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Histology and Immunohistochemistry of Tigers and Birds Naturally Infected with H5N1 Highly Pathogenic Avian Influenza Virus in Thailand

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Abstract

Tigers and birds infected with H5N1 highly pathogenic avian influenza virus in Thailand in 2004 and 2005 were investigated histologically and immunohistochemically. Histologically, tigers had interstitial pneumonia and hepatocytic necrosis, and a native chicken had focal necrosis of the parenchyma of brain. Immunohistochemically, influenza virus antigens were demonstrated in the necrotic foci of the liver in tigers. Influenza virus antigens were detected in the necrotic area of brain, necrosis of the spleen, and vascular endothelium of the whole body in the native chicken and open-billed storks.

Discipline: Animal health
Additional key words: hepatocytic necrosis, interstitial pneumonia, native chicken, stork

Introduction

Highly pathogenic avian influenza virus (HPAIV) (H5N1) has been prevalent in chickens, wild birds and humans in Asian countries since 2004[2,7]. More than 64 million chickens died or were killed as a result of HPAIV infections in Thailand (January 2004 to November 2005)[1]. Tigers and leopards were affected with HPAIV in a zoo in Suphanburi in January 2004[3], and many tigers in Sri Racha Tiger Zoo were affected with HPAIV in October 2004[10] in Thailand. The tigers were fed chicken carcasses infected with HPAIV and died after they showed clinical symptoms including high fever and respiratory distress. Wild birds were infected with HPAIV in Thailand[2]. There is little information available on the pathology of animals naturally infected with HPAIV in Thailand. We describe the pathology of dead animals (tigers, wild birds and a native chicken) from which HPAIV was isolated in Thailand.

Materials and methods

Four tigers and seven birds; one cockatoo (*Cacatua* sp.), one pigeon (*Columba* sp.), one egret (*Egretta* sp.), four open-billed storks (*Anastomus oscitans*), and one native chicken (*Gallus domesticus*) were investigated pathologically. All animals were dead cases. Bengal tigers (*Panthera tigris tigris*) kept at the Sri Racha Tiger Zoo in Thailand died in October 2004. Dead birds were discovered in 2005. All specimens were submitted to our laboratory for examination of HPAIV. HPAIV (H5N1) was isolated from tigers and birds. Avian influenza-infected chicken carcasses were the sources of infection for the tiger cases.

The liver, spleen, kidney, heart, lung, brain, pancreas, trachea, intestine, lymph node, and others were fixed with 10% neutral phosphate buffered formalin, sectioned, stained with hematoxylin and eosin (HE), and investigated histologically.

The influenza virus antigens were detected by immunohistochemistry (IHC) using monoclonal antibodies against nucleoprotein of A type influenza virus. Mouse-derived monoclonal antibody specific for type-A influenza virus nucleoprotein (OBT0104, Oxford Biotechnology Ltd) were used as the primary antibodies in the immunoperoxidase technique for detection of HPAIV in formalin-fixed, paraffin-embedded sections. A Histofine simple stain PO (M) kit (Nichirei Inc., Tokyo, Japan) was used according to the manufacturer's instruc-

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tions[5]. The labeled polymer was prepared by combining amino acid polymers with peroxidase and goat anti-mouse Ig, which are reduced to Fab'. The sections were digested for 15 min at 37ºC by 0.1% actinase E (Kaken Seiyaku, Tokyo) in phosphate-buffered solution (PBS). After quenching the endogenous peroxidase with 3% solution of hydrogen peroxidase in absolute methanol, we added the primary antibodies (1:1,000 dilution) to the sections and incubated them for overnight in a refrigerator (4ºC). After rinsing them in PBS, we added the Histofine simple stain PO (M) and then incubated them for 30 min. We next rinsed them in PBS, added chromogen/substrate reagent, and incubated them for 3–5 min. After staining, the sections were counter-stained with hematoxylin.

