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## **Histopathological findings in Dalmatian pelicans (*Pelecanus crispus*) naturally infected with avian influenza subtype A H5N1 in Bulgaria**

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**ABSTRACT.** The aim of this study is to estimate the histopathological changes in visceral organs of naturally infected with the avian influenza virus (AIV) subtype A H5N1 dalmatian pelicans in Bulgaria. The identified gross lesions are: haemorrhagic small intestine, sparse content in gizzard and proventriculus, well defined hyperemia of the tracheal mucosa associated with petechiae, as well as meningeal and brain congestion. The infected birds exhibited the following histopathological changes: edema of the tracheal mucosa with loss of mucosal glands, mild to moderate congestion with focal necrosis and multifocal non suppurative encephalitis and gliosis, mononuclear infiltration in the cecum, and diffuse mononuclear infiltration in the submucosa of the small intestine. The virus was detected by virus isolation (VI) and RT-PCR from tissue samples (lung, trachea, small intestine, brain, proventriculus, cloaca) from the infected birds.

**Keywords:** H5N1, Bulgaria, histopathology, natural infection.

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## Abbreviations

AIV	avian influenza virus
ECE	embryonated chicken eggs
HA	hemagglutinin
HA assay	hemagglutination assay
H&E	haematoxylin and eosin
HI	hemagglutination inhibition
HP	highly pathogenic
HPAI	high pathogenicity avian influenza
IVPI	intravenous pathogenicity index
LP	low pathogenic
LPAI	low pathogenicity avian influenza
LPAIV	low pathogenicity avian influenza virus
MEM	minimum essential media
NA	neuraminidase
OIE	World Organisation for Animal Health
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRT-PCR	real-time reverse transcription polymerase chain reaction
VI	virus isolation

## INTRODUCTION

Avian influenza, commonly known as fowl plague, is caused by viruses with segmented, negative-sense, single-stranded RNA genomes belonging to Influenzavirus A genus of the Orthomyxoviridae family (Cox et al., 2000). AIV are divided into subtypes based on the antigenic surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To the knowledge of the authors, 18 HA subtypes (H1-H18) and 11 NA subtypes (N1-N11) have been recognized thus far. Waterfowl and terrestrial birds have been identified as a reservoir of all influenza viruses except H17N10 and H18N11. These types are found in bats in Guatemala and Peru (Tong et al., 2012). Based on their ability to cause clinical disease with high mortality in domestic chickens, influenza subtypes are divided into high pathogenic (HP), and low pathogenic (LP).

On the one hand, AIVs are classified as HP for poultry when the intravenous pathogenicity index (IVPI) in six-week-old chickens is either greater than

1.2 or causes at least 75% mortality in four-to-eight-week-old chickens infected intravenously. The same classification applies when the characteristic motif of basic amino acids in the cleavage site of HA (PQRESRRKK/GLF) is identified after sequence analysis (OIE Terrestrial Manual, Chapter 2.3.4, 2015). Diseases caused by high pathogenicity avian influenza viruses (HPAI) are usually associated with higher virulence and mortality, which can reach up to 100%. On the other hand, low pathogenic avian influenza viruses (LPAIV) are mostly of low virulence. Nevertheless, LP strains should be controlled in farms due to their potential to become “building material” for new HPAI strains.

Naturally occurring infections with AIV have been reported in free-living birds from 26 families, representing 105 species (Olsen et al., 2006). Isolations have been reported on every continent except Antarctica, where only serological evidence is present (Austin and Webster, 1993). The infected species are

mainly associated with aquatic habitat. More specifically, with representatives of the orders Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and shorebirds). Thus far, naturally occurring viruses that cause HPAI have been associated with subtypes H5 and H7. However, H5 and H7 viruses are also present in LP forms (Alexander, 2007).

The HPAI virus H5N1 first appeared in 1997 in Hong Kong where 16 cases of infection in humans, resulting in six deaths, were registered (Shortridge et al., 2000). The viruses isolated from humans were identical to those found in birds. They had the characteristic motif of basic amino acids in the cleavage site of HA (Suarez and Perdue, 1998). Data about the predecessor of this virus causing the death of a large numbers of geese was collected in 1996 in China (Tang et al., 1998). Since then, for 18 years until 2014, H5N1 has undergone significant evolution and has generated many different clades due to antigenic drift in the H5 gene (Sonnberg et al., 2013). The occurrence of H5N1 in Bulgaria was first reported in 2006 in swans and geese (Goujgoulova and Oreshkova, 2007). In 2010, H5N1 was reported in common buzzard (Marinova-Petkova et al., 2012), and in 2015 in dalmatian pelicans, pigeon and black-headed gulls (1 REF OIE 17083, World Organisation for Animal Health (OIE), 2015).

