of the infectious virus was determined by the positivity of allantoic fluids for HA activity or by commercial antigen detection kit QuickVue Influenza A+B. Blind passage of allantoic fluids was performed in study 8 when the chicken embryo was confirmed dead with negative results for virus recovery. The virus titer was expressed as EID₅₀/ ml.

RT-PCR. One-step RT-PCR was performed on the total RNA extracted from the same samples as in virus isolation to detect the H5 AI virus gene (SuperScript One-Step RT-PCR System; Invitrogen, Carlsbad, CA, USA). The 1:10 dilution of RNA templates was used for feather samples in study 7. The primers used were H5–248–270F and H5–671–647R (83). The PCR product was electrophoresed, and viral RNA detection was confirmed by the expected band size of 424 bp.

Immunohistochemistry. Immunohistochemical testing in study 7 was performed to detect AI virus nucleoprotein in the feather tissue by using a rabbit polyclonal antibody (diluted 1:500; ab22285, Abcam Ltd., Cambridge, U.K.). A Histofine Simple Stain MAX-PO (R) kit (Nichirei Inc., Tokyo, Japan) was used as secondary antibodies. The sections were pretreated with 10 mmol of citrate buffer at pH 6.0 in a microwave oven at 500 W for 10 minutes for antigen retrieval.

Results

Study 7

Clinical signs. Domestic ducks inoculated with Ck/Miya/K11/07 did not show any clinical signs except for persistent corneal opacity on day 3 PI or later. Similarly, all Ws/Akita/1/08-inoculated ducks exhibited corneal opacity on day 3 PI or later. Duck F exhibited the neurologic symptoms followed by mortality on day 6 PI.

Rapid tests. Feather samples of all birds were positive for rapid tests. Especially, 5 of 6 birds were positive on days 3 and 4 PI (Table 7 and Fig. 8B). A few swabs were positive for the rapid test. However, the result for the cloacal swabs was considered a possible false-positive reaction because samples taken at the same sampling time were negative for isolation and RT-PCR.

Virus isolation. Viruses were isolated from the oropharyngeal swabs, cloacal swabs, and feather calami of all birds, and feathers tested positive for the virus for a longer period than did the swabs except for samples of duck F (Table 7). Viruses were isolated from the feathers mostly up to day 6 PI. The isolation was also positive in the feathers of duck E on day 10 PI.

The degree and duration of viral replication in the feathers of domestic ducks (A–C) infected with Ck/Miya/K11/07 was similar to results of ducks (D–F) infected with Ws/Akita/1/08 (Table 7). The virus titers of feather calami ranged from $10^{1.7}$ to 10^7 EID₅₀/ml, although there was variability in titer depending on each bird. Relatively high loads of viruses were detected on days 3 and 4 PI. The viral titer of feathers from a dead duck F did not differ greatly from the results of other ducks.

Although the feather samples used for virus isolation differed from those used in rapid tests, the higher virus titers of feathers in each bird appeared to have corresponded to the positive period of feathers for rapid tests.

When the results of isolation were compared between oropharyngeal and cloacal swabs, which indicate viral excretion directly from the body, viruses were isolated mostly from oropharyngeal swabs, while the cloacal swabs were negative.

RT-PCR. The sensitivity of RT-PCR was slightly higher than that of virus

isolation except for the results with cloacal swabs (Table 7).

Immunohistochemistry. Virus antigens were detected in feather epidermal cells from days 3 through 6 PI, and a few stromal cells in the feather pulp on days 3 and 4 PI (Fig. 8C).

Table 7. Results of virus isolation, RT-PCR and rapid tests with the feathers (F), oropharyngeal swabs (O) and cloacal swabs (C) in domestic ducks inoculated with H5N1 HPAI viruses.^a

dpi ^c	Ck/Miya/K11/07									Ws/Akita/1/08								
	Duck A			Duck B			Duck C			Duck D			Duck E			Duck F ^b		
	F	O	C	F	O	C	F	O	C	F	O	C	F	O	C	F	O	C
2	4.2 ^d (+) ^e	2.0 (+)	-(-)	1.7 (+)	1.7 (+)	-(+)	2.8 (+)	2.0 (+)	1.7 (-)	<u>5.5 (+)</u>	<u>4.3 (+)</u>	<u>-(-)</u>	<u>3.5 (+)</u>	5.3 (+)	-(-)	<u>5.0 (+)</u>	4.5 (+)	-(+)
3	<u>6.9 (+)</u>	2.7 (+)	-(-)	<u>4.5 (+)</u>	3.0 (+)	2.5 (+)	<u>4.8 (+)</u>	1.7 (+)	2.8 (-)	<u>6.5 (+)</u>	4.3 (+)	-(-)	5.2 (+)	3.8 (+)	-(-)	<u>6.0 (+)</u>	4.3 (+)	-(-)
4	<u>6.8 (+)</u>	3.7 (+)	1.7 (-)	<u>2.3 (+)</u>	3.5 (+)	1.7 (-)	<u>4.8 (+)</u>	3.3 (+)	-(-)	<u>5.5 (+)</u>	3.8 (+)	-(-)	3.0 (+)	3.5 (+)	-(-)	<u>5.5 (+)</u>	3.3 (+)	-(-)
5	<u>6.5 (+)</u>	-(+)	-(-)	1.7 (+)	-(+)	-(-)	2.5 (+)	1.7 (+)	-(-)	<u>5.5 (+)</u>	2.3 (+)	-(-)	3.2 (+)	-(+)	-(-)	<u>5.8 (+)</u>	3.5 (+)	-(-)
6	<u>5.6 (+)</u>	-(+)	-(-)	-(-)	-(-)	-(-)	4.0 (+)	-(-)	-(-)	<u>4.5 (+)</u>	-(+)	-(-)	5.0 (+)	-(-)	-(-)	<u>7.0 (+)</u>	2.3 (+)	<u>-(-)</u>
7	3.8 (+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)
8	5.8 (+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)
9	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)
10	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	2.7 (+)	-(-)	-(-)	-(-)	-(-)	-(-)

^a Samples positive for rapid tests were underlined.

^b Duck F died on day 6 postinoculation.

^c dpi, days postinoculation.

^d Viral titer expressed as EID₅₀/ml; - = negative for virus isolation (<10^{1.5} EID₅₀/ml).

