

**PATHOMORPHOLOGICAL LESIONS AND DISTRIBUTION OF VIRAL ANTIGEN IN BIRDS
INFECTED WITH THE PATHOGENIC STRAIN OF H5N1 AVIAN INFLUENZA VIRUS**

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*The aim of this study was to determine pathomorphological lesions in the organs of birds infected with H5N1 avian influenza virus, presence and distribution of the viral antigen in tissue samples and the degree of their correlation. Examination was conducted on the carcasses of seven mute swans (*Cygnus olor*) and one domestic rooster (*Gallus gallus domesticus*). After necropsy, trachea, lungs, liver, spleen, pancreas, kidney and intestine tissues were sampled for histopathology and immunohistochemical examination. LSAB method and H5N1 avian influenza virus nucleoprotein polyclonal antibodies were used for detecting the viral antigen in the examined tissues.*

The most frequent gross lesions were hemorrhages and necroses of the pancreas. Major histological lesions were multifocal necroses in the pancreas, liver and spleen, non purulent encephalitis, with neuronal dystrophy and neuronophagia. Viral nucleoprotein was immunohistochemically proven in the pancreas, brain, liver, lungs, and in one swan in the kidney. A high correlation between pathomorphological lesions and distribution of the viral antigen was determined.

Key words: brain, H5N1, immunohistochemistry, pancreas, pathomorphological lesions, swan

INTRODUCTION

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. At that moment, a large number of European countries, including our neighboring states, reported the occurrence of this virus in wild birds and domestic poultry to the OIE. The public was focused on avian influenza, because of possible major economic losses, as well as possible threats to the health of the human population. During the following two months, a large number of wild aquatic birds and domestic poultry from all over Serbia were examined for the avian influenza virus. For most of birds, including swans, the

cause of death was exhaustion, and probably lack of food, common for the weather conditions and migration patterns at that time, but also mechanical trauma, bacterial and fungal infections. The presence of avian influenza virus was confirmed in the carcasses of one domestic rooster and seven mute swans. According to available data literature, mortality caused by avian influenza was relatively rare in wild waterfowl, usually restricted to individual cases. Swans, as representatives of the order Anseriformes, together with wild geese and ducks, are marked as the main reservoir of the virus, in which the disease usually runs asymptotically and the mortality is low (Hinshaw *et al.*, 1986; Perkins and Swayne, 2002; Ellis *et al.*, 2004). However, several recent studies (Ellis *et al.*, 2004; Teifke *et al.*, 2007; Brown *et al.*, 2008), as well as reports of a relatively large number of dead swans in several European countries in which the avian influenza virus was isolated, testifies to the fact that the swans are one of the least resistant to the virus of all waterfowl birds of the order Anseriformes, and may represent a very important species for studying the epidemiology of avian influenza. Some authors even say that swans are not a biologically important vector of avian influenza, but rather "closing hosts" in its transmission (Nagy *et al.*, 2007), and that they could be used, after complete clarification of disease pathogenesis and species susceptibility, as avian influenza indicators in wildlife (Teifke *et al.*, 2007).

MATERIALS AND METHODS

Gross examination

Examination was done on the organs (brain, trachea, lungs, liver, spleen, pancreas, kidney and intestine) of seven mute swans (*Cygnus olor*) and one domestic rooster (*Gallus gallus domesticus*) in which the highly pathogenic avian influenza virus, subtype H5N1, was previously proven by virological methods, virus isolation in chicken embryos and molecular techniques. The carcasses of these birds were shipped to the Veterinary Institute Kraljevo laboratory from two locations in western and north – western Serbia.

Histopathology

After necropsy and gross examination, tissues of the brain, trachea, lungs, liver, spleen, pancreas, kidney and intestine were taken for pathohistological and immunohistochemical studies. Samples were fixed in 10% buffered formalin for 48 hours, and after standard processing in an automated tissue processor were cast in paraffin blocks. Paraffin sections, 4-5 μm thick, were stained with haematoxylin and eosin (HE).

