# *Comparative pathological findings in mute swans (*Cygnus olor*) naturally infected with highly pathogenic Avian influenza viruses H5N1 and H5N8 in Serbia*

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H5N1,
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Mute swans,
Pancreas,
Serbia.

**Summary**
The aim of this study was to compare pathological lesions and viral antigen expression in the organs of mute swans (*Cygnus olor*) naturally infected with highly pathogenic avian influenza virus subtypes H5N1 and H5N8. The examination was conducted on the carcasses of 22 mute swans which died during the avian influenza outbreaks in Serbia in 2006 and 2016-2017. Avian influenza virus subtype H5N8 isolated from mute swans in 2016-2017 was clustered within the 2.3.4.4 clade group B. After necropsy, lung, liver, spleen, pancreas, kidney and brain tissues were sampled for histopathology and immunohistochemical examination. Avian influenza virus nucleoprotein polyclonal antibodies were used for detecting the viral antigen in the examined tissues. The most significant gross lesions were necrosis and haemorrhages in the pancreas. Major histological lesions were multifocal necroses in the pancreas, spleen and liver, non-purulent encephalitis, lung congestion and oedema. Immunohistochemical demonstration of HPAIV nucleoprotein in pancreas and brain was strongly consistent with histological lesions in both infected groups. Our findings showed that pancreas was the most affected organ in all examined mute swans. In addition to increased mortality rate, similar pathological findings were detected in mute swans naturally infected with highly pathogenic avian influenza viruses H5N1 and H5N8.

## *Lesioni macro e microscopiche rilevate in cigni reali infettati con virus influenzali ad alta patogenicità sottotipi H5N1 e H5N8 in Serbia*

**Parole chiave**
Cigno reale,
H5N1,
H5N8,
Lesioni macro- e microscopiche,
Pancreas,
Serbia.

**Riassunto**
Scopo di questo studio è stato confrontare le lesioni macro e microscopiche e l'espressione dell'antigene virale negli organi di cigni reali naturalmente infetti con i sottotipi H5N1 e H5N8 del virus dell'influenza aviaria ad alta patogenicità. L'esame è stato condotto su 22 carcasse di cigni reali morti durante i focolai di influenza aviaria in Serbia nel 2006 e nel 2016-2017. Il ceppo H5N8, isolato nel biennio 2016-2017, appartiene al clade genetico 2.3.4.4 gruppo B. Dopo la necroscopia, polmone, fegato, milza, pancreas, reni e tessuti cerebrali sono stati campionati per gli esami istopatologici e immunoistochimici. Per rilevare l'antigene virale sono stati utilizzati anticorpi policlonali nei confronti della nucleoproteina del virus dell'influenza aviaria. Le lesioni macroscopiche più significative sono state necrosi ed emorragie nel pancreas. Le principali lesioni istologiche sono state necrosi multifocali nel pancreas, milza e fegato; encefalite non purulenta; congestione polmonare ed edema. In entrambi i gruppi infetti, i risultati dell'indagine immunoistochimica confermavano le lesioni istologiche del pancreas e del cervello. I risultati hanno dimostrato che il pancreas è stato l'organo più colpito in tutti i cigni reali esaminati e che oltre ad un aumento della mortalità, le lesioni patologiche rilevate nei cigni reali naturalmente infetti con virus influenzali ad alta patogenicità sottotipi H5N1 e H5N8 sono simili.

Highly pathogenic avian influenza (HPAI) viruses are considered to be a major concern worldwide because of their ability to cause significant mortality rates in the poultry industry, their pandemic potential and ability to infect humans. The diversity of influenza viruses in avian species and viral transmission between poultry and wild birds can result in genomic reassortment, making the new generations of influenza virus subtypes. Currently, the influenza A virus has been subtyped into 18 hemagglutinin (H1-18) and 11 neuraminidase (N1-11) (Tong *et al.* 2013). To date, all the avian influenza virus subtypes were isolated from aquatic birds, with the exception of H17N10 and H18N11 that are found in bats (Shi *et al.* 2014).