The internal organs (lung, heart, spleen, liver, kidney, intestine, and brain) were homogenized and diluted with PBS containing antibiotics (penicillin and streptomycin), and cloacal swabs were placed in viral transport media. The samples were centrifuged at high speed (12,000 rpm, 5 min) and the supernatants were collected and inoculated into 9-day-old embryonated chicken eggs via the allantoic cavity. Allantoic cavity fluid from inoculated eggs was tested for hemagglutination (HA), and hemagglutination inhibition (HI) using antibodies specific to H5N1 virus, and Newcastle disease virus. Meanwhile they were confirmed by reverse transcription PCR (RT-PCR) and real-time RT-PCR[8] using the primers and probes routinely used in the National Institute of Animal Health, Thailand (NIAH Thailand protocol, 2004).

Results

Three of four tigers had interstitial pneumonia and/or multifocal hepatocytic necrosis (Table 1). The alveolar septa were thickened (Fig. 1) due to the increase of macrophages (Fig. 2) and swelling of alveolar epithelial cells with vascular congestion in the lung. Macrophages engulfing destroyed erythrocyte fragments were observed within the blood vessels of the interstitium of the lung (Fig. 3). There was serous exudation in the alveolar spaces of the areas affected with the interstitial pneumonia (Fig. 4). Megakaryocytes were rarely seen in the lungs. Multifocal necrosis of hepatocytes with proliferation of macrophages or fibrin exudation was present in the livers (Fig. 5). Marked congestion, megakaryocytes and yellow pigments (hemosiderin) were seen in the spleen. Focal hemorrhages were seen in the myocardium. Vascular congestion, fibrin thrombi and blood absorption were seen in the lymph nodes.

The histological lesions were observed in the native chicken and storks (Table 2). There were hepatocytic necrosis, splenic necrosis, interstitial pneumonia, and necrosis of the lamina propria of the intestine in the storks. The native chicken had cerebral necrosis and splenic necrosis. There were vasculitis and perivascular hemorrhages (Fig. 6) and perivascular necrosis (Fig. 7) in the cerebrum of the native chicken. Rarely focal necrosis of neurons was seen in the cerebrum (Fig. 8). Occasional necrosis of follicles with fibrinous exudation was seen in the spleen (Fig. 9). The cockatoo, pigeon and egret had no histological lesions.

Immunohistochemically, influenza virus antigens were detected in hepatocytic necrosis in the liver (Fig. 10) of the tigers (Table 1). There was no positive immunohistochemical reaction in the other organs of the tigers. The influenza virus antigens were present in the storks and native chicken (Table 2). The antigens were observed in the perivascular necrotic area (Fig. 11) and focal necrosis

Table 1. Histology and immunohistochemistry of four dead Bengal tigers infected with AIV

Organs	No. 1 (47A155-3)	No. 2 (47A155-4)	No. 3 (47A155-6)	No. 4 (47A155-7)
Liver	*	N++, C+++, A++	N+++, Y++, A+++	*
Spleen	C+, A–	C++, M+, A–	Y+, A–	*
Kidney	–, A–	–, A–	–, A–	–, A–
Heart	–, A–	H+, A–	H+, A–	*
Lung	*	IP++, A–	*	IP+, A–
Trachea	–, A–	–, A–	*	*
Pancreas	–, A–	–, A–	–, A–	*
Brain	*	*	–, A–	*
Intestine	*	*	*	–, A–
Lymph node	–, A–	B+, C+, A–	B+, C+, T+, A–	*

N: necrosis, C: congestion, M: megakaryocyte, Y: yellow pigment, H: hemorrhage,
IP: interstitial pneumonia, B: blood absorption, T: thrombus, *: not examined.
A: detection of influenza virus antigen.
Severity of lesions and antigen distribution; –: no, +: mild, ++: moderate, +++: severe.

Table 2. Histology and immunohistochemistry of eight dead birds infected with AIV