On the 25<sup>th</sup> of March 2015, the H5N1 was confirmed (after VI and RT-PCR) to be the cause of death of 21 dalmatian pelicans in Srebarna reserve. Several days later, over 100 dalmatian pelicans from the Danube Delta in Romania were also found dead. Furthermore, during 2015, HPAI H5N1 (the isolate which had been detected in dalmatian pelicans) had been reported in Russia, Bulgaria, Romania and Kazakhstan (<http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2015/>). Therefore, the aim of this study is to estimate histopathological changes in visceral organs derived from dalmatian pelicans, naturally infected with AIV subtype A H5N1 in Bulgaria.

## MATERIALS AND METHODS

**Collection of samples.** A total of 21 recently perished dalmatian pelicans at the Srebarna reserve were available for the purposes of the study. However, only 4 of them were in an adequate post mortem condition

as the majority dalmatian pelicans exhibited advanced stages of putrefaction and thus rendering them unsuitable for the performed analysis this study. The criteria for inclusion of carcasses in the study were based upon: low degree of autolysis; detection of HPAI nucleic acid by polymerase chain reaction (PCR) in tissue samples and availability of brain and at least one other target organ for histopathologic investigation (lung, trachea, intestines, cloaca, and proventriculus). Although H5N1 can invade multiple organs and tissues, the organs were chosen as most significant for the limitations of the study. Nevertheless, organs from each carcass (heart, liver, spleen, gizzard and kidney) were tested bacteriologically to exclude bacteriological infection as a cause for mass mortality in dalmatian pelicans.

Carcasses were weighed, necropsied and their visceral organs and brains were examined macroscopically for gross lesions. Tissues in good post mortem condition and low degree of autolysis were collected aseptically. From each selected organ, one half was used for virus isolation (VI), and the other for histopathology. For VI, a 10% suspension (w/v) of ground sample was prepared in Minimum Essential Media (MEM) (pH 7.2-7.4), supplemented with Streptomycin (200 mg/l), Penicillin G ( $2 \times 10^6$  IU/l), Nystatin dehydrate ( $0.5 \times 10^6$  IU/l), Polymyxin B ( $2 \times 10^6$  IU/l), Gentamicin sulfate (250 mg/l), and Sulphamethoxazole (200 mg/l).

## Virus isolation and identification

After homogenization, the samples were centrifuged at 800g for 10 minutes at 4°C. This was followed by 200µl of supernatant from each organ sample inoculated into the allantoic cavity of three 10-day-old embryonated chicken eggs (ECE). The infected embryos were incubated at 36°C for up to 120 hours and checked daily. All chicken embryos were found dead after 24-48 hours and their allantoic fluids were tested for hemagglutination activity via a hemagglutination assay (HA). The HA positive allantoic fluids were examined for hemagglutination inhibition (HI) using 4 hemagglutination units per well and hyperimmune standard serum (H5N1, H5N3) produced from Instituto Zooprofilattico delle Venezie (Comin et al., 2012; Molesti et al., 2014). The standard OIE procedure was followed for both the HA and HI assays (OIE Terrestrial Manual, Chapter 2.3.4, 2015).

### Nucleic acid detection

The supernatants of the tissue homogenates were tested using real time reverse transcription polymerase chain reaction (rRT-PCR) by matrix AIV gene. RNA extractions were performed with the QIAamp® Viral RNA Mini Kit (Qiagen, Venlo, Netherlands). We used one step rRT-PCR with AcuFlock® Influenza A Virus real-time RT-PCR Kit (specific primers and probe included in kit) following the manufacturer's protocol (AnDia Tec®, Kornwestheim, Germany). For virus subtyping (H5, H7) RT-PCR-QIAGEN one step RT-PCR Kit with DNA samples positive for the M-gene according to Spackman et al. (2002) was used.

### Histopathology

Tissue samples from dalmatian pelicans were immediately fixed in 10% buffered formalin for subsequent histopathological examination. The tissues were routinely dehydrated, paraffin embedded, sectioned at 5 µm and stained with hematoxylin and eosin (H&E). Histopathological lesions were observed and documented with a Leica DM 5000 B microscope equipped with a digital camera and original software.