^e (RT-PCR result); + = positive; - = negative.

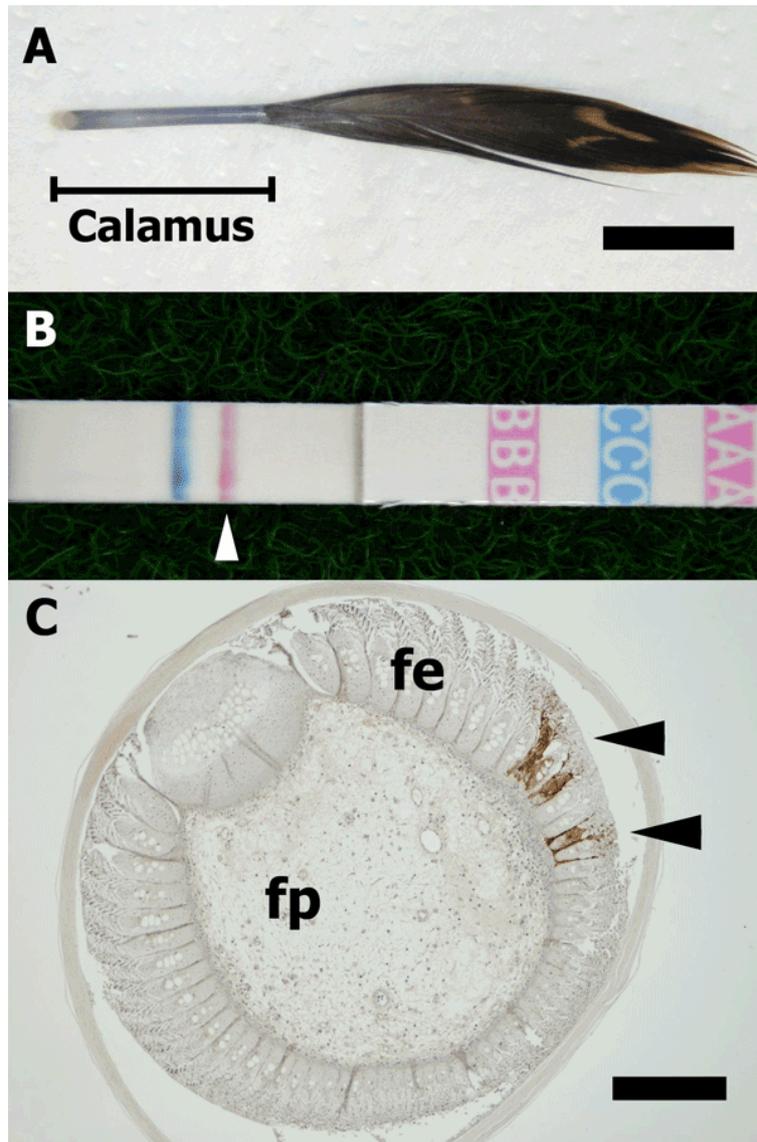


Figure 8. Results of the rapid test and immunohistochemistry using biopsied duck feathers.

A: Developing contour feather. The calamus was used for examination. Bar = 1 cm.

B: Result of the rapid test using feathers. A pink line (arrowhead) indicates a positive result for influenza A virus.

C: Immunohistochemical stain of a biopsied feather composed of feather epidermis (fe) and feather pulp (fp). AI virus nucleoprotein was detected in the feather epidermal cells (arrowheads). Immunohistochemistry. Hematoxylin counterstain. Bar = 200 μ m.

Study 8

Clinical signs observed were corneal opacity in 2 Ck/Miya/K11/07- and in 1 Ws/Akita/1/08-inoculated duck. Other ducks were clinically healthy until their euthanization 3 days after inoculation. The initial titers for the pooled feather calami were $10^{6.5}$ EID₅₀/ml for Ck/Miya/K11/07 and $10^{4.5}$ EID₅₀/ml for Ws/Akita/1/08 (Table 8).

Infectious virus was isolated up to day 160 from feathers stored at 4°C (Table 8). Viral titers of $10^{4.3}$ EID₅₀/ml or greater were detected for 120 days in feathers stored at 4°C. In feathers stored at 20°C, viruses were isolated for a maximum period of 15 days. Although the feather samples showed some variability in viral titer over time, this variability is believed to have resulted from using pooled feathers from a number of infected birds with unequal viral distribution among feathers. RT-PCR using the same samples as for viral isolation revealed that viral RNA in feathers was more stable than viral infectivity (Table 8). Viral RNA was detected in feathers at 4°C up to day 360 (the time of study completion).

Initial viral titers in drinking water were $10^{1.8}$ EID₅₀/ml for Ck/Miya/K11/07 and $10^{2.3}$ EID₅₀/ml for Ws/Akita/1/08. Low viral titers not exceeding $10^{2.3}$ EID₅₀/ml were inconsistently detected in drinking water at 4°C over a maximum period of 30 days. No virus was isolated from drinking water at 20°C from day 3 and from feces on the sampling day (day 0). HPAI virus was only isolated from a fecal sample at 4°C on day 6 with a titer of $10^{2.5}$ EID₅₀/ml. The inconsistent results reported for drinking water and feces over the first 30 days post-sampling is believed to be due to the unequal distribution of a small amount of virus in the samples.

Table 8. Results of virus isolation and RT-PCR in stored samples derived from domestic ducks inoculated with H5N1 HPAI viruses.^a