Immunohistochemistry

The labeled streptavidin-biotin (LSAB) method was used for immunohistochemical staining (ChemMate, DAKO, Denmark). After dewaxing and rehydration, antigen retrieval (citric buffer, 560 W, 21 min) was done. Inhibition of endogenous peroxidase was obtained by treatment of sections in 3% solution of hydrogen peroxide in methanol for 15 minutes. Sections were incubated with normal goat serum for 30 minutes at room temperature. In order to

detect the avian influenza antigen, sections were incubated with a rabbit antinucleoprotein serum kindly provided by Dr. Jens P. Teifke (the Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany) (1:500 dilution in TBS). A biotinylated goat anti-rabbit immunoglobulin (ChemMate, DAKO, Denmark) was used as the secondary antibody. All sections were incubated with streptavidin-peroxidase complex (ChemMate, DAKO, Denmark) for 20 minutes at room temperature. Reaction was visualised by using diaminobenzidine tetrahydrochloride (Liquid DAB, DAKO, Denmark) or 3-amino-9-ethylcarbazole (AEC, ChemMate, DAKO, Denmark) and counterstained with Mayer's haematoxylin. As a negative control, slides were incubated with PBS instead of primary antibodies.

RESULTS

Gross examination

The dominant findings were lesions in the pancreas, which were found in all birds, in the form of disseminated and patchy, demarcated hemorrhages and necrotic fields of irregular shapes, different sizes, from a few millimeters to one and a half centimeters in diameter (Fig. 1a). In two swans, petechiation of subcutaneous tissue and seroses of the sternum was present. Blood vessels of the brain and meninges were initiated (Fig. 1b). Lung congestion and edema were common findings. Subepicardial hemorrhages were found in two swans. In most cases, the liver and spleen were enlarged and congested, some of them with subcapsular hemorrhages and focal necroses. In two swans, kidneys were enlarged and congested. The mucosae of the small intestine and rectum were diffusely hyperemic, in some cases hemorrhages were present in the small intestine, rectal mucosa and cecal tonsils. In the rooster, cyanosis of the comb and distal parts of the legs were observed. Blood vessels of the brain and meninges were initiated. Tracheal mucosa was diffusely hyperemic, a blood clot was present in the lumen. The lungs and the liver were congested. Hemorrhages and necroses of pancreas were observed. Hemorrhages were also observed on duodenal, rectal and proventricular mucosae.

Histopathology

Non-purulent encephalitis with perivascular accumulation of lymphocytes and plasmacytes was found in the cerebrum. Dystrophy, necroses of nerve cells and neuronophagia were also present. Lung congestion and edema were observed. In the liver, hemorrhages and focal areas of hepatocellular necrosis with mononuclear cells and heterophils, were found (Fig. 1c). Surrounding hepatocytes were swollen and infiltrated with mononuclears and heterophiles. A similar finding was noted in the spleen, where multifocal coagulative necrosis of lymphoid tissue was observed. In necrotic debris and surrounding tissue, lymphocytes, macrophages and heterophils were present. Karyorexis of lymphoid cells was observed. The predominant findings in the pancreas were also multifocal hemorrhages and necroses of exocrine acinar cells (Fig. 1d). Areas of coagulative necrosis were infiltrated with mononuclears and heterophils.

These cells were also found in the surrounding tissue that showed signs of mild to moderate inflammation. In the small intestine, desquamation of surface epithelium was observed. Propria mucosae and submucosa were infiltrated with heterophils, which were also present in the exudate between the intestinal villi. Trachea and kidneys had no histological lesions.

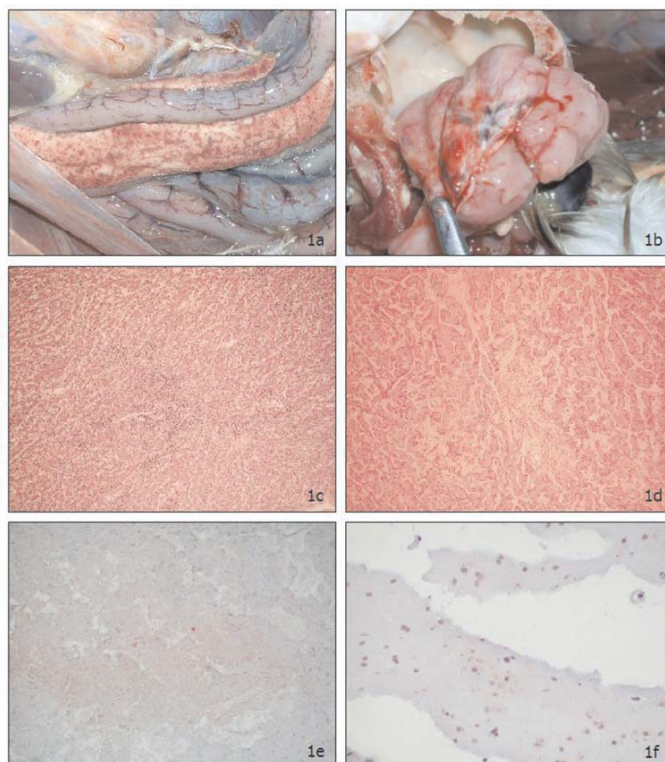


Figure 1. a) Pancreas; mute swan. Multifocal necrosis and hemorrhages. b) Brain; mute swan. Initiated blood vessels. c) Liver; mute swan. Necrosis, HE, x100. d) Pancreas; mute swan. Necrosis, HE, x100. e) Pancreas; mute swan. Against AIV nucleoprotein, LSAB, x400. f) Brain; mute swan. Against AIV nucleoprotein, LSAB, x400