## RESULTS

### Virus isolation and identification

H5N1 virus was isolated from the lung, brain, small intestine, proventriculus, trachea and cloaca of dalmatian pelicans. Only haemagglutinating viruses including influenza A strains were isolated from these birds. In all cases of isolated H5N1 virus, the virus killed chicken embryos within 24-48h following allantoic cavity inoculation on the first passage. Upon inspection petechial hemorrhage throughout the body and delays in development were observed as well. The allantoic fluid from dead embryos was tested for hemagglutination activity. The Isolate *A / dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1)* resulted in agglutination of erythrocytes. After HI with different positive serums, it was found that *A / dalmatian pelican / Srebarna / Bulgaria / 2015 (H5N1)* is of the H5 subtype.

### Nucleic acid detection

PCR for M gene and H5N1-specific rRT-PCR analysis showed that the H5 AIV was present in the lung, trachea, proventriculus, cloaca and brain tissue of all birds examined in this study.

### Gross lesion

All of the tested animals showed symptoms of disease. Carcasses were cachectic, weighing approximately 5kg, amounting to a nearly 50% reduction from the normal weight of 9-9.5kg. Pathological changes in the skin were not detected. Upon gross examination, the infected birds had a haemorrhagic small intestine, abnormally low content in the gizzard and proventriculus, well defined hyperemia of the tracheal mucosa associated with single petechiae and meninges' and brain congestion (Figure A). Gross pathologic changes were not observed in the lungs, heart, liver, spleen, gizzard and kidney.

### Histopathological findings

Histopathological examination of the dalmatian pelicans naturally infected by H5N1 revealed hyperemia and edema of the tracheal mucosa with loss of mucosal glands, desquamation of epithelial cells, and mononuclear infiltration in the propria (Figure B). The cerebral hemispheres are the most affected parts in the brain. In this location a mild to moderate congestion with focal necrosis, gliosis, multifocal infiltration by lymphocytes (non suppurative encephalitis), as well as perivascular proliferations of glial cells (Figure C) was observed. Hyperemia and mononuclear infiltration in cecum, diffuse mononuclear infiltration in the propria and submucosa of the small intestine, complicated by desquamation of enterocytes and necrosis (Figure D) and mononuclear infiltration in the proventriculus (Figure E) were present as well.

## DISCUSSION

Historically, high mortality in wild aquatic birds due to infection with HP AIV has been a rare event, restricted to few individual birds adjacent to holdings in which poultry infected with HP AIV were kept (Becker, 1966). However, during the recent outbreaks in different regions across Europe in 2015, dalmatian pelicans were the most affected species (1 REF OIE 17083, World Organisation for Animal Health (OIE), 2015). To the knowledge of the authors, the literature provides very limited data on influenza virus pathology in this species.

During the recent outbreak in Bulgaria, the most consistent gross lesions observed in dalmatian pelicans naturally infected with HP AIV (H5N1) were