Day	Ck/Miya/K11/07						Ws/Akita/1/08						
	4°C			20°C			4°C			20°C			
	Feathers	Water	Feces	Feathers	Water	Feces	Feathers	Water	Feces	Feathers	Water	Feces	
0 ^b				6.5 ^c (+) ^d	1.8 (+)	- (-)					4.5 (+)	2.3 (+)	- (-)
3	5.3 (+)	2.3 (+)	- (-)	4.0 (+)	- (+)	- (-)	5.5 (+)	2.3 (-)	- (-)	5.8 (+)	- (+)	- (+)	
6	5.5 (+)	2.3 (+)	2.5 (-)	4.5 (+)	- (+)	- (-)	6.8 (+)	1.8 (+)	- (-)	6.5 (+)	- (+)	- (-)	
10	5.5 (+)	- (+)	- (-)	2.3 (+)	- (-)	- (-)	6.3 (+)	- (-)	- (-)	5.3 (+)	- (-)	- (-)	
15	4.3 (+)	1.8 (+)	- (-)	- (+)	- (-)	- (-)	6.5 (+)	- (-)	- (-)	3.0 (+)	- (-)	- (-)	
20	5.8 (+)	- (-)	- (-)	- (+)	- (-)	- (-)	7.4 (+)	- (-)	- (-)	- (+)	- (-)	- (-)	
30	5.5 (+)	1.8 (+)	- (-)	- (-)	- (-)	- (-)	6.8 (+)	- (+)	- (-)	- (+)	- (-)	- (-)	
40	4.5 (+)	- (-)	- (-)	- (-)	- (-)	- (-)	5.8 (+)	- (+)	- (-)	- (+)	- (-)	- (-)	
50	4.8 (+)	- (-)	- (-)	- (-)	- (-)	- (-)	6.8 (+)	- (-)	- (-)	- (-)	- (-)	- (-)	
60	5.3 (+)	- (-)		- (-)	- (-)		5.5 (+)	- (-)		- (-)	- (-)		
70	4.8 (+)	- (-)		- (-)	- (-)		5.8 (+)	- (-)		- (-)	- (-)		
80	4.5 (+)	- (-)		- (-)	- (-)		6.5 (+)	- (-)		- (-)	- (-)		
100	4.3 (+)	- (-)		- (-)	- (-)		4.8 (+)	- (-)		- (-)	- (-)		
120	5.3 (+)						7.5 (+)						
160	3.3 (+)						3.3 (+)						
200	- (+)						- (+)						
240	- (+)						- (+)						
280	- (+)						- (+)						
320	- (+)						- (+)						
360	- (+)						- (+)						

^a Investigation was completed when each sample produced repeated negative results in virus isolation and RT-PCR.

^b Day 0 means a sampling day from domestic ducks 3 days after inoculation. The initial titers were determined on a sampling day at room temperature and described in cells of 20 °C.

^c Viral titer expressed as EID₅₀/ml; -, negative for virus isolation (<10^{1.6} EID₅₀/ml).

^d (RT-PCR result); +, positive; -, negative.

Discussion

Domestic ducks infected with Asian lineage H5N1 HPAI virus can asymptotically excrete the virus (26, 59, 66). Therefore, early detection of infected ducks that are shedding the virus would reduce the risk of virus transmission. Except for the serum samples for antibody detection, swab and fecal samples are commonly used as clinical samples for virus detection from live birds (56, 69). In study 7, larger amounts of viruses were isolated for a longer time from feather calami than from swabs of infected domestic ducks. Therefore, these results suggest that the feather calamus can be considered useful clinical samples for surveillance or diagnostic examination of H5N1 HPAI virus in domestic ducks. The epidermis, the outer layer of the feather, is a tissue that has poor host immune response against viral replication (19). As has been observed in virus isolation, viruses may be able to survive longer in differentiated epidermal tissue such as contour feathers.

Immunohistochemical analysis confirmed the presence of viral antigens in host cells that constitute the feather shaft. This finding indicates that, by using tissue samples such as biopsied feathers for antigen detection, people can make use of the virus proteins being produced in infected host cells, as well as those in the virions.

The sensitivity of the rapid tests was not adequate for swabs, a finding similar to that of other studies (14, 81). However, positive results for rapid tests of feather samples only may shed light on the on-site field detection of H5N1 HPAI in asymptomatic domestic ducks. When virus shedding from the oral cavity and cloaca of domestic ducks is maintained at a low level of viral load during the infection, selecting the sample with higher viral load and antigens in tissues, such as feathers, can increase the detection rate obtained from on-site examination. The results in study 6 show the potential of feathers as candidates for early H5N1 HPAI virus detection.

Nevertheless, as reported in chapter II, the potential risk of viral transmission through waterfowl feathers cannot be disregarded. The results of study 8 further emphasize the possible role of feathers in environmental contamination. When

feathers detach from the body during the active phase of viral replication, the infectious virus can be recovered from the stored feather tissue for a time period dependent on the storage temperature. The most interesting finding is that $10^{4.3}$ EID₅₀/ml or more of infectious virus persisted for at least 120 days in feather tissue stored at 4°C. Viral infectivity persisted up to 15 days in feathers stored at 20°C, and the virus could be detected for the longest duration in feathers stored at both 4°C and 20°C compared to feces from infected birds or in water contaminated during the experimental infection. Whereas contaminated water or feces can be quickly diluted in the environment, contaminated tissues such as feathers with high viral loads can exist as solid materials in the field. Direct environmental contamination from these infected feathers may be limited to a local area because of the nature of solid materials, but could also occur in waterfowl habitats where H5N1 HPAI virus exists. Similarly, other contaminated tissues such as the carcass pose a possible risk for environmental contamination.

The results suggest that infected feathers detached from the body for whatever reason could contaminate the environment. The following are some possible situations: mass culling of infected ducks after outbreaks of H5N1 HPAI virus, defeathering of domestic ducks with asymptomatic infection at slaughter, and trading of unprocessed waterfowl feathers leading to virus spread via contaminated feathers. Furthermore, although clinical symptoms such as feather loss were not observed in domestic ducks in the experimental infection, molting can be induced in birds by stress or critical disease conditions in the field.

From another perspective, the epidemiological importance of contaminated feathers may be their potential as a source of direct infection for other animals or humans. Down feathers plucked from an infected duck had the infectivity to healthy domestic ducks orally inoculated with those affected feathers (chapter II). Avian origin influenza A virus (H5N1) infection in humans in the Republic of Azerbaijan is suspected to have been caused by defeathering of infected wild swans (21). The author reported that H5N1 HPAI virus can replicate in wild swan feathers, demonstrating the zoonotic potential of feathers as a source of H5N1 HPAI virus infection (chapter II). In addition to waterfowl, histological evidence of the presence of H5N1 HPAI virus in feathers has been reported for

chickens experimentally infected with the virus (86).