Immunohistochemistry

The viral nucleoprotein was most frequently detected in tissues of the pancreas, brain and liver. In the pancreas, it was detected in all samples, in areas of necrosis, in necrotic debris, and in cytoplasm and nucleus of exocrine acinar cells (Fig. 1e). In the cerebrum, the viral antigen was observed in the cytoplasm and nucleus of neurons and glial cells (Fig. 1f). In the lungs, it was detected in the nucleus and cytoplasm of endothelial cells of blood vessels. Positive immunohistochemical signal was noted in the liver, in the necrotic areas, in nuclei

and cytoplasm of hepatocytes, blood vessels endothelial cells and macrophages. In the spleen, it was also noted in the areas of necrosis, the nucleus and cytoplasm of mononuclear cells and blood vessels endothelial cells. In one sample, the viral antigen was detected in the cytoplasm and nucleus of tubular epithelial cells and blood vessels endothelium of the kidneys. In examined tissues of the trachea and small intestine, there was no positive immunohistochemical signal of the presence of avian influenza virus antigen.

Table 1. Major histological lesions and distribution of influenza virus antigen

Organ	Histopathology	IHC positive	Cell types
Brain	Non-purulent encephalitis, neuronophagia	1/1	Neurons, glial cells
Trachea	No lesions	0/8	No lesions
Lung	Edema and congestion	4/8	Vascular endothelium
Liver	Multifocal necrosis	5/8	Hepatocytes
Spleen	Multifocal necrosis	3/8	Lymphoid cells
Pancreas	Multifocal necrosis	8/8	Exocrine acinar cells
Small intestine	Catarrhal enteritis	0/8	No lesions
Kidney	No lesions	1/8	Tubular epithelium

DISCUSSION

In all examined birds, the lesions in the pancreas, in the form of multifocal necrosis and hemorrhages, were the most intensive and the most frequent gross finding. This finding was confirmed by histological examination: coagulative necrosis of exocrine acinar cells was found in all samples, including the rooster (100%). Same results were obtained in naturally infected swans in Germany (Teifke *et al.*, 2007), while in different species of experimentally infected swans the dominant finding were pancreatic hemorrhages, and multifocal necrosis was found in a much smaller percentage (Kalthoff *et al.*, 2008). In experimentally infected galliform birds, multifocal coagulative necrosis of pancreatic acinar cells was found in a high percentage (55 – 86%) (Perkins and Swayne, 2001). These lesions were not obtained in the area of Langerhans islets, but only in exocrine cells areas, and were surrounded with mild to moderate inflammation. Lesions in the brain, including viral encephalitis, which were obtained in the examined birds, coincide with findings of other authors in naturally and experimentally infected swans (Teifke *et al.*, 2007; Kalthoff *et al.*, 2008) and domestic fowl (Perkins and Swayne, 2001; Nakatani *et al.*, 2005).

Hemorrhages in the subcutaneous tissue and the sternal serosa were relatively frequent (57% and 43%). Theifke *et al.* (2007) did not observe this kind of lesion in naturally infected swans, but they were found in swans experimentally

infected with avian influenza virus (Kalthoff *et al.*, 2008). In the rooster, subcutaneous hemorrhages were not found, but cyanosis of the comb, bibs, and the skin of the distal parts of the legs was noticed. Similar lesions, including facial edema, hemorrhages and necrosis of pectoral muscles, were found in a high percentage (80%) in experimentally infected domestic fowl (Perkins and Swayne, 2001).

Lung congestion and edema were found in four swans (57%) and the rooster. Identical findings, including hemorrhages in lungs parenchyma, were reported by other authors (Perkins and Swayne, 2001; Teifke *et al.*, 2007; Kalthoff *et al.*, 2008).

Subepicardial hemorrhages were observed in two swans (29%). In naturally infected swans it was a sporadic finding (Teifke *et al.*, 2007), while in experimentally infected swans these lesions were much more frequent and pronounced (Kalthoff *et al.*, 2008). Such lesions were not found in the rooster, although they were reported in experimentally infected galliform birds, mostly in quails and pheasants (Perkins and Swayne, 2001).