Organs	Cockatoo	Pigeon	Egret	Stork 1	Stork 2	Stork 3	Stork 4	Native chicken
Liver	–, A–	–, A–	–, A–	C+, A+	N+, A+	–, A–	–, A–	–, A+++
Spleen	–, A–	–, A–	–, A–	N+++, A+	N+++, A–	–, A–	–, A–	–, A++
Kidney	–, A–	–, A–	–, A–	*	*	*	–, A–	–, A++
Heart	–, A–	–, A–	–, A–	–, A–	–, A–	–, A–	–, A–	–, A++
Lung	–, A–	–, A–	–, A–	IP++, A–	–, A–	IP+, A–	–, A–	–, A–
Trachea	–, A–	–, A–	–, A–	–, A–	–, A–	–, A–	–, A–	–, A–
Pancreas	*	–, A–	–, A–	*	*	*	*	–, A–
Brain	–, A–	*	–, A–	*	*	–, A–	–, A–	N+, H++, A++
Intestine	–, A–	*	–, A–	–, A–	N+, A+	–, A–	–, A–	P+, A+
Gizzard	–, A–	–, A–	–, A–	*	*	*	*	–, A–
Proventriculus	–, A–	*	*	*	*	–, A–	–, A–	*

N: necrosis, H: hemorrhage, P: parasite, *: not examined.
A: detection of influenza virus antigen, IP: interstitial pneumonia.
Severity of lesions and antigen distribution; –: no, +: mild, ++: moderate, +++: severe.

(Fig. 12) of the cerebrum of the native chicken and in the lamina propria of the intestine of the stork (Table 2). In addition, virus antigens were noted in the intact cells; renal tubular epithelial cells (Fig. 13), myocardial cells (Fig. 14), and vascular endothelium (Figs. 13 & 15) of various organs in the native chicken or storks. Viral antigens were noted mainly in the nucleus of affected cells. There were no positive reactions in any organs of the cockatoo, pigeon and egret.

Discussion

Histological changes observed in our cases of tigers were similar to those of the tigers and leopards observed by Keawcharoen et al.[4] or Thanawongnuwech et al.[10]. They reported encephalitis and pneumonia in the tigers and leopards that were fed fresh chicken carcasses from a local slaughterhouse. Unfortunately we did not take brain samples in the pathological examination of three tigers, although small pieces of the cerebrum of a tiger (No. 3) were just examined. Keawcharoen et al.[4] did not refer to hepatocytic necrosis in their paper, while Thanawongnuwech et al.[10] reported multifocal necrotizing hepatitis.

Keawcharoen et al.[4] and Thanawongnuwech et al.[10] demonstrated influenza virus nucleoprotein antigen in the alveolar epithelial cells and bronchial epithelial cells. We only detected influenza virus nucleoprotein antigens in the livers of tigers.

Proliferation of macrophages in the lungs was reported in the chickens affected with infectious bursal disease, avian influenza and avian adenovirus causing acute fatal infections[6]. In them the erythrocytes were destroyed by the infection of these viruses followed by the proliferation of macrophages for engulfing the destroyed erythrocytes (virus-associated hemophagocytic syndrome). This syndrome was described in human cases affected with HPAIV[11]. Proliferation of macrophages that engulf destroyed erythrocytes was observed in the lungs of tigers in the present cases and may be similar to the changes in these reports. "Alveolar damage" including the exudation of fibrin, serum and neutrophiles in the alveolar spaces is characteristic of the changes that occur in the human HPAIV infection[3]. Serous exudation in the alveolar spaces was associated with the increase of macrophages in alveolar septa in the tigers of the present cases.

The histological lesions and immunohistochemistry of the native chicken and open-billed storks in the present cases were almost the same as those of natural reports of chickens[7,9]. H5N1 HPAIV induces the necrosis of neurons in the brain and liver necrosis with virus antigens[7]. We could not demonstrate HPAI infection in the cockatoo, pigeon and egret histologically and immunohistochemically. It is probable that the birds had just ingested something containing the virus without virus replication occurring in their bodies.

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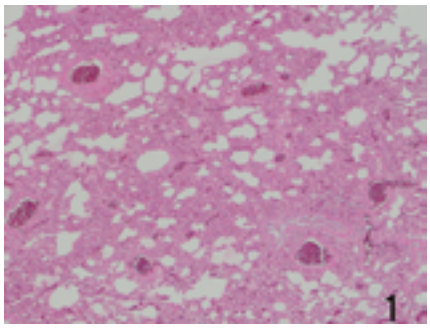

Fig. 1. Thickening of alveolar septa
Tiger No. 2. HE.

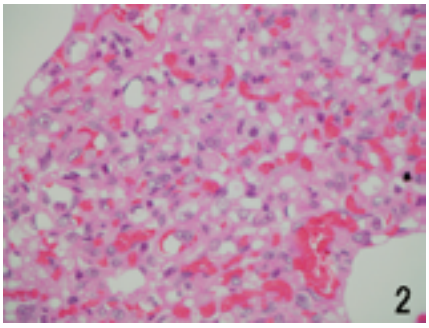

Fig. 2. Increase of macrophages in alveolar septa
Tiger No. 4. HE.

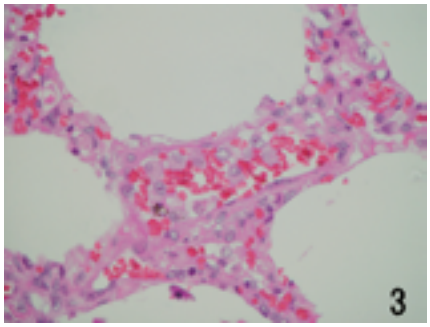

Fig. 3. Macrophages in the blood vessel of alveolar septa
Tiger No. 4. HE.

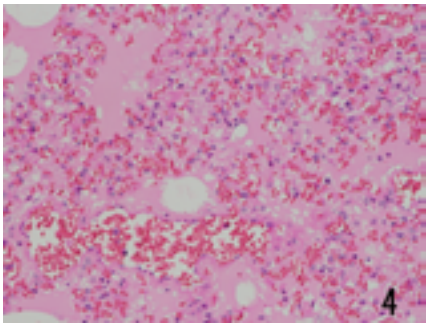

Fig. 4. Serous exudation in alveolar spaces
Tiger No. 2. HE.

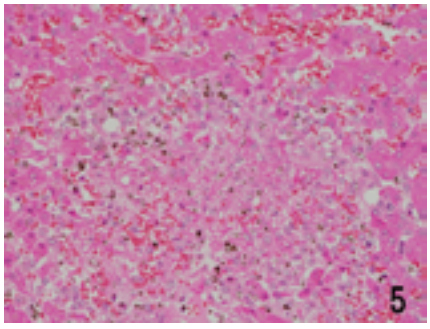

Fig. 5. Focal necrosis of hepatocytes
Tiger No. 3. HE.

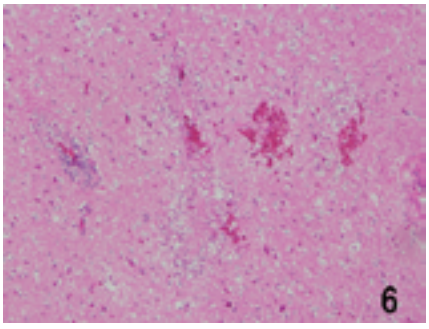

Fig. 6. Hemorrhage, vasculitis and perivascular necrosis in the cerebrum
Native chicken. HE.

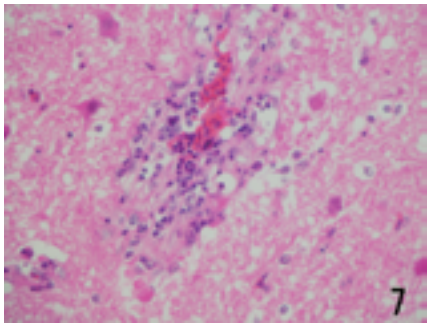

Fig. 7. Vasculitis and perivascular necrosis in the cerebrum
Native chicken. HE.

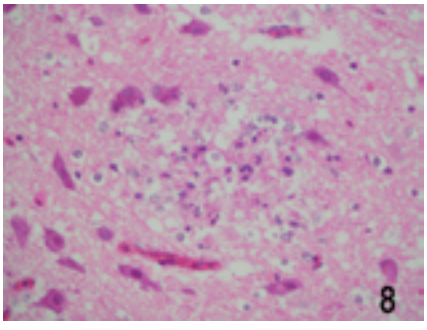

Fig. 8. Focal necrosis of the parenchyma in the cerebrum
Native chicken. HE.

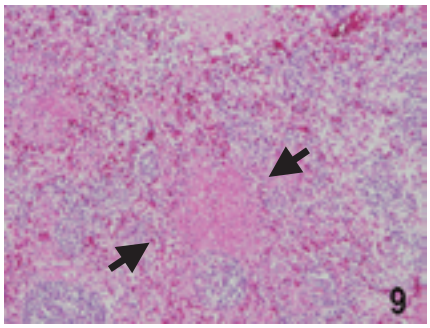

Fig. 9. Necrosis of the follicles in the spleen (arrows)
Native chicken. HE.

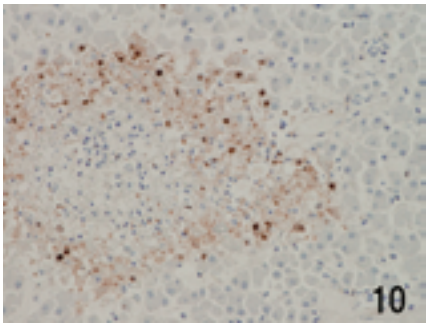

Fig. 10. Influenza virus antigen in necrotic area of liver
Tiger No. 3. IHC and hematoxylin.

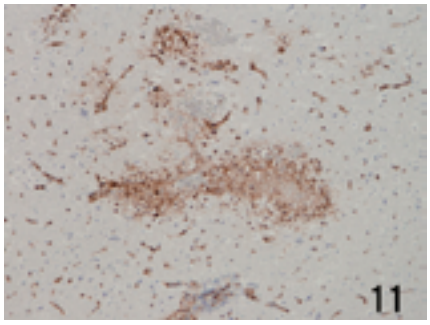

Fig. 11. Influenza virus antigen in the perivascular necrotic area of the cerebrum
Native chicken. IHC and hematoxylin.

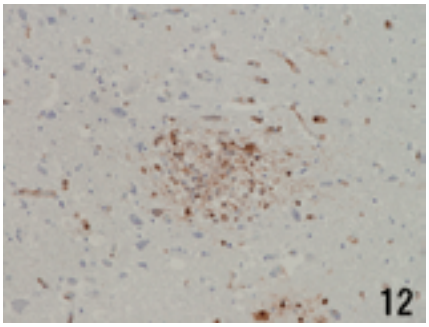

Fig. 12. Influenza virus antigen in necrotic area of the cerebrum
Native chicken. IHC and hematoxylin.

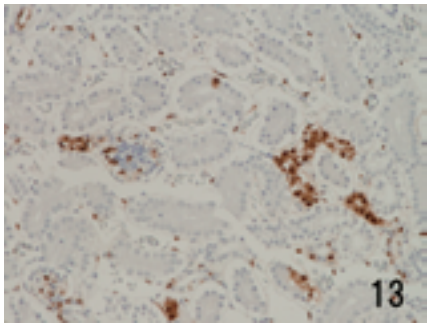

Fig. 13. Influenza virus antigen in the tubular epithelium and vascular endothelium of the kidney
Native chicken. IHC and hematoxylin.

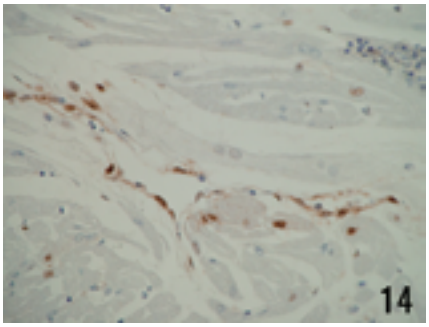

Fig. 14. Influenza virus antigen in the vascular endothelium and myocardial cells of the heart
Native chicken. IHC and hematoxylin.

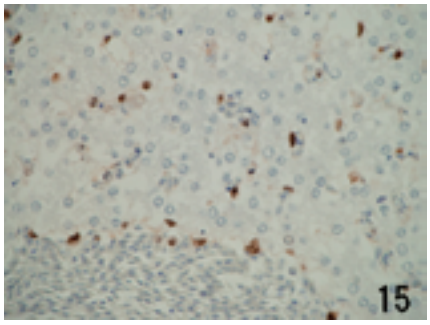

Fig. 15. Influenza virus antigen in the vascular endothelium of the liver
Native chicken. IHC and hematoxylin.

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