The lack of a standard protocol for analyzing the persistence of influenza virus in the environment makes it difficult to compare results from related studies. Several factors such as temperature, relative humidity, salinity, and pH can influence the persistence of AI virus in a variety of media such as distilled water, allantoic fluids, and river water (8, 9, 61, 62, 76, 77, 82). Similar to the studies above, a low temperature of 4°C effectively increased viral persistence in feathers and drinking water. Although the actual effect of relative humidity was not determined in the present study, all samples are suspected to have been maintained in relatively high humidity as they were completely sealed in a tube or bottle. As has been observed with water and fecal samples in the present study, the initial amount of virus in the samples can also affect the experimental result in terms of viral persistence.

One limitation of the present study is that the data were collected under experimental conditions may not be applicable to the field conditions. Further studies are needed to clarify the extent of viral transmission by feathers contaminated with H5N1 HPAI virus.

In conclusion, it was reported that the feathers of domestic ducks infected with Asian lineage H5N1 HPAI virus can be used as useful clinical sample for detection of infected birds. At the same time, feathers of infected ducks can also be a source of environmental contamination. The possible epidemiological consequence is that contaminated feathers may manifest as fomites containing high viral loads in the environment.

Summary

The author evaluated the usefulness of plucked contour feathers of domestic ducks as clinical samples to detect H5N1 HPAI virus. Feather calami and swabs of experimentally infected ducks were examined for 10 days after inoculation. Larger amounts of viruses were isolated for a longer time from feather calami than from swabs. The higher virus titers of feathers in each bird appeared to correspond to the virus positive period of feathers for rapid tests. The results indicate that feather samples are useful candidates for the detection of the virus in domestic ducks in terms of the recoverable viral load and positive duration. The author also investigated H5N1 HPAI virus persistence in feathers detached from bodies of infected ducks to evaluate their potential risk for environmental contamination. Four-week-old domestic ducks were inoculated with H5N1 HPAI viruses. Feathers, drinking water, and feces were collected on day 3 PI and stored at 4°C or 20°C. Persistence of the infectivity of the virus in samples was investigated for 360 days by virus isolation. Infectious viruses persisted for the longest period in feathers compared with drinking water and feces, at both 4°C and 20°C. Viral infectivity persisted in the feathers for 160 days at 4°C and for 15 days at 20°C. Viral titers of $10^{4.3}$ EID₅₀/ml or greater were detected for 120 days in feathers stored at 4°C. These results indicate that feathers detached from domestic ducks infected with H5N1 HPAI virus can be a source of environmental contamination, and may function as fomites with high viral loads in the environment.

Conclusions

Since the first outbreak occurred in poultry in Hong Kong in 1997, Asian lineage H5N1 HPAI virus has spread from Asia to Europe and Africa, resulting in serious economic losses in many countries. Several reports of virus detection in wild and domestic waterfowl indicate that waterfowl can serve as carriers and amplifying hosts of this virus. Therefore, the author hypothesized that the detailed analysis on viral replication in waterfowl would provide information to reduce the risk of viral transmission from infected waterfowl.

In the present thesis, the author demonstrated that Asian lineage H5N1 HPAI virus could replicate in the feather tissue of waterfowl, and that feathers could be a possible route of transmission of virus shedding from infected birds. It was also shown that feathers of infected domestic ducks contained viruses infectious to other birds and mammals, and possibly to humans as a zoonotic infection. At the same time, the results indicate that feathers are useful clinical samples for detection of the virus in infected domestic ducks. Finally, it was demonstrated that contaminated feathers of infected domestic ducks may serve as fomites with high viral loads in the environment, leading to a possible spread of the disease. In conclusion, the author presented a new viewpoint on an epidemiological role of waterfowl feathers in H5N1 HPAI virus transmission. The details of 3 chapters are outlined as follows.

In chapter I, the author examined the pathogenesis in domestic ducks inoculated with Japanese H5N1 HPAI virus Ck/Yama/7/04. The virus caused mortality, neurologic symptoms, and pathologic changes such as the encephalitis, pancreatic necrosis, corneal opacity and epidermal necrosis of the feathers. High mortality in ducklings indicates that the age of birds is a factor influencing on the viral replication. The feather lesions caused by virus replication raise the potential of feathers as new route of virus shedding from infected waterfowl. These findings suggest that feathers of ducks infected with H5N1 HPAI can be a potential source of spread of the virus to unaffected birds in nature.

In chapter II, the experimental infection was performed to examine whether the feather lesion observed in the previous chapter is common to other H5N1 HPAI

virus strains or other waterfowl species. Two different H5N1 HPAI virus clades of Ck/Yama/7/04 and Ck/Miya/K11/07 replicated in the feather epidermis of domestic ducks and geese in experimental infection, indicating that virus replication in feathers can be common among waterfowl species infected with Asian lineage H5N1 HPAI viruses. Electron microscopic examination provided the direct evidence of viral replication in feather epidermal cells. Viral replication in the feather tissue was also found in whooper swans naturally infected with the virus. In addition, it was confirmed the viral transmission in domestic ducks by oral inoculation of down feathers taken from a duck infected with the virus. These results indicate that feathers of domestic ducks infected with Asian lineage H5N1 HPAI viruses could be infectious to birds, mammals, and possibly to humans.

In chapter III, it was reported that feathers of infected domestic ducks can be useful samples for virus detection as well as their swabs. In the experimental infection using 2 H5N1 HPAI viruses and domestic ducks, feather calami and swabs were examined by commercial rapid tests, virus isolation, RT-PCR and immunohistochemistry. As a result of virus isolation, larger amounts of viruses were isolated for longer period from feathers than from the swabs. Therefore, feathers are considered to be useful clinical samples for surveillance or diagnostic examination of H5N1 HPAI virus in domestic ducks.

The author also investigated the viral persistence in feathers detached from bodies of domestic ducks infected with H5N1 HPAI viruses to evaluate the possible environmental contamination by infective feathers. Feathers were plucked from experimentally infected domestic ducks, stored at 4°C or 20°C, and examined by virus isolation and RT-PCR for 360 days. Drinking water and feces were included in this study for comparison. Infectious viruses persisted for the longest period in feather calami compared with drinking water and feces at both 4°C and 20°C. Viral infectivity persisted in the feathers for 160 days at 4°C and for 15 days at 20°C. Viral RNA in feathers was more stable than the infectivity. These results indicate that feathers detached from domestic ducks infected with H5N1 HPAI virus can function as fomites containing high viral loads in the environment.

