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Comparative histopathological characteristics of highly pathogenic avian influenza (HPAI) in chickens and domestic ducks in 2008 Korea

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Summary. We compared characteristic lesions occurring in chickens and domestic ducks naturally infected with H5N1 HPAI virus in April and May 2008. Infected chickens generally exhibited pale-green, watery diarrhoea, depression, neurological signs and cyanosis of wattles and combs, and infected ducks generally exhibited neurological signs and watery diarrhoea. Gross petechial or ecchymotic haemorrhage affected the heart, proventriculus, liver, muscle, fat, and pancreas in chickens, and muscle in ducks. Necrotic foci were primarily present in the pancreas of both species and in the heart of domestic ducks. Histopathologically, chickens exhibited multifocal encephalomalacia, multifocal lymphohistiocytic myocarditis, multifocal necrotic pancreatitis and haemorrhage of several organs and tissues; ducks exhibited lymphohistiocytic meningoencephalitis with multifocal haemorrhages, multifocal necrotic pancreatitis, and severe necrotic myocarditis with mineralisation. The characteristic histopathologic findings of 2008 HPAI were multifocal encephalomalacia and necrotic pancreatitis accompanied by lymphohistiocytic myocarditis, and haemorrhage in various organs and tissues in chickens, whereas in ducks, they were severe necrotic myocarditis with mineralisation and necrotic pancreatitis, accompanied with lymphohistiocytic meningoencephalitis. The high mortality of domestic ducks may be intimately associated with heart failure resulting from increased H5N1 HPAI viral cardiotropism.

Key words: Avian influenza, Cardiotropism, Chicken, Duck, HPAI

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Introduction

Influenza viruses are classified as type A, B, or C according to the antigenic characteristics of their nucleoprotein and matrix proteins. The avian influenza viruses are type A viruses and are further classified according to their haemagglutinin (HA) and neuraminidase (NA) antigen subtypes (Webster et al., 1992). At present, sixteen HA and nine NA subtypes are known (Fouchier et al., 2005; Alexander, 2007). Highly pathogenic avian influenza (HPAI) viruses belong to subtypes H5 and H7, but not all viruses of these subtypes induce HPAI (Alexander, 2007).

HPAI has a mortality rate as high as 100% in chickens. Although infection of aquatic birds, including ducks, with influenza viruses has long been known, infected birds have generally shown no characteristic symptoms of influenza (Keawcharoen et al., 2008). And aquatic birds have been thought to serve as a natural reservoir of influenza viruses and to play a critical role in recombination and propagation of these viruses (Li et al., 2004; Olsen et al., 2006; Keawcharoen et al., 2008). However, since 1999, mortality in aquatic birds, including ducks, infected by HPAI virus has been demonstrated (Perkins and Swayne, 2002; Ellis et al., 2004; Kwon et al., 2005a; Chen et al., 2006a,b).

The first documented outbreak of HPAI occurred during fall/winter of 2003-2004 in Korea; since then, two more outbreaks have occurred. The latest outbreak of H5N1 HPAI, which occurred in April and May 2008, killed more than 50% of infected ducks, whereas previously the HPAI mortality rate in domestic ducks was low (Kwon et al., 2005a). The histopathological findings of the latest outbreak were also more severe in ducks, whereas findings in chickens were similar to those observed previously (Kwon et al., 2005b). The findings in ducks differed somewhat from those in

chickens. Therefore, we analysed the histopathological differences in infected ducks and chickens.

Materials and methods

Animals

On April 1, 2008, suspected HPAI cases were submitted to the National Veterinary Research and Quarantine Service from a layer farm in Chonbuk Province and confirmed as H5 HPAI on the day they were submitted. HPAI outbreaks were identified in 42 farms (48 cases) consisting of 10 duck farms (10 cases), 26 chicken farms (26 cases), and six farms (12 cases) rearing chickens and domestic ducks together. Four of the duck farms and five of the chicken farms existed within a 3 km radius of the outbreak farm and were subjected to culling to prevent the spread of HPAI. One to fifteen animals per case were submitted from 42 farms for diagnosis. After necropsy, specimens such as trachea, ceacal tonsil and kidney were inoculated into embryonated chicken eggs for virus isolation. Simultaneously, to prevent the spread of HPAI, the H5 subtype was subjected to RT-PCR to identify suspected flocks for culling. Virus isolated from eggs was identified by the haemagglutinin activity test, RT-PCR and sequence analysis, the haemagglutinin inhibition test, and the neuraminidase inhibition test with reference antisera, and the plaque assay without trypsin treatment according to Korean Standard Procedures for HPAI Diagnosis. Chickens (32 cases) and domestic ducks (16 cases) with HPAI infections were collected from all areas of Korea during April and May 2008 (Table 1). Chickens included layer, broiler, and Korean native breeds.

Histopathology

Gross findings were recorded at necropsy. Abnormal and normal portions of all organs and tissues, including brain, lungs, heart, liver, spleen, kidneys, thymus, trachea, proventriculus, gizzard, intestine, bursa, skeletal muscles (breast and leg), skin and reproductive organs were removed, fixed in 10% buffered formalin, and embedded in paraffin. The paraffin-embedded tissues were sectioned at 4 µm, stained with haematoxylin and eosin, and assessed histopathologically.