Avian influenza virus can infect domestic poultry and a wide range of avian species (Webster *et al.* 1992, Alexander 2000). Wild birds of the orders *Anseriformes* and *Charadriiformes* are considered as natural reservoirs of low pathogenic avian influenza (LPAI) viruses. LPAI viruses of subtypes H5 and H7 once they are introduced into poultry flocks may evolve into HPAI (Highly Pathogenic Avian Influenza) viruses. Up to worldwide massive HPAI H5N1 outbreaks in 2005-2006, HPAI viruses have been rarely detected in wild birds, followed by high mortality rate. Since these outbreaks, HPAI H5N1 has continued to cause illness and death in a variety of wild birds in Asia as well as in Europe (Feare 2010). Mute swans belong to the order *Anseriformes* and represent natural, asymptomatically infected reservoir for influenza viruses. However, during the HPAI H5N1 outbreaks in Europe, mute swans were one of the most frequently affected species of wild birds (Nagy *et al.* 2007, Brown *et al.* 2008, Feare 2010). Some authors characterized them as indicator species for avian influenza virus (Teifke *et al.* 2007, Terregino *et al.* 2006). Also, reports from many European countries clearly show that the European mute swan population was predominantly affected with the HPAI H5N8 infection (Brown *et al.* 2017). This findings suggests a significant role of mute swans in the ecology of HPAI H5 viruses (Nagy *et al.* 2007, Feare 2010).

Currently circulating HPAI virus subtype H5N8 in Europe originates as a product of several reassortment events. During worldwide massive outbreaks of HPAI H5N8 from 2014 to 2016, two distinct groups of H5N8 viruses were identified: group A (Buan-like) and group B (Gochang-like) (Lee *et al.* 2017). First outbreak of HPAI H5N8 in Europe was reported in 2014 and these viruses belong to group A. The second introduction of HPAI H5N8 in Europe began via the autumnal migration of wild birds in 2016 and these viruses belong to group B. Phylogenetic analyses of H5N8 clade 2.3.4.4 group B strains detected in 2016 in Europe showed significant differences between viruses from H5N8 clade 2.3.4.4 group A (Beerens *et al.* 2017, Pohlmann *et al.* 2017).

To date, outbreaks of highly pathogenic avian influenza viruses (HPAI) occurred twice in Serbia: in 2006 and 2016. These outbreaks occurred in wild birds as well as in domestic poultry. During these outbreaks, there were no significant economic losses in the poultry industry. In February 2006, in the laboratory of the Veterinary Institute "Kraljevo", Serbia, the presence of the highly pathogenic avian influenza virus, subtype H5N1, was confirmed in a swan carcass (Vasković *et al.* 2011). Ten years later, increased mortality among mute swans (*Cygnus olor*) has been detected since the end of November 2016, in Vojvodina province. On November 30$^{th}$, 2016 in the laboratory for virology of the Scientific Veterinary Institute "Novi Sad", Serbia, the presence of the HPAI subtype H5N8 was confirmed in a swan carcass. Genetic analyses of HPAI H5N8 showed that this virus belongs to clade 2.3.4.4 group B. This report of the outbreak was the first official notification of HPAI in Serbia since the epidemic of H5N1 in 2006. During these outbreaks, swans appeared to be highly susceptible and represented mainly reported affected species. Also, mute swans were the only wild bird species showing neurologic symptoms, including torticollis, ataxia, and incoordination (Božić *et al.* 2016). The purpose of this study was to describe and compare pathological findings, presence and type of histological lesions and viral antigen distribution in mute swans that were naturally infected with HPAI viruses H5N1 and H5N8, in the Republic of Serbia.

Fifteen adult mute swans (*Cygnus olor*) that died during the HPAI H5N8 outbreak have been analysed in this study. Mute swan carcasses were collected during avian influenza epidemic outbreaks from November 2016 to January 2017. All of the cases occurred either in the area of Special Nature Reserve "Koviljsko - Petrovaradinski Rit" or in nearby areas, along with the bank of the river Danube in Northern Serbia, Province of Vojvodina.

Morphologic lesions and viral nucleoprotein expression in seven mute swans naturally infected with HPAI H5N1 (tissue samples collected during the H5N1 outbreak 2006) were re-evaluated (Vasković *et al.* 2011) in order to be compared with morphological lesions and viral nucleoprotein expression in fifteen H5N8+ swans (H5N8 outbreak, 2016-2017).