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References

1. **Alexander, D. J., G. Parsons, and R. J. Manvell.** 1986. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol.* **15**:647–662.
2. **Alexander, D. J.** 2000. A review of avian influenza in different bird species. *Vet. Microbiol.* **74**:3–13.
3. **Alexander, D. J.** 2007. Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. *Avian Dis.* **51**:161–166.
4. **Bean, B., B. M. Moore, B. Sterner, L. R. Peterson, D. N. Gerding, and H. H. Balfour, Jr.** 1982. Survival of influenza viruses on environmental surfaces. *J. Infect. Dis.* **146**:47–51.
5. **Beigel, J. H., J. Farrar, A. M. Han, F. G. Hayden, R. Hyer, M. D. de Jong, S. Lochindarat, T. K. Nguyen, T. H. Nguyen, T. H. Tran, A. Nicoll, S. Touch, and K. Y. Yuen.; The writing committee of the world health organization (WHO) consultation on human influenza A/H5.** 2005. Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.* **353**:1374–1385.
6. **Bragstad, K., P. H. Jørgensen, K. Handberg, A. S. Hammer, S. Kabell, and A. Fomsgaard.** 2007. First introduction of highly pathogenic H5N1 avian influenza A viruses in wild and domestic birds in Denmark, Northern Europe. *Virology* **4**:43.
7. **Brown, J. D., D. E. Stallknecht, J. R. Beck, D. L. Suarez, and D. E. Swayne.** 2006. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. *Emerg. Infect. Dis.* **12**:1664–1670.
8. **Brown, J. D., D. E. Swayne, R. J. Cooper, R. E. Burns, and D. E. Stallknecht.** 2007. Persistence of H5 and H7 avian influenza viruses in water. *Avian Dis.* **51**:285-289.
9. **Brown, J. D., G. Goekjian, R. Poulson, S. Valeika, and D. E. Stallknecht.** 2009. Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature. *Vet. Microbiol.* **136**:20–26.
10. **Calnek, B. W., H. K. Adldinger, and D. E. Kahn.** 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from

Marek's disease. *Avian Dis.* **14**:219–233.

11. Chen, H., G. J. D. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. Peiris, and Y. Guan Y. 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. *Nature.* **436**:191–192.

12. Chen, H., Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, and Y. Kawaoka. 2006. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. *J. Virol.* **80**:5976–5983.

13. Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, O. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nature Med.* **7**:1306–1312.

14. Chua, T. H., T. M. Ellis, C. W. Wong, Y. Guan, S. X. Ge, G. Peng, C. Lamichhane, C. Maliadis, S. W. Tan, P. Selleck, and J. Parkinson. 2007. Performance evaluation of five detection tests for avian influenza antigen with various avian samples. *Avian Dis.* **51**:96–105.

15. Cooley, A. J., H. van Campen, M. S. Philpott, B. C. Easterday, and V. S. Hinshaw. 1989. Pathological lesions in the lungs of ducks infected with influenza A viruses. *Vet. Pathol.* **26**:1–5.

16. Dovas, C. I., M. Papanastassopoulou, M. P. Georgiadis, E. Chatzinasiou, V. I. Maliogka, and G. K. Georgiades. 2010. Detection and quantification of infectious avian influenza A (H5N1) virus in environmental water by using real-time reverse transcription-PCR. *Appl. Environ. Microbiol.* **76**:2165–2174.

17. Ellis, T. M., R. B. Bousfield, L. A. Bissett, K. C. Dyrting, G. S. Luk, S. T. Tsim, K. Sturm-Ramirez, R. G. Webster, Y. Guan, and J. S. Malik Peiris. 2004. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathol.* **33**:492–505.

18. Feare, C. J. 2007. The role of wild birds in the spread of HPAI H5N1. *Avian Dis.* **51**:440–447.

19. Flint, S. J., L. W. Enquist, V. R. Racaniello, and A. M. Skalka. 2004. Patterns of infection. *In: Principles of virology: molecular biology, pathogenesis, and control of animal viruses.* 2nd ed. Washington, D. C. American Society for

Microbiology, p. 596–621.

20. **Gilbert, M., P. Chaitaweesub, T. Parakamawongsa, S. Premashthira, T. Tiensin, W. Kalpravidh, H. Wagner, and J. Slingenbergh.** 2006. Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerg. Infect. Dis.* **12**:227–234.
21. **Gilsdorf, A., N. Boxall, V. Gasimov, I. Agayev, F. Mammadzade, P. Ursu, E. Gasimov, C. Brown, S. Mardel, D. Jankovic, G. Pimentel, I. A. Ayoub, E. M. Ellassal, C. Salvi, D. Legros, C. Pessoa da Silva, A. Hay, R. Andraghetti, G. Rodier, and B. Ganter.** 2006. Two clusters of human infection with influenza A/H5N1 virus in the Republic of Azerbaijan, February-March 2006. *Eurosurveillance.* **11**:122–126.
22. **Graiver, D. A., C. L. Topliff, C. L. Kelling, and S. L. Bartelt-Hunt.** 2009. Survival of the avian influenza virus (H6N2) after land disposal. *Environ. Sci. Technol.* **43**:4063–4067.
23. **Harrison, G. J.** 1986. Feather disorders. *Vet. Clin. North Am. Small Anim. Pract.* **14**:179-199.
24. **Hayden, F., and A. Croisier.** 2005. Transmission of avian influenza viruses to and between humans. *J. Infect. Dis.* **192**:1311–1314.
25. **Hinshaw, V. S., R. G. Webster, and B. Turner.** 1979. Water-bone transmission of influenza A viruses? *Intervirology.* **11**:66–68.
26. **Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc. Natl. Acad. Sci. U. S. A.* **102**:10682–10687.
27. **Isoda, N., Y. Sakoda, N. Kishida, G. R. Bai, K. Matsuda, T. Umemura, and H. Kida.** 2006. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. *Arch. Virol.* **151**:1267–1279.
28. **Kalthoff, D., A. Breithaupt, J. P. Teifke, A. Globig, T. Harder, T. C. Mettenleiter, and M. Beer.** 2008. Highly pathogenic avian influenza virus

(H5N1) in experimentally infected adult mute swans. *Emerg. Infect. Dis.* **14**:1267–1270.