Immunohistochemistry

To detect the influenza virus, paraffin-embedded sections were immunostained using a fully automatic immunohistochemical system (Ventana Discovery XT; Ventana Medical Systems, Inc., Tucson, AZ, USA) and the DAB Detection System (Ventana Medical Systems Inc.) according to the manufacturer's instructions. Brain, heart and pancreas of chicken from the 2006 outbreak in Korea were used to confirm positive immunoreactivity of the primary antibody in tissues. The optimal conditions for the automatic immunohistochemical system were also determined using positive and negative controls. Briefly, paraffin sections, including controls, were deparaffinised at 75°C for 8 min, treated with protease 1 for 8 min at 37°C, and exposed to the mouse anti-influenza A nucleoprotein antibody (1:3000 dilution; AbD Serotec, Oxford, UK) in antibody diluent (Dako North America, Inc., Carpinteria, CA, USA) for 32 min at 37°C. As a negative control for immunoreactivity, normal mouse serum was applied to positive control tissue at the same dilution instead of the primary antibody. The universal secondary antibody, detection reagent, and DAB chromogen were then applied sequentially at 37°C. The sections were counterstained with haematoxylin and bluing reagent (Ventana Medical Systems, Inc.) and cover-slipped for microscopic examination.

Results

Clinical signs

In the chicken farm first confirmed with HPAI, morbidity (96%) was observed in one flock (64 weeks old) of seven flocks and mortality increased markedly at day 3, after showing clinical signs such as severe watery diarrhoea, depression, cyanosis and neurological signs. Cumulative mortality was 26% by day 5 after the birds showed clinical signs. However, as the outbreak continued, the morbidity and mortality rate generally decreased due to the early destruction of suspicious animals. In 32 chicken cases, the most common manifestations of infection were cyanosis of the comb, wattles or legs, and neurological signs such as incoordination, torticollis, and seizures. Respiratory distress and pale brown or white watery diarrhoea were also observed. Additionally, breeders and layers manifested severe decreases in egg production.

At the duck farm confirmed as the second HPAI outbreak in 2008, the mortality was initially observed from March 31st. Diseased animals (34 days old) showed depression, neurological signs and watery diarrhoea. Morbidity and mortality reached 60% and 50% on the day of submission, respectively. Additionally, the mortality rate reached 64% at some duck farms. In 16 duck cases, the primary clinical signs were depression and neurological signs, such as incoordination, torticollis, and seizures. Feed consumption was markedly decreased.

Gross findings

In chickens, petechial haemorrhages or ecchymoses were observed in various organs and tissues: muscle (16 cases), epicardium (4 cases), proventriculus (12 cases) and thymus (2 cases). The tracheal mucosa was congestive (8 cases), and white or pale grey foci of various sizes were present in the pancreas (19 cases; Fig. 1) and spleen (15 cases). The liver was enlarged and