The detection of H5N8 HPAI virus presence was performed in pooled 10% tissue suspensions (lungs, brain, duodenum, and kidney) and selected cloacal content in PBS by molecular diagnostic methods. Briefly, total RNA was extracted using TRIzol® reagent (Invitrogen, Life Technologies) according to the manufacturer recommendations. The presence of avian influenza virus and confirmation of virus subtype H5 was detected by identification of

the matrix (M) and H5 gene by real-time reverse transcription PCR (real-time RT-PCR) as described by Spackman and colleagues (Spackman *et al.* 2002), and by using RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Life Technologies). In addition, N8 gene was detected by conventional RT-PCR technique as described by Fereidouni and colleagues (Fereidouni *et al.* 2009), by using OneStep RT-PCR kit (Qiagen, Germany). A highly pathogenic pathotype was confirmed by Sanger sequencing of the hemagglutinin (HA) gene as described by Slomka and colleagues (Slomka *et al.* 2012).

Tissues available for histopathology from the selected H5N8+ swans included kidney, spleen, pancreas, heart, lungs, liver and brain. Collected tissues were fixed for 24 hours in 10% buffered neutral formaldehyde and processed for paraffin embedding. Paraffin-embedded sections were cut (4-5 µm) and stained with haematoxylin and eosin (HE). In addition, serial sections were immunohistochemically analysed to determine the distribution of influenza virus antigens in individual tissues.

Immunohistochemical (IHC) technique was performed using the Novolink Polymer Detection System (Novocastra, Newcastle-upon-Tyne, UK). Antigen retrieval was achieved by heating the sections in a microwave oven at 560 W for 21 minutes in a citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation of the slides with Novocastra Peroxidase Block, and nonspecific binding sites were blocked with Novocastra Protein Block. After antigen retrieval and inactivation of endogenous peroxidase, the sections were incubated with the rabbit anti-nucleoprotein serum kindly provided by Dr. Jens P. Teifke (the Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany) diluted in phosphate buffer saline at ratio 1:1000 for 1 h in a humid chamber at room temperature. After primary and secondary antibody treatment (Novolink polymer; Novocastra), the immunoreaction was visualized using diaminobenzidine tetrahydrochloride (DAB) solution and counterstained with Mayer's haematoxylin.

In general, all of fifteen mute swans infected with HPAI H5N8 virus were in good body condition, with sufficient body fat reserves. Noticeably, all swans showed no external gross lesions. Four infected swans had diarrhoea and showed dark brown discoloured feathers around the cloaca. In three swans, bleeding from beak and nostrils was present. At necropsy, the most common lesions were multifocal, sharply demarcated necrosis and haemorrhages in the pancreas that were found in all swans (Figure 1a). Haemorrhages in mesenteric adipose tissue were also a consistent finding. Characteristic but not present in all infected swans was a congestion and oedema of the lungs. In trachea and nasal cavity no macroscopic lesions were revealed. Haemorrhages in subepicardium with scattered myocardial ecchymosis were present in all swans. In many cases, liver and spleen were moderately enlarged and congested. Haemorrhages in the duodenal mucosa and presence of bloody mucinous exudate in the lumen of small intestine were present in five examined swans. In two swans, haemorrhagic exudate was found in the lumen of the oesophagus. Haemorrhages in muscles of the neck, intercostal and pectoral muscles were present in few cases. No macroscopic visible lesions in kidneys were found. Hyperaemia was clearly visible in the brain and meninges (Figure 1b).

Histopathological examination revealed lesions in the lungs, heart, spleen, kidneys, pancreas and brain in the majority of swans infected with HPAI H5N8 virus. The most prominent lesions were haemorrhages and necrosis of pancreas (15/15), as well as non-purulent encephalitis (15/15), haemorrhages in subepicardium (15/15) followed by necrosis of liver and spleen (10/15 cases). Haemorrhages were observed in the lungs, heart, spleen, kidneys and brain. In addition to haemorrhages, the lungs showed evidence of congestion and oedema. In one case, interstitial pneumonia was observed. Endothelial cells and macrophages in the lumen of alveoli were positive to viral nucleoprotein. In the liver parenchyma focal areas of hepatocellular necrosis and lymphocytic infiltrate and macrophages were present. Presence of virus antigen was evident in the necrotic hepatocytes and in the sinusoidal endothelium of the liver. The spleen showed multifocal necrosis of the lymphoid tissues and hemosiderosis as a result of massive haemorrhages. Huge amounts of anti-nucleoprotein antibody were distributed in the necrotic areas of the spleen, as well as in the macrophages and endothelial cells of blood vessels. As for the kidney, necrosis of tubules was evident. Virus antigen was observed in the tubular epithelium and glomerular capillary endothelium of kidney. Punctate to massive haemorrhages were located subepicardially and myocardially, too. Lymphocytic infiltrate was also present in perimysium in several cases. A small number of cardiomyocytes was positive. The pancreas showed multifocal acinar necrosis with mononuclear cell infiltration (Figure 2a). Viral antigen was detected in the cytoplasm and nucleus of necrotic cells as well as in the macrophages (Figure 2b). Furthermore, histopathological examination showed neuronal satellitosis, neuronophagia and mild lymphocytic perivascular cuffing in the cerebrum. In two cases encephalomalacia was observed. The cerebellum showed massive haemorrhages, focal necrosis and lymphocytic perivascular cuffing (Figure 2c). Lymphocytes infiltrate meninges in almost all the