Lesions in the liver and spleen were also significant. Hemorrhages, congestion and multifocal coagulative necrosis of hepatocytes and lymphoid cells, were found in naturally infected swans and domestic fowl (Perkins and Swayne, 2001; Teifke *et al.*, 2007; Kalthoff *et al.*, 2008). The same findings, including a mild inflammation, were observed in four (57%) and three (43%) swans in our study.

In two swans, kidney swelling and congestion were macroscopically observed, while no histological lesions were found. They were not found in the kidneys of naturally and experimentally infected swans either (Teifke *et al.*, 2007; Kalthoff *et al.*, 2008). However, the discrete necrosis of tubular epithelial cells has been noticed in experimentally infected pheasants, quails and turkeys (Perkins and Swayne, 2001).

Hemorrhages of proventricular mucosa were found in the rooster, and the same lesions were observed by other authors in experimentally infected domestic fowl (Perkins and Swayne, 2001).

Avian influenza virus was detected in all eight samples (100%) in exocrine acinar cells of pancreas. In the same cells it was detected in a very high percentage in naturally and experimentally infected swans and domestic fowl (Perkins and Swayne, 2001; Teifke *et al.*, 2007; Brown *et al.*, 2008; Kalthoff *et al.*, 2008).

Intensive immunohistochemical reaction was also detected in neurons and glial cells of the cerebrum, which is in accordance with other findings in swans and domestic fowl (Perkins and Swayne, 2001; Teifke *et al.*, 2007; Kalthoff *et al.*, 2008).

The viral antigen was frequently detected in the lungs (50%), liver (62%) and spleen (38%), which is also very similar to findings of other authors (Perkins and Swayne, 2001; Teifke *et al.*, 2007; Brown *et al.*, 2008; Kalthoff *et al.*, 2008). Viral antigen was detected in the kidney tubules in one swan (14%), while the same finding, but in a higher percentage, was reported by other authors (Teifke *et al.*, 2007; Kalthoff *et al.*, 2008). It was not detected in the small intestine and the trachea, and similar results were obtained in naturally and experimentally infected

swans, in which the virus was only detected in the trachea, in a minor percent (Teifke *et al.*, 2007; Kalthoff *et al.*, 2008).

Distribution of the viral antigen was in high correlation with the morphological lesions. It was most consistent in the brain and the pancreas. Having in mind the gross lesions in the pancreas, which were intensive and found in all samples, their association with the presence of the virus, and literature data (Selleck *et al.*, 2003), it can be concluded that the pancreas is one of the most important organs for the diagnosis of avian influenza in swans. Lesions in mute swans and domestic rooster were similar in character and distribution. Established findings are very similar to the findings in swans infected with H5N1 avian influenza virus in other European countries.

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PATOMORFOLOŠKE PROMENE I DISTRIBUCIJA VIRUSNOG ANTIGENA KOD PTICA INFCIRANIH PATOGENIM SOJEM H5N1 VIRUSA AVIJARNE INFLUENCE

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SADRŽAJ

Cilj ovog ispitivanja je bio utvrđivanje patomorfoloških promena na organima ptica inficiranih H5N1 virusom avijarne influence, imunohistohemijsko utvrđivanje prisustva i distribucije virusnog antigena u parafinskim isečcima uzoraka njihovih organa, kao i utvrđivanje stepena njihove korelacije. Ispitivanje je izvršeno na organima sedam labudova grbaca (*Cygnus olor*) i jednog petla (*Gallus gallus domesticus*). Nakon izvršene obdukcije i makroskopskog pregleda svih organskih sistema, izvršeno je uzorkovanje tkiva mozga, traheje, pluća, jetre, slezine, pankreasa, bubrega i creva za patohistološka i imunohistohemijska ispitivanja. Korišćena je LSAB imunohistohemijska metoda sa poliklonskim antitelima na nukleoprotein virusa avijarne influence podtipa H5N1.

Dominantne promene utvrđene makroskopskim pregledom bile su krvavljenja i nekroze pankreasa. Histološkim pregledom su ustanovljene multifokalne nekroze pankreasa, jetre i slezine, a u velikom mozgu promene u tipu negnojnog encefalitisa, praćene distrofijom i nekrozom nervnih ćelija i pojavom neuronofagije. Virusni nukleoprotein je imunohistohemijski dokazan u pankreasu, mozgu, jetri, plućima, slezini, a kod jednog labuda i u bubrezima. Utvrđen je visok stepen korelacije između ustanovljenih patomorfoloških promena i distribucije virusnog antigena.