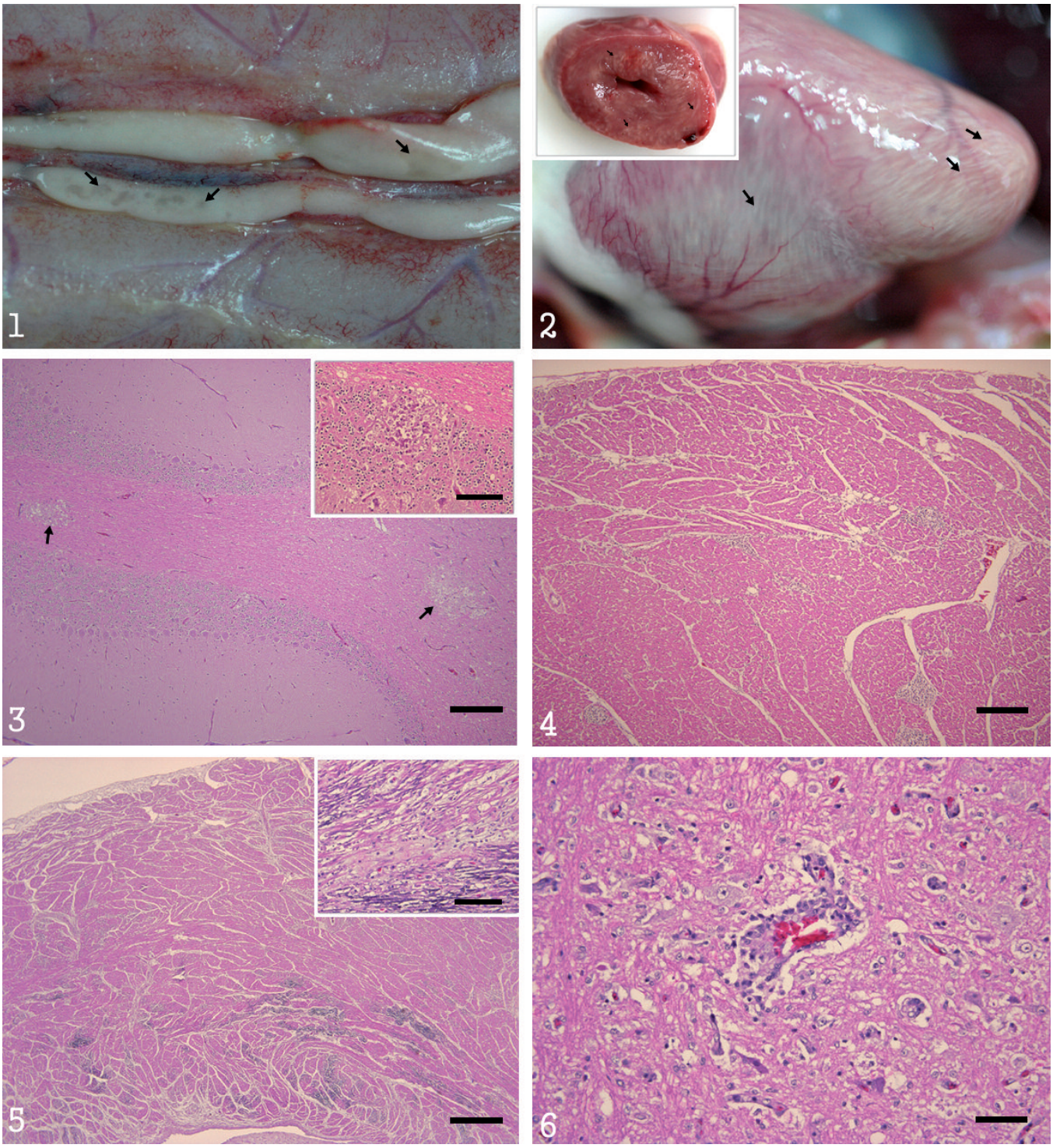

Fig. 1. Chicken pancreas. Variously sized pale grey spots are multifocally observed in the pancreas (arrows).

Fig. 2. Duck heart. Many white stripes are present on the epicardium (arrows). Insert: The same stripes and appearance are scattered on the cut surface of the myocardium (arrows).

Fig. 3. Chicken cerebellum. Malacic foci are multifocally observed in the cerebellum (arrows). Haematoxylin and eosin. Bar: 500μm. Insert: Necrosis exists in the granular layer. Bar: 100 μm.

Fig. 4. Chicken heart. Infiltration foci of lymphocytes and macrophages are multifocally observed in myocardium. Haematoxylin and eosin. Bar: 100 μm.

Fig. 5. Chicken heart. Severe degenerative and necrotic foci accompanied by macrophage infiltration and calcification are seen in myocardium. Haematoxylin and eosin. Bar: 500 μm. Insert: A necrotic area with calcification. Bar: 100 μm.

Fig. 6. Duck cerebrum. Lymphocytes and macrophages infiltrate the Virchow-Robin space. Haematoxylin and eosin. Bar: 50 μm.

friable (18 cases) or ruptured; ruptured livers (10 cases) were covered with blood clots. In breeders and layers, ovarian follicles (10 cases) were often flaccid, hemorrhagic, or ruptured. In cases of ovarian follicle rupture (5 cases), peritonitis was occasionally observed.

In ducks, the pericardium was moderately full of clear yellow fluid (4 cases), and many white stripes were present on the epicardium and on the cut surface of the myocardium (12 cases; Fig. 2). Hemorrhagic foci were present in muscle tissue (5 cases), and the brain blood vessels were congestive (4 cases). As in chickens, white or pale grey foci of various sizes were present in the pancreas (10 cases) and spleen. The spleen was dark red and enlarged (4 cases), and the liver was enlarged and friable (4 cases).

Histopathological findings

In chickens, commonly observed histopathological findings included haemorrhage in various organs and

Table 1. List of natural highly pathogenic avian influenza outbreaks in 2008 Korea.