cases. Viral antigen was detected in the cytoplasm and nucleus of neurons and glial cells of the cerebrum and cerebellum (Figure 2d).

Major macroscopic, histological lesions and distribution of influenza virus antigen in mute swans naturally infected with HPAI (H5N8 subtype) are summarized in Table I.

During HPAI outbreaks in Serbia in 2006 and 2016, mute swans were the most affected wild bird species. The introduction of H5N8 clade 2.3.4.4 group B in Serbia in 2016 also resulted in great proportion of diseased mute swans showing central nervous system involvement, such as incoordination and ataxia (Božić *et al.* 2016). These clinical signs were

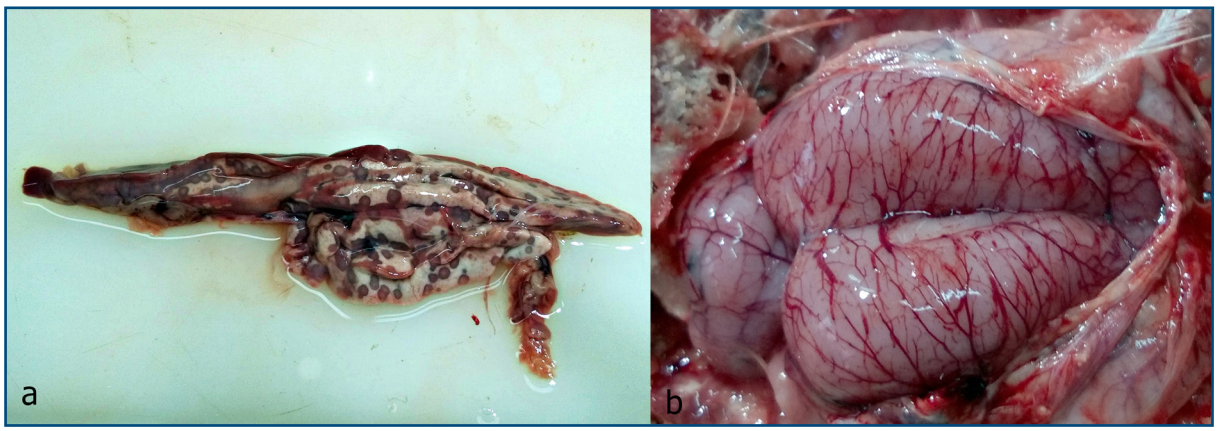


**Figure 1. a)** *Pancreas, mute swan, H5N8+*. Multifocal necrosis and haemorrhages. **b)** *Brain, mute swan, H5N8+*. Hyperaemia.