29. Keawcharoen, J., K. Oraveerakul, T. Kuiken, R. A. Fouchier, A. Amonsin, S. Payungporn, S. Noppornpanth, S. Wattanodorn, A. Theambooniers, R. Tantilertcharoen, R. Pattanarangsarn, N. Arya, P. Ratanakorn, D. M. Osterhaus, and Y. Poovorawan. 2004. Avian influenza H5N1 in tigers and leopards. *Emerg. Infect. Dis.* **10**:2189–2191.

30. Kida, H., R. Yanagawa, and Y. Matsuoka. 1980. Duck influenza lacking evidence of disease signs and immune response. *Infect. Immun.* **30**:547–553.

31. Kilpatrick, A. M., A. A. Chmura, D. W. Gibbons, R. C. Fleischer, P. P. Marra, and P. Daszak. 2006. Predicting the global spread of H5N1 avian influenza. *Proc. Natl. Acad. Sci. U. S. A.* **103**:19368–19373.

32. Kishida, N., Y. Sakoda, N. Isoda, K. Matsuda, M. Eto, Y. Sunaga, T. Umemura, and H. Kida. 2005. Pathogenicity of H5 influenza viruses for ducks. *Arch. Virol.* **150**:1383–1392.

33. Komar, N., and B. Olsen. 2008. Avian influenza virus (H5N1) mortality surveillance. *Emerg. Infect. Dis.* **14**:1176–1168.

34. Kwon, Y. K., S. J. Joh, M. C. Kim, H. W. Sung, Y. J. Lee, J. G. Choi, E. K. Lee, and J. H. Kim. 2005. Highly pathogenic avian influenza (H5N1) in the commercial domestic ducks of South Korea. *Avian Pathol.* **34**:367–370.

35. Lee, C. W., D. L. Suarez, T. M. Tumpey, H. W. Sung, Y. K. Kwon, Y. J. Lee, J. G. Choi, S. J. Joh, M. C. Kim, E. K. Lee, J. M. Park, X. Lu, J. M. Katz, E. Spackman, D. E. Swayne, and J. H. Kim. 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J. Virol.* **79**:3692–3702.

36. Li, K. S., Y. Guan, J. Wang, G. J. D. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature.* **430**:209–213.

37. Liu, J., H. Xiao, F. Lei, Q. Zhu, K. Qin, X. W. Zhang, X. L. Zhang, D.

- Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, and G. F. Gao.** 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. *Science*. **309**:1206.
- 38. Lu, H., A. E. Castro, K. Pennick, J. Liu, Q. Yang, P. Dunn, D. Weinstock, and D. Henzler.** 2003. Survival of avian influenza virus H7N2 in SPF chickens and their environments. *Avian Dis.* **47**:1015–1021.
- 39. Markwel, I. D. D., and K. F. Shortridge.** 1982. Possible waterborne transmission and maintenance of influenza viruses in domestic ducks. *Appl. Environ. Microbiol.* **43**:110–115.
- 40. Martin, V., L. Sims, J. Lubroth, D. Pfeiffer, J. Slingenbergh, and J. Domenech.** 2006. Epidemiology and ecology of highly pathogenic avian influenza with particular emphasis on South East Asia. *Dev. Biol (Basel)*. **124**:23–36.
- 41. Mase, M., J. H. Kim, Y. J. Lee, K. Tsukamoto, T. Imada, K. Imai, and S. Yamaguchi.** 2005. Genetic comparison of H5N1 influenza A viruses isolated from chickens in Japan and Korea. *Microbiol. Immunol.* **49**:871–874.
- 42. Mase, M., K. Tsukamoto, T. Imada, K. Imai, N. Tanimura, K. Nakamura, Y. Yamamoto, T. Hitomi, T. Kira, T. Nakai, M. Kiso, T. Horimoto, Y. Kawaoka, and S. Yamaguchi.** 2005. Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. *Virology*. **332**:167–176.
- 43. Melville, D. S., and K. F. Shortridge.** 2006. Spread of H5N1 avian influenza virus: an ecological conundrum. *Lett. Appl. Microbiol.* **42**:435-437.
- 44. Nagy, A., J. Machova, J. Hornickova, M. Tomci, I. Nagl, B. Horyna, and I. Holko.** 2007. Highly pathogenic avian influenza virus subtype H5N1 in Mute swans in the Czech Republic. *Vet. Microbiol.* **120**:9–16.
- 45. Nakatani, H., K. Nakamura, Y. Yamamoto, M. Yamada, and Y. Yamamoto.** 2005. Epidemiology, pathology, and immunohistochemistry of layer hens naturally affected with H5N1 highly pathogenic avian influenza in Japan. *Avian Dis.* **49**:436–441.
- 46. Nishiguchi, A., T. Yamamoto, T. Tsutsui, T. Sugizaki, M. Mase, K. Tsukamoto, T. Ito, and N. Terakado.** 2005. Control of an outbreak of highly pathogenic avian influenza, caused by the virus sub-type H5N1, in Japan in 2004. *Rev. Sci. Tech. Off. Int. Epizoot.* **24**:933–944.
- 47. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenström, A. D. Osterhaus,**

- and **R. A. Fouchier**. 2006. Global patterns of influenza A virus in wild birds. *Science*. **312**:384–388.
- 48. Pantin-Jackwood, M. J., D. L. Suarez, E. Spackman, and D. E. Swayne**. 2007. Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks. *Virus Res*. **130**:151–161.
- 49. Perkins, L. E. L., and D. E. Swayne**. 2001. Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. *Vet. Pathol*. **38**:149–164.
- 50. Perkins, L. E. L., and D. E. Swayne**. 2002. Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. *Avian Dis*. **46**:53–63.
- 51. Perkins, L. E. L., and D. E. Swayne**. 2003. Comparative susceptibility of selected avian and mammalian species to a Hong Kong-origin H5N1 high-pathogenicity avian influenza virus. *Avian Dis*. **47**:956–967.
- 52. Rappole, J. H., and Z. Hubálek**. 2006. Birds and influenza H5N1 virus movement to and within North America. *Emerg. Infect. Dis*. **12**:1486–1492.
- 53. Rice, E. W., N. J. Adcock, M. Sivaganesan, J. D. Brown, D. E. Stallknecht, and D. E. Swayne**. 2007. Chlorine inactivation of highly pathogenic avian influenza virus (H5N1). *Emerg. Infect. Dis*. **13**:1568–1570.
- 54. Ritchie, B. W., F. D. Niagro, K. S. Latimer, W. L. Steffens, D. Pest, J. Ancona, and P. D. Lukert**. 1991. Routes and prevalence of shedding of psittacine beak and feather disease virus. *Am. J. Vet. Res*. **52**:1804–1809.
- 55. Ritchie, B. W.** 1995. Papovaviridae. *In: Avian viruses: function and control*. Lake Worth, FL. Wingers Publishing Inc. p.127–170.
- 56. Rose, K., S. Newman, M. Uhart, and J. Lubroth**. 2006. *In: Wild bird highly pathogenic avian influenza surveillance*. Food and Agriculture Organization of the United Nations, Animal Production and Health Manual No. 4. Rome, Italy. Available at: <ftp://ftp.fao.org/docrep/fao/010/a0960e/a0960e00.pdf> (Accessed 10 June 2010)
- 57. Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster**. 1998. Characterization of avian H5N1