Case no	Species	Breed	Age (weeks)	Sex	Submitted Head	Remarks
1	Chiken	Layer	64	F	10 (D, 5; E, 5)	Farm
2	Duck	Commercial	5, 7	ND	4 (D)	Farm
3	Duck	Commercial	2	ND	10 (D, 5; E, 5)	Farm
4	Duck	Commercial	1, 4	ND	11 (D, 6; E, 5)	Farm
5	Duck	Commercial	1, 6	ND	15 (D, 10; E, 5)	Farm
6	Duck	Commercial	5, 6	ND	9 (D, 5; E, 4)	Farm
7	Duck	Commercial	6	ND	5 (D)	Farm
8	Chiken	Korean native	17	F	4 (D)	Farm
9	Chiken	Layer	65	F	6 (D, 3; E, 3)	Farm
10	Chiken	Layer	19, 30	F	6 (D, 3; E, 3)	Farm
11	Chiken	Layer	15	F	6 (D, 3; E, 3)	Farm
12	Chiken	Layer	50	F	8 (D, 4; E, 4)	Farm
13	Chiken	Layer	50	F	6 (D, 3; E, 3)	Farm
14	Chiken	Breeder	30	F	5 (D)	Farm
15	Chiken	Layer	36	F	4 (D)	Farm
16	Chiken	Layer	23	F	6 (D, 3; E. 3)	Farm
17	Chiken	Layer	24, 31	F	6 (D, 3; E. 3)	Farm
18	Chiken	Layer	27	F	6 (D, 3; E. 3)	Farm
19	Chiken	Korean native	13	F	6 (D, 3; E. 3)	Farm
20	Duck	Commercial	3	ND	10 (D, 5; E, 5)	Farm
21	Chiken	Korean native	Unknown	F	4 (D)	Restaurant*
22	Duck	Commercial	Unknown	ND	4 (D)	Restaurant*
23	Duck	Commercial	3	ND	5 (D)	Farm
24	Chiken	Layer	57, 86	F	10 (D, 5; E, 5)	Farm
25	Chiken	Layer	17, 65	F	6 (D, 3; E, 3)	Farm
26	Chiken	Layer	46	F	6 (D, 3; E, 3)	Farm
27	Chiken	Breeder	29	F	5 (D)	Farm
28	Chiken	Korean native	11	F	6 (D, 3; E, 3)	Farm
29	Chiken	Korean native	17	F	9 (D, 5; E, 4)	Farm
30	Chiken	Breeder	25	F	10 (D, 5; E, 5)	Farm
31	Duck	Breeder	32, 45	F	5 (D)	Farm
32	Chiken	Korean native	17	F	7 (D, 4; E, 3)	Self consumption
33	Chiken	Layer	6, 10	F	5 (D)	Self consumption
34	Chiken	Korean native	4	F	6 (D, 3; E, 3)	Self consumption
35	Chiken	Korean native	Unknown	F, M	2 (D, 1; E, 1)	Park
36	Chiken	Korean native	9	F	10 (D, 5; E, 5)	Farm
37	Duck	Commercial	Unknown	ND	1 (D)	Self consumption
38	Duck	Commercial	Unknown	ND	1 (D)	Self consumption
39	Chiken	Korean native	35	F	5 (D)	Farm
40	Duck	Breeder	50	F	4 (D)	Farm
41	Chiken	Korean native	16	F	2 (D)	Self consumption
42	Duck	Commercial	8	ND	1 (D)	Self consumption
43	Duck	Commercial	Unknown	ND	1 (D)	Self consumption
44	Duck	Commercial	8	ND	2 (D)	Farm
45	Chiken	Korean native	32	F	5 (D)	Self consumption
46	Chiken	Layer	26, 34	F	10 (D, 5; E, 5)	Farm
47	Chiken	Korean native	8	F	10 (D, 5; E, 5)	Farm
48	Chiken	Breeder	34	F	10 (D, 5; E, 5)	Farm

ND: not determined; F: female; D: dead; E: euthanized; *: animals raised for sale in restaurants, #: small sized farm raised for food or pets.

tissues, multifocal encephalomalacia (30 cases; Fig. 3), necrotic pancreatitis (21 cases), and lymphohistiocytic myocarditis (25 cases; Fig. 4). Malacia was most frequently observed in the brain stem, less frequently in the cerebellum and even less frequently in the cerebrum. Multifocal necrosis was occasionally observed in the liver (6 cases) and white pulp of the spleen (10 cases; Table 2).

In ducks, the predominant lesions occurred primarily in the heart, brain, and pancreas. Severe necrosis occasionally accompanied by calcification was observed in the heart (16 cases; Fig. 5). In the brain, there was haemorrhage and the meninges and the areas around blood vessels were infiltrated with lymphocytes and macrophages (10 cases; Fig. 6). Multifocal necrosis was seen in pancreatic acinar cells (10 cases) but not in islet cells. Additionally, necrotic splenitis (2 cases), necrotic hepatitis (2 cases), and lymphohistiocytic myositis (7 cases) were observed (Table 2).

Immunohistochemical findings

The distributions of viral antigens are summarised in Table 3. In both chickens and ducks, viral antigens were almost always immunolocalised at the sites of histopathological change. Immunoreactivity was localised in both the nucleus and cytoplasm of cells in normal areas surrounding lesions, as well as intralesionally. In particular, although histopathological lesions were not observed in the kidney, a strong reaction was shown in the renal tubular epithelium during immunohistopathological investigations, and the signal was more intense and broadly distributed in ducks than in chickens (Fig. 7). The antigen distribution in heart and pancreas was multifocal in ducks but diffuse in chickens (Fig. 8). Additionally, an immunopositive reaction was observed in the pancreatic islet cells despite their seeming histopathological normalcy. Viral antigens were also detected in the intestinal mucosal epithelia and

Table 2. Histopathological findings of highly pathogenic avian influenza in chickens and domestic ducks in the 2008 outbreak.