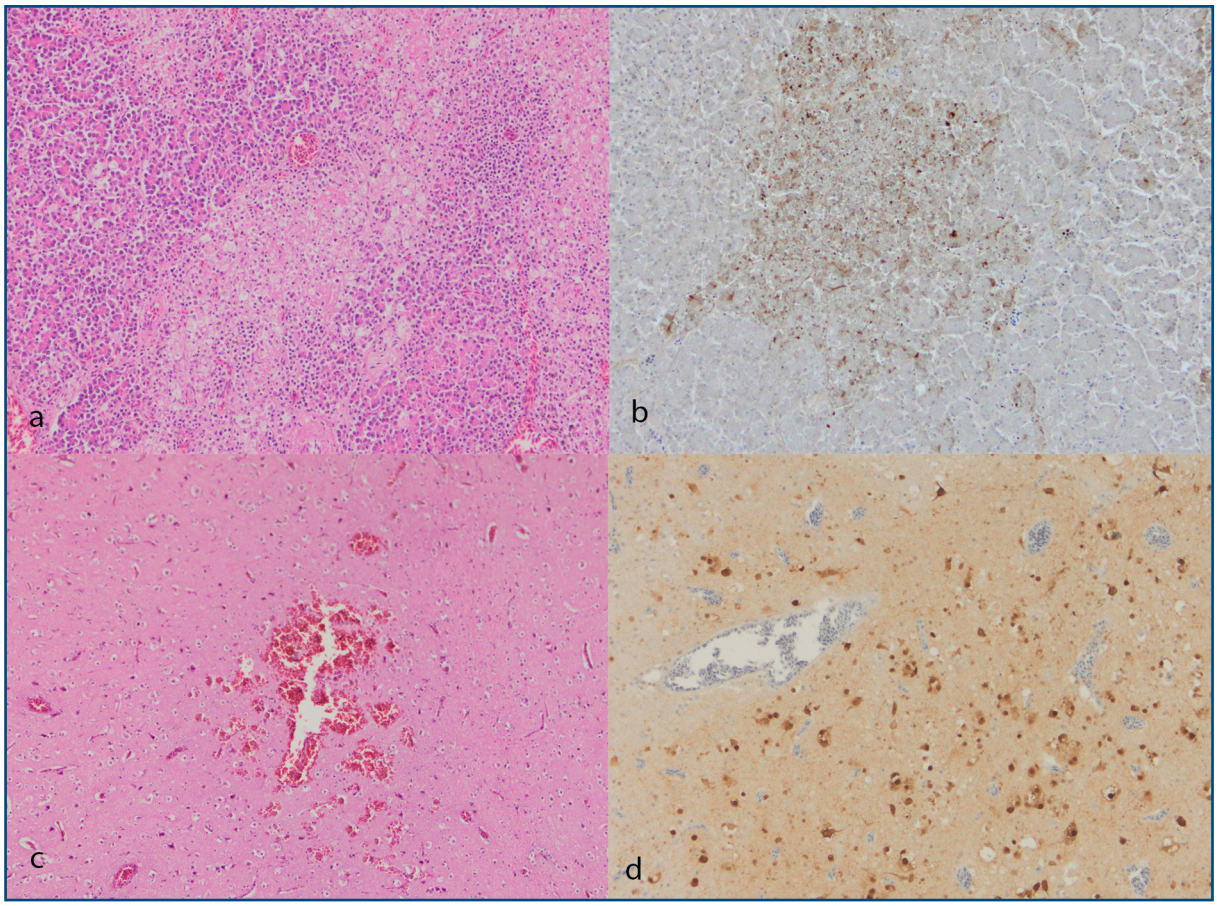


**Figure 2. a)** *Pancreas, mute swan, H5N8+*. Necrosis, HE, original magnification x200. **b)** *Brain, mute swan, H5N8+*. Haemorrhages, HE, original magnification x100. **c)** *Pancreas, mute swan, H5N8+*. Intranuclear and intracytoplasmic staining for AIV nucleoprotein, Novolink™ Polymer Detection System, original magnification x200. **d)** *Brain, mute swan, H5N8+*. Intracytoplasmic staining for AIV nucleoprotein in cortical neurons, Novolink™ Polymer Detection System, original magnification x200.

not noticed in swans infected with HPAI H5N1. Vasković and colleagues (Vasković *et al.* 2011) first described pathomorphologic lesions in 7 mute swans infected with HPAI H5N1 in Serbia. The most frequent gross lesions included haemorrhages and necrosis in the pancreas, lung congestion and oedema, congestion and enlargement of liver and spleen, congestion of kidneys, haemorrhages in the small intestine, rectal mucosa and tonsils in the caecum. The most consistent histological lesions were non-purulent encephalitis with a perivascular accumulation of lymphocytes, lung congestion and oedema, haemorrhages and hepatocellular necrosis in liver and spleen, haemorrhages and necrosis of the exocrine pancreas cells. Histological lesions were not found in kidneys. The viral nucleoprotein was most frequently detected in the pancreas (7/7), liver (5/7), lung (4/7), spleen (3/7) and brain (1/1). Viral antigen was detected in the kidney tubules in one swan (1/7).