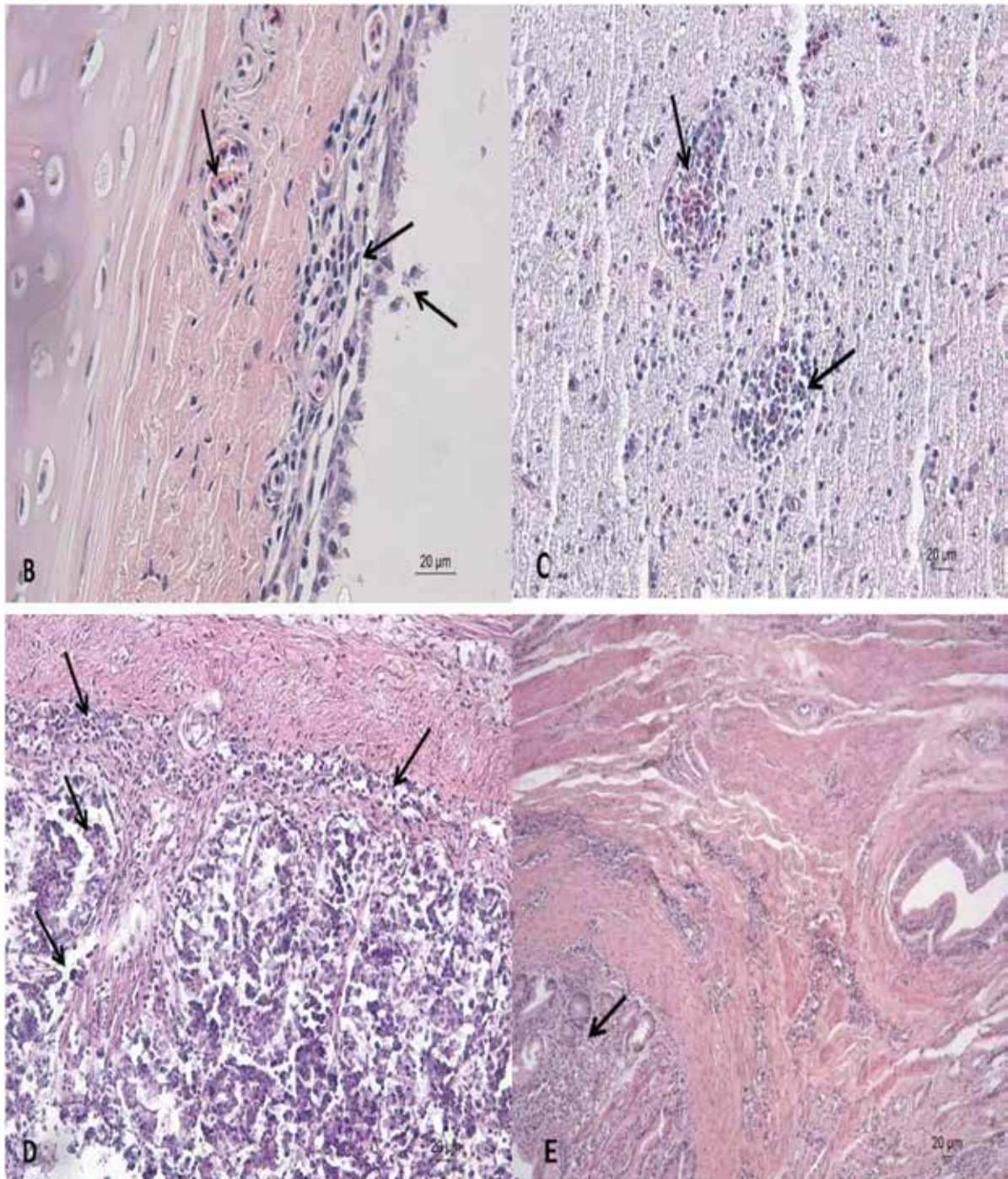


**Figure A.** Macroscopic. Brain, dalmatian pelican. Congestion (arrow).

meningeal and brain congestion and hyperemia of the tracheal mucosa complicated with petechiae. Teifke et al. (2007) report gross and histopathological lesions in populations of adult mute and whooper swans which appeared either emaciated or well-nourished with sufficient body fat reserves at time of death during an outbreak in Germany. The most consistent and predominant lesions in adult swans of this study were haemorrhages with necrosis in the pancreas. In a more recent study, Bröjer and colleagues (2009) reported few only gross lesions in tufted ducks naturally infected with H5N1 in Sweden in which lung congestion, red-brown mottling of the pancreas and moderately enlarged spleens have been observed. The exhibited differences in gross lesions between our findings and those by Teifke (2007) and Bröjer (2009) may be due to the different susceptibility of different bird species that have been evaluated. In naturally infected free-living birds, the clinical and pathologic picture of viral infection is influenced by several factors, such as the age of the bird, the amount and routes of viral expo-

sure, the presence of concomitant infections, the levels of immunity acquired during previous exposure to influenza viruses, and the time course of the infection (Kalthoff et al., 2008).

The most consistent lesion in our study was multifocal non suppurative encephalitis. This is in agreement with natural H5N1 infection in other wild bird species (Ellis et al., 2004; Kwon et al., 2005; Liu et al., 2005; Pálmai et al., 2007; Bröjer et al., 2012), and with experimental infection of wild birds (Keawcharoen et al., 2008). The number of dalmatian pelicans with encephalitis, in association with high levels of virus as detected by rRT-PCR, suggests that the virus is highly neurotropic, as shown by previous studies (Brown et al., 2006; Pasick et al., 2007). The mechanisms of dissemination of the virus in the brain in wild birds are not yet fully understood. However, the explanation that the route of infection also affects the route of dissemination is plausible. Experimental studies on mice, ferrets, and chickens suggest that influenza viruses can enter the central nervous system hematoge-



**Figure B.** Trachea, dalmatian pelican. Mononuclear infiltration in the propria and epithelial cells desquamation (arrows).

**Figure C.** Brain, dalmatian pelican. Perivascular infiltration of glial cells and lymphocytes (arrows).

**Figure D.** Small intestine, Dalmatian pelican. Desquamation and necrosis of enterocytes, mononuclear infiltration in the mucosa and submucosa (arrows).

**