Organ or tissue/Lesion		Chiken	Domestic duck
Brain**	Necrosis	++ ~ ++++*	-
	Cerebrum	(++)	
	Cerebellum	(+++)	
	Brain stem	(++++)	
	Lymphohistiocytic meningoencephalitis	-	+ ~ +++
	Cerebrum		(+)
	Cerebellum		(++)
	Brain stem		(+++)
	Hemorrhage	-	++
Heart**	Necrosis	-	+++ ~ ++++
	Lymphohistiocytic myocarditis	+ ~ +++	-
	Calcification	-	++
Pancreas	Necrosis	- ~ +++	- ~ +++
Liver	Necrosis	- ~ ++	- ~ ++
	Hemorrhage	- ~ ++	-
Spleen	Necrosis	- ~ ++	- ~ ++
	Lymphoid depletion	- ~ +++	- ~ +++
Lung	Interstitial pneumonia	- ~ +	- ~ +
	Congestion	- ~ ++	- ~ ++
Trachea	Lymphocytic tracheitis	- ~ +	- ~ +
	Congestion	- ~ +++	- ~ +++
Fat tissue	Hemorrhage	- ~ +++	-
	Lymphocytes infiltration	- ~ ++	-
	Necrosis	+	-
Skeletal muscle	Hemorrhage	- ~ +++	- ~ ++
	Lymphocytes infiltration	-	- ~ ++
Other lymphoid tissues	Lymphoid depletion	- ~ +++	- ~ +++

* -: no lesion; +: minimal lesion; ++: mild lesion; +++: moderate lesion; ++++: severe lesion; ** Graded histological lesions except cases with no lesions.

Table 3. Distribution of influenza viral antigens by immunohistochemistry in chickens and ducks in the 2008 outbreaks.

Organ or tissue/positive cell or area		Chiken	Domestic duck
Brain	Neuron	+ ~ +++*	+ ~ ++
	Glial cell	+	+ ~ +++
	Purkinje cell	+ ~ ++	+
	Granular cell	+ ~ ++	+
	Choroid plexus	+	+ ~ +++
	Ependymal cell	+ ~ +++	+
Heart	Myocyte	++ ~ +++	++ ~ +++
	Tunica media	- ~ ++	-
	Endothelium of blood vessel	- ~ +	-
Pancreas	Acinar epithelium	++ ~ +++	- ~ ++
	Islet cell	- ~ +	-
	Ductular epithelium	- ~ ++	-
Liver	Hepatocytes	- ~ ++	- ~ +
	Kupffer cell	- ~ +++	- ~ ++
	Endothelium of blood vessel	- ~ ++	-
Spleen	White pulp	- ~ ++	- ~ ++
	Endothelium of blood vessel	- ~ +	- ~ +
Lung	Bronchiolar epithelium	- ~ +	- ~ +
	Atrial epithelium	- ~ ++	- ~ ++
	Tunica media	- ~ +	-
	Endothelium of blood vessel	-	- ~ +
Trachea	Epithelium	- ~ +	- ~ +
	Lamina propria	- ~ ++	- ~ +
Kidney	Tubular epithelium	+ ~ ++	+ ~ +++
Intestine	Mucosal epithelium	- ~ ++	- ~ ++
	Lamina propria	- ~ ++	- ~ ++
	Nerve	- ~ +	- ~ +
Fat tissue	Adipocyte	- ~ ++	-
Skeletal muscle	Muscle fiber	- ~ +	- ~ +
	Endothelium of blood vessel	- ~ +	-

* -: none; +: few; ++: moderate; +++: numerous.