Comparing these two groups, it has been found that the most dominant gross lesions found in all infected mute swans were multifocal pancreatic necrosis and haemorrhages. Pancreatic necrotic fields were irregularly shaped, different sizes, ranging from a few millimetres to one and a half centimetres in diameter in both infected groups. Necroses of the exocrine acinar cells were detected in all tested samples of pancreatic tissue. An identical finding was obtained in swans naturally infected with H5N1 in Germany (Teifke *et al.* 2007) as well as in wild birds naturally infected with H5N8 in South Korea (Kim *et al.* 2015). Acute necrotizing pancreatitis is a common finding of HPAIV infections as a result of the systemic replication (Teifke *et al.* 2007, Bertran *et al.* 2011, Božić *et al.* 2018) and possibly the main cause of death. Immunohistochemical findings revealed the presence of HPAI H5N1 and H5N8 virus antigen in the cytoplasm and nucleus of necrotic cells as well as in the macrophages, in common with previous reports (Kwon *et al.* 2005, Kim *et al.* 2015).

Subepicardial haemorrhages were dominant finding in swans infected with H5N8, while it was less common finding in swans infected with H5N1 (Vasković *et al.* 2011). Haemorrhages of subcutaneous tissue and serosa were present in a few infected swans. Lung congestion and oedema were common findings, while it was more severe in swans infected with HPAI H5N8. A very striking macroscopic lesion found in mute swans infected with H5N8 was haemorrhages in mesenteric adipose tissue. These pathological lesions were not detected in swans infected with H5N1 subtype (Vasković *et al.* 2011). Half of the investigated H5N8 and H5N1 infected mute swans had enlarged and congested liver and spleen. In both infected groups, nasal mucosa, cloaca, gonads and skin were without lesions.

In swans infected with HPAI H5N1, kidneys were enlarged and congested, while there was no visible macroscopic lesion in kidneys of swans infected with HPAI H5N8. The histopathologic findings of renal tissue in swans infected with H5N1 and H5N8 differed. Renal tubular necrosis was evident and virus antigen was observed in the tubular epithelium and glomerular capillary endothelium in swans infected with H5N8. The antigenic staining patterns were similar and lesions detected in kidneys corresponded to those observed in wild birds infected with H5N8 (Kim *et al.* 2015). There were no histopathologic lesions in kidneys of swans infected with H5N1, and virus antigen expression was detected in only one case. Teifke and colleagues (Teifke *et al.* 2007) and Kwon and colleagues (Kwon *et al.* 2010) reported that was no evidence of histopathologic lesions in the kidneys of waterfowl infected with H5N1, though experimental infection

**Table I.** *Frequency of occurrence of macroscopic, histological lesions and distribution of viral antigen it the tissues from 15 mute swans naturally infected with HPAI H5N8.*

| Organ | Histopathological lesions | IHC positive/ analyzed organs | Viral antigen positive cells |
|---|---|---|---|
| Pancreas | Haemorrhages<br>Multifocal acinar necrosis | 15/15 | Acinar necrotic cells, macrophages |
| Heart | Haemorrhages in subepicardium, Intramyocardial ecchymoses<br>Lymphocytic infiltrate in perimysium | 6/15 | Cardiomyocytes |
| Lung | Congestion Oedema Haemorrhages | 11/15 | Endothelial cells and macrophages |
| Spleen | Multifocal necrosis<br>Massive haemorrhages and hemosiderosis | 12/15 | Macrophages and endothelial cells of blood vessels |
| Liver | Hepatocellular necrosis<br>Lymphocytic infiltrate | 12/15 | Necrotic hepatocytes |
| Kidneys | Necrosis of tubules | 13/15 | Tubular epithelium and glomerular capillary endothelium |
| Brain | Neuronal satellitosis neuronophagia<br>Lymphocytic perivascular cuffing<br>Encephalomalacia | 15/15 | Neurons and glial cells |

studies have shown that H5N1 virus infects the tubular epithelium. Distinctly initiated blood vessels of the brain and meninges were consistently present in most swans infected with both H5N8 and H5N1. In the cerebrum, a large number of degenerating neurons and glial cells showed strong nuclear and cytoplasmatic reaction of virus antigen, in both infected groups.

Most HPAI viruses replicate into endothelial cells, leading to increasing vascular permeability and causing oedema and haemorrhage in the different organs (Pantin-Jackwood and Swayne 2009). Pancreatic lesions are the most prominent finding in swans naturally and experimentally infected with highly pathogenic avian influenza viruses (Pálmai *et al.* 2007, Kalthoff *et al.* 2008, Vasković *et al.* 2011, Teifke *et al.* 2007, Kim *et al.* 2015). Our findings also showed that pancreas was the most affected organ in all examined mute swans infected with both HPAI H5N1 and H5N8 suggesting that mute swans are highly susceptible to infection with both HPAI H5N1 and H5N8 viruses.

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