influenza viruses from poultry in Hong Kong. *Virology*. **252**:331–342.

58. Sims, L. D, J. Domenech, C. Benigno, S. Kahn, A. Kamata, J. Lubroth, V. Martin, and P. Roeder. 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. *Vet. Rec.* **157**:159–164.

59. Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster. 2006. Domestic ducks and H5N1 influenza epidemic, Thailand. *Emerg. Infect. Dis.* **12**:575–581.

60. Songserm, T., A. Amonsin, R. Jam-on, N. Sae-Heng, N. Pariyothorn, S. Payungporn, A. Theamboonlers, S. Chutinimitkul, R. Thanawongnuwech, and Y. Poovorawan. 2006. Fatal avian influenza A H5N1 in a dog. *Emerg. Infect. Dis.* **12**:1744–1747.

61. Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. 1990. Persistence of avian influenza viruses in water. *Avian Dis.* **34**:406–411.

62. Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. 1990. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. *Avian Dis.* **34**:412–418.

63. Stallknecht, D. E., and J. D. Brown. 2009. Tenacity of avian influenza viruses. *Rev. Sci. Tech.* **28**:59–67.

64. Starick, E., Beer, M., Hoffmann, B., Staubach, C., Werner, O., Globig, A., Strebelow, G., Grund, C., Durban, M., Conraths, F. J., Mettenleiter, T. and Harder, T. 2008. Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus. *Vet. Microbiol.* **128**:243–252.

65. Sturm-Ramirez, K. M., T. Ellis, B. Bousfield, L. Bissett, K. Dyrting, J. E. Rehg, L. Poon, Y. Guan, M. Peiris, and R. G. Webster. 2004. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J. Virol.* **78**:4892–4901.

66. Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster. 2005. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? *J. Virol.* **79**:11269–11279.

- 67. Teifke, J. P., R. Klopfleisch, A. Globig, E. Starick, B. Hoffmann, P. U. Wolf, M. Beer, T. C. Mettenleiter, and T. C. Harder.** 2007. Pathology of natural infections by H5N1 highly pathogenic avian influenza virus in mute (*Cygnus olor*) and whooper (*Cygnus cygnus*) swans. *Vet. Pathol.* **44**:137–143.
- 68. Terregino, C., A. Milani, I. Capua, A. M. Marino, and N. Cavaliere.** 2006. Highly pathogenic avian influenza H5N1 subtype in mute swans in Italy. *Vet. Rec.* **158**:491.
- 69. Terregino, C.** 2009. *In: Avian influenza and newcastle disease. A field and laboratory manual.* I. Capua, and D. J. Alexander. eds. Milan, Italy. Springer. p. 35–44.
- 70. Thomas, Y., G. Vogel, W. Wunderli, P. Suter, M. Witschi, D. Koch, C. Tapparel, and L. Kaiser.** 2008. Survival of influenza virus on banknotes. *Appl. Environ. Microbiol.* **74**:3002–3007.
- 71. Tiwari, A., D. P. Patnayak, Y. Chander, M. Parsad, and S. M. Goyal.** 2006. Survival of two avian respiratory viruses on porous and nonporous surfaces. *Avian Dis.* **50**:284–287.
- 72. Tollis, M., and L. di Trani.** 2002. Recent developments in avian influenza research: epidemiology and immunoprophylaxis. *Vet. J.* **164**:202–215.
- 73. Tumpey, T. M., D. L. Suarez, L. E. L. Perkins, D. A. Senne, J. G. Lee, Y. J. Lee, I. P. Mo, H. W. Sung, and D. E. Swayne.** 2002. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. *J. Virol.* **76**:6344–6355.
- 74. Uchida, Y., M. Mase, K. Yoneda, A. Kimura, T. Obara, S. Kumagai, T. Saito, Y. Yamamoto, K. Nakamura, K. Tsukamoto, and S. Yamaguchi.** 2008. Highly pathogenic avian influenza virus (H5N1) isolated from whooper swans, Japan. *Emerg. Infect. Dis.* **14**:1427–1429.
- 75. United States Environmental Protection Agency.** 2006. Disposal of domestic birds infected by avian influenza – an overview of considerations and options. Washington, District of Columbia. Available at: <http://www.epa.gov/osw/inforesources/pubs/flu.pdf> (Accessed 10 June 2010)
- 76. Weber, T. P., and N. I. Stilianakis.** 2008. Inactivation of influenza A viruses in the environment and modes of transmission: a critical review. *J. Infect.*

57:361–373.

77. Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti. 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology*. **84**:268–278.

78. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**:152–179.

79. Webster, R. G., M. Peiris, H. Chen, and Y. Guan. 2006. H5N1 outbreaks and enzootic influenza. *Emerg. Infect. Dis.* **12**:3–8.

80. Wobeser, G., and A. G. Wobeser. 1992. Carcass disappearance and estimation of mortality in a simulated die-off of small birds. *J. Wildl. Dis.* **28**:548–554.

81. Woolcock, P. R., and C. J. Cardona. 2005. Commercial immunoassay kits for the detection of influenza virus type A: evaluation of their use with poultry. *Avian Dis.* **49**:477–481.