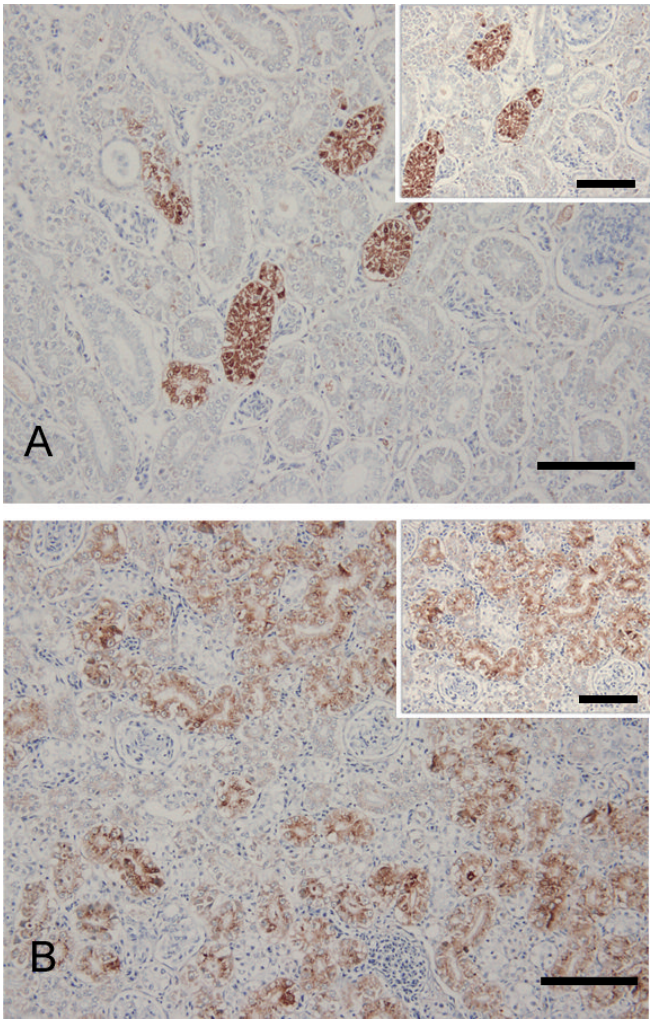

Fig. 7. Kidney. Immunohistochemical staining for influenza A nucleoprotein and DAB chromogen. The renal tubular epithelium was strongly immunopositive. **A.** Chicken. An immunoreaction is observed in some tubules. Immunoperoxidase with hematoxylin and bluing reagent counterstain. Insert: High magnification. **B.** Duck. Immunopositive reactions are seen in many tubules. Immunoperoxidase with hematoxylin and bluing reagent counterstain. Insert: High magnification. Bars: A, B, 200 μm; inserts, 100 μm.

lamina propria (Fig. 9).

Discussion

HPAI virus (H5N1) began to cause epidemic disease in ducks during the Asian outbreak of 2002-2005, although some clinical signs and mortality were documented in ducks and geese infected by H7N1 HPAI virus in Italy during 1999 and 2000 (Capua and Mutinelli, 2001; Ellis et al., 2004; Kwon et al., 2005a; Chen et al., 2006a,b). Epidemic infection of domestic ducks in Korea was noted at the time of the first Korean outbreak of HPAI during the fall/winter of 2003-2004 (Kwon et al., 2005a,b). In all, three HPAI outbreaks have occurred in Korea; the second outbreak occurred during the winter and early spring of 2006-2007, and the most recent occurred during the spring of 2008.

In chickens, the clinical signs and histopathological findings in the spring 2008 outbreak were similar to those of previous outbreaks and included high morbidity with neurological signs and necrosis and haemorrhage in various organs and tissues (Kwon et al., 2005b). However, mortality in the 2008 outbreak was much lower than that of previous outbreaks, suggesting that it was closely associated with the immediate report of suspected animals, quick confirmation of HPAI and the stamping out policy. In ducks, on the other hand, the clinical signs and histopathological findings of the recent outbreak were different from those observed previously (Kwon et al., 2005a). The duck mortality rate increased

markedly to higher than 50% in this outbreak; in the first and second outbreaks, the mortality rates were less than 15% and 0.4%, respectively. Severe necrotic myocarditis, often accompanied by calcification, was observed in duck hearts, suggesting that an increasing cause of death in ducks might be heart failure from extensive myocardial necrosis. Although necrotic pancreatitis and encephalitis were observed in previous outbreaks (Kwon et al., 2005a), the lesions appeared more severe and were more broadly distributed in the 2008 outbreak. Two-week-old ducks intranasally infected with A/chicken/Korea/IS/06 show no clinical signs, except mild depression and mild respiratory signs (Jeong et al., 2009), whereas all of the 2-week-old ducks intranasally infected with A/chicken/Korea/Gimje/08 and A/duck/Korea/JE2/08, died at 4.6 days and 4.9 days after inoculation, respectively (unpublished data). Furthermore, the mortality rate was 50% in 30-week-old ducks intranasally infected with A/chicken/Korea/Gimje/08 (unpublished data).

H5N1 HPAI viruses were pathobiologically divided into four groups according to pathogenesis and lesions in 2-week-old domestic ducks; severe systemic injuries (group 1), nervous and cardiovascular injuries (group 2), increased pathogenicity in the respiratory tract (group 3) and no injury or mild respiratory problem (group 4) (Swayne, 2007). Thus, the above phenomena may be closely related to the observed shift from low (group 3)

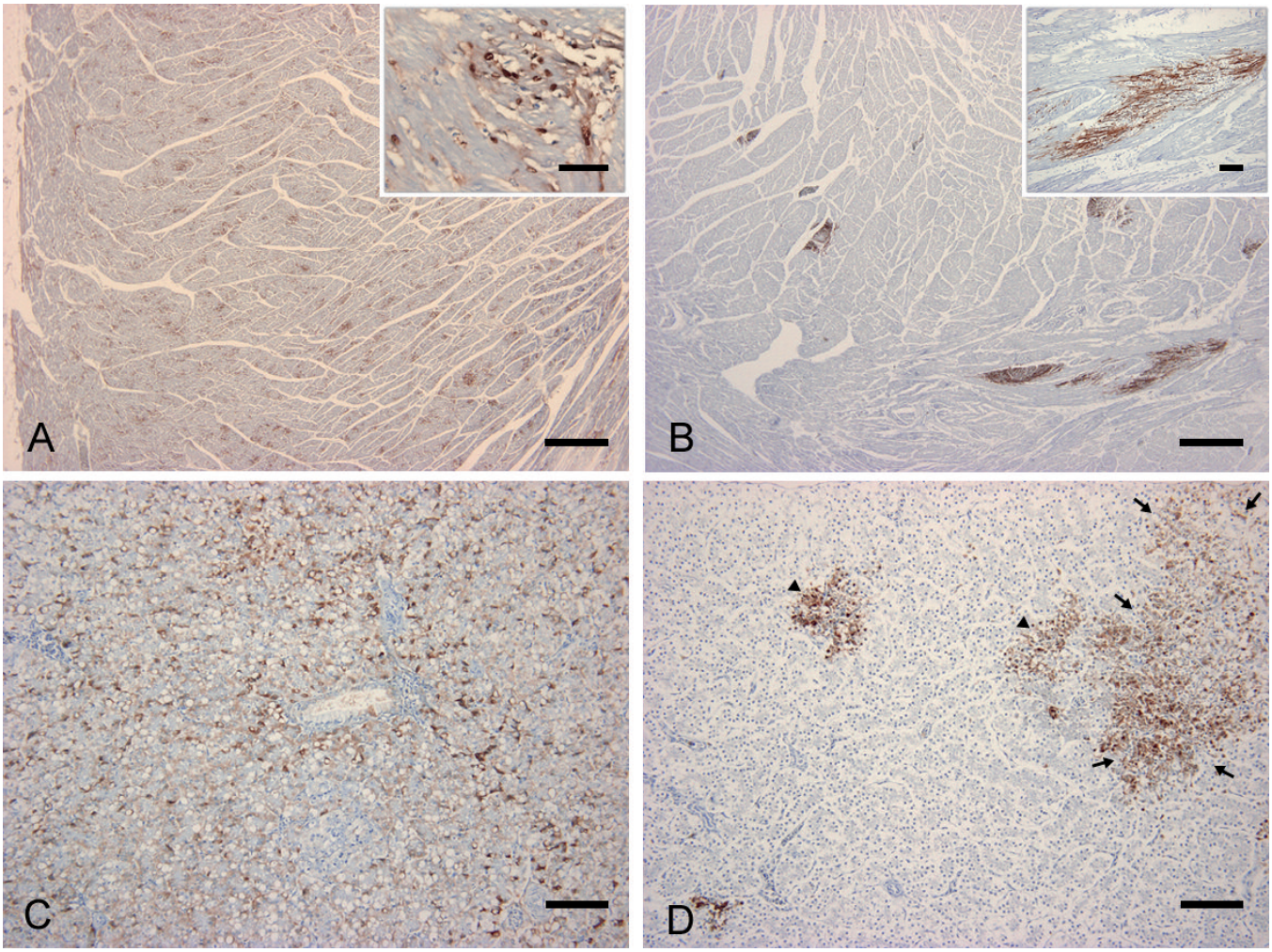

Fig. 8. Immunohistochemical staining for influenza A nucleoprotein and DAB chromogen. **A.** Chicken heart. Diffuse immunoreactivity is observed in the myocardium. Immunoperoxidase with hematoxylin and bluing reagent counterstain. Insert: Many nuclei and some myocardiocyte cytoplasms are strongly positive. **B.** Duck heart. Immunopositive reactions are multifocally seen in the necrotic area. Immunoperoxidase with hematoxylin and bluing reagent counterstain. Bar: 500μm. Insert: Immunoreactivity is diffusely located in the necrotic area. **C.** Chicken pancreas. Diffuse immunoreactivity in pancreatic acini. Immunoperoxidase with hematoxylin and bluing reagent counterstain. **D.** Duck pancreas. Immunopositive reactions are seen in normal pancreatic acini (arrowheads) and in the necrotic area (arrows). Immunoperoxidase with hematoxylin and bluing reagent counterstain. Bars: A, B, 500 μm; inserts A, B, 50 μm; C, D, 100 μm.

to high pathogenicity and mortality (group 1) in ducks.

In previous studies, various HPAI-induced lesions in chickens and ducks were demonstrated in natural outbreaks and in inoculation experiments (Brown et al., 1992; Kobayashi et al., 1996; Mo et al., 1997; Swayne, 1997; Capua and Mutinelli, 2001; Perkins and Swayne, 2002; Ellis et al., 2004; Kishida et al., 2005; Kwon et al., 2005a,b; Pantin-Jackwood and Swayne, 2007; Vascellari et al., 2007). Although the histopathological findings of the experimental studies varied somewhat with the particular viral isolate, inoculum dose, host age, and inoculation route, the most commonly affected organs were brain, pancreas, heart, and spleen in chickens (Kobayashi et al., 1996; Mo et al., 1997; Swayne, 1997), and brain and heart or pancreas in ducks (Kishida et al., 2005; Pantin-Jackwood and Swayne, 2007; Vascellari et al., 2007). In the present study, histopathological changes were systemically observed in all organs and tissues of chickens and ducks, but the brain and heart histopathology revealed different patterns in the two species. Based on the characteristic histopathological patterns of the two species, it may be possible to distinguish between chickens and ducks in the 2008 HPAI outbreak. The increased mortality of ducks was associated with insults to multiple systems, especially severe damage to the heart, such as extensive myocardial necrosis with/without calcification. Previous studies have reported that the high mortality may be related to host age in ducks (Kwon et al., 2005a; Pantin-Jackwood and Swayne, 2007; Swayne, 2007). However, the ages of the ducks that died were 1- to 50-weeks-old in the present study, suggesting high lethality regardless of age as chickens infected by HPAI. In the experimental inoculation using 2008 HPAIV, however, there was a difference in mortality according to age; the mortality rate reached 50% in adult ducks (unpublished data). Additionally, viral antigens were immunohistochemically detected in the intestinal mucosal epithelia and lamina propria in ducks, as in chickens. Thus, these results indicated that the pathogenicity and the propagation of the HPAI virus in ducks may have increased.

HPAI viral antigens have been immunohistochemically demonstrated in various organs and tissues of naturally or experimentally infected birds (Brown et al., 1992; Kobayashi et al., 1996; Mo et al., 1997; Swayne, 1997; Capua and Mutinelli, 2001; Perkins and Swayne, 2002; Kishida et al., 2005; Kwon et al., 2005a,b; Pantin-Jackwood and Swayne, 2007; Vascellari et al., 2007). They are detected most frequently in brain, heart, pancreas, spleen, liver, lung, and kidney, and in normal areas as well as in histopathological lesions, particularly in histologically normal organs in experimental studies (Kobayashi et al., 1996; Swayne, 1997; Vascellari et al., 2007). In the present study, immunostaining for viral antigens yielded positive signals in regions without lesions, such as pancreatic islet cells and kidney, as well as in various lesions.

In conclusion, our characterisation of the histopathological findings in chickens and domestic ducks naturally infected with H5N1 HPAI virus in 2008 showed that the virus produced systemic infection and

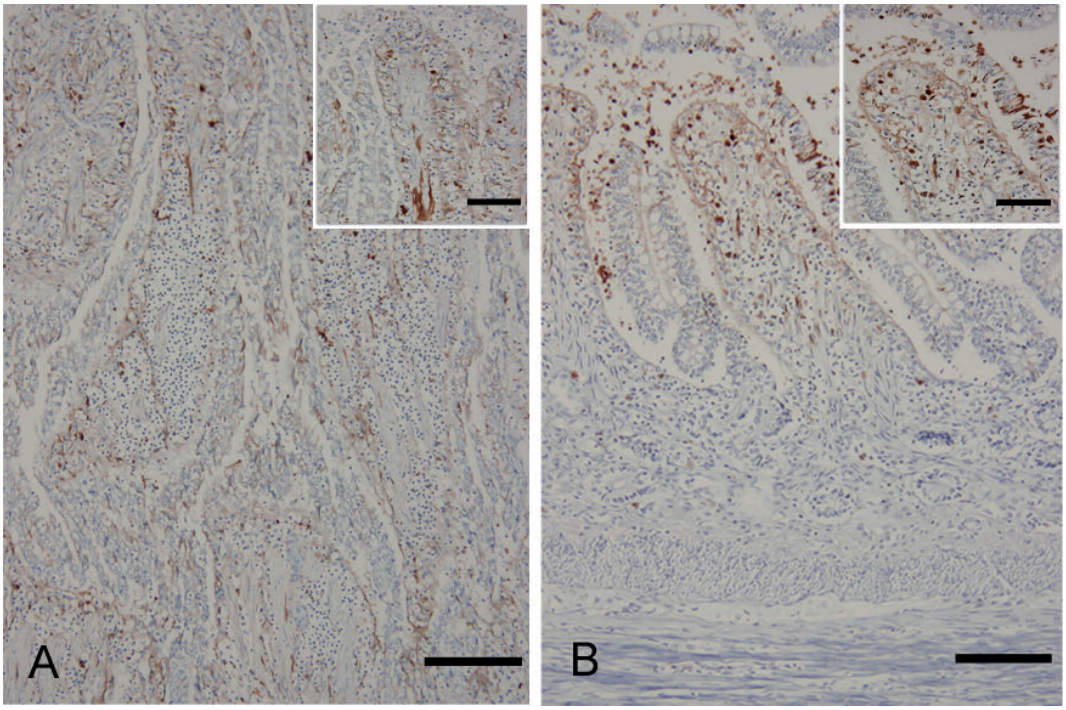

Fig. 9. Intestine. Immunohistochemical staining for influenza A nucleoprotein and DAB chromogen. Immunoreactivity was observed in the intestinal mucosal epithelia and lamina propria of chicken **(A)** and duck **(B).** Immunoperoxidase with hematoxylin and bluing reagent counterstain. Bars: A, B, 200 μm; Inserts, 100 μm.

lesions in most organs and tissues. Infected chickens exhibited high morbidity, and the HPAI virus produced necrosis in the brain and pancreas, and haemorrhage in various organs and tissues in chickens. In domestic ducks, HPAI virus induced increased mortality and severe necrosis in the heart and pancreas. Further experimental studies will be required to determine the pathogenicity-enhancing mechanism in ducks.

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