82. World Health Organization. 2006. Review of latest available evidence on risks to human health through potential transmission of avian influenza (H5N1) through water and sewage. Last updated 10/10/2007, Geneva, Switzerland. Available at: http://www.who.int/water_sanitation_health/emerging/avianflu/en/ (Accessed 10 June 2010)

83. World Health Organization. 2007. Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases. Geneva, Switzerland. Available at: http://www.who.int/csr/disease/avian_influenza/guidelines/labtests/en/index.html (Accessed 10 June 2010)

84. World Organisation for Animal Health. 2004. Highly pathogenic avian influenza. *In: Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees)*, 5th ed. vol. 1, pp. 258–269.

85. World Organisation for Animal Health. 2008. Update on highly pathogenic avian influenza in animals (type H5 and H7), Follow-up report No.:4
OIE Ref: 7082 Available at:
www.oie.int/wahis/reports/en_fup_0000007082_20080602_170028.pdf. (Accessed

10 June 2010)

86. Yamamoto, Y., K. Nakamura, M. Yamada, and M. Mase. 2010. Comparative pathology of chickens and domestic ducks experimentally infected with highly pathogenic avian influenza viruses (H5N1) isolated in Japan in 2007 and 2008. *J. Agric. Res. Quart.* **44**:73-80.

87. Yu, M., Z. Yue, P. Wu, D. Y. Wu, J. A. Mayer, M. Medina, R. B. Wideltitz, T. X. Jiang, and C. M. Chuong. 2004. The developmental biology of feather follicles. *Int. J. Dev. Biol.* **48**:181-191.

Summary in Japanese (和文要旨)

1997年、アジア系統 H5N1 亜型ウイルスによる高病原性鳥インフルエンザ (HPAI) が香港の家禽に発生した。その後、本ウイルスは、アジアからヨーロッパやアフリカへ伝播し、多くの国々に深刻な経済的損失をもたらした。この H5N1 HPAI ウイルスの主要な伝播経路として、野鳥や家禽の移動、汚染鶏肉の流通、生鳥市場等の関連が指摘されている。特に、東南アジアにおける流行には、鶏群における HPAI の発生と水生家禽との疫学的関連が報告されている。さらに、斃死した野生水禽からの H5N1 HPAI ウイルス分離が多数報告されている。以上の知見から、著者は、水禽の体内でのウイルス増殖に関する解析が、水禽を介する H5N1 HPAI ウイルスの伝播経路の解明に寄与すると考え研究を行った。

本研究において、著者は H5N1 HPAI ウイルスが水禽の羽組織で増殖することを見出し、羽が感染水禽からのウイルス排泄経路に重要であることを提示した。次に、ウイルスに汚染された羽が、健康な鳥や哺乳動物への感染源となりうることを示し、公衆衛生の観点から重要であることを指摘した。また、感染水禽の羽が、ウイルス検出のための有用な組織サンプルとして利用できる可能性を示した。最後に、ウイルスに汚染された感染水禽の羽が、環境中において、ウイルス媒介物としての役割を果たす可能性を指摘した。本研究は、H5N1 HPAI ウイルス伝播に水禽の羽組織が関わるという新しい見解を提示した。上記の研究成果が、今後の HPAI の制圧に貢献することを期待する。本論文の各章の概要を以下に記載する。

第 I 章では、H5N1 HPAI ウイルス山口株を接種したアイガモにおける病理発生について解析した。ウイルスを接種されたアイガモは斃死や神経症状を示した。脳炎、脾臓壊死、角膜混濁、羽を構成する上皮組織の壊死等の病理所見が認められた。幼アイガモでは高致死率が観察され、水禽の日齢が体内でのウイルス増殖に影響することが示唆された。アイガモの羽でウイルスが増殖することを明らかにし、羽が感染水禽からのウイルス排泄経路となる可能性を提示した。

第 II 章では、前章で観察された山口株によるアイガモの羽における増殖に関して、H5N1 HPAI ウイルス清武株やガチョウでの本現象の再現性について検索した。H5N1 HPAI ウイルスを実験感染させたアイガモやガチョウの

羽を免疫組織化学、電子顕微鏡検索、およびウイルス分離に供し、山口株および清武株が、アイガモおよびガチョウの羽上皮細胞で増殖したことを確認した。この結果、羽における H5N1 HPAI ウイルスの増殖が、感染水禽に共通する現象である可能性が示された。

さらに、H5N1 HPAI ウイルスに自然感染した白鳥の羽組織を、病理組織学的、ウイルス学的に検索した。羽軸根からウイルスが分離され、免疫組織化学の結果、羽上皮細胞および羽包上皮細胞にウイルス抗原が検出された。さらに羽のパラフィン包埋標本から H5N1 HPAI ウイルスの遺伝子が検出された。この結果、ウイルスに自然感染した水禽の羽にウイルスが存在しうる事が明らかとなった。

感染アイガモの綿羽を健康なアイガモに経口投与したウイルス伝播試験の結果、投与アイガモの感染を確認した。この結果、山口株に感染したアイガモの羽が、鳥に対する感染性を有する事が示された。以上の成績に基づき、感染水禽の羽が、動物や人への感染源となりうる可能性について指摘した。

第Ⅲ章では、ウイルス検出における、感染アイガモの羽の臨床診断における有用性を評価した。H5N1 HPAI ウイルス清武株および秋田株をアイガモに経鼻接種し、簡易抗原検出キット、ウイルス分離、免疫組織化学、および逆転写 PCR を用いて、羽の羽軸根、咽喉頭スワブおよびクロアカスワブからのウイルス検出を経時的に行った。ウイルス分離の結果、スワブと比較して、羽から大量のウイルスが長期間検出された。感染アイガモの羽が、スワブと同様に、ウイルス検出のための有用な試料となりうる可能性を提示した。

最後に、感染水禽の羽を介した環境中へのウイルス汚染を評価した。清武株および秋田株を実験感染させたアイガモの羽、飲用水、糞便を採材し、異なる 2 つの温度条件下で、試料中のウイルスの感染性の存続期間を解析した。飲用水や糞便と比較して、羽は大量の感染性ウイルスを長期間保持していた。羽組織中からの感染性ウイルスの分離期間は、摂氏 4 度で保存された場合 160 日間、摂氏 20 度の場合 15 日間であった。以上の成績から、感染水禽の羽が、野外環境において高力価の H5N1 HPAI ウイルスを含有する感染媒介物として成立する可能性が示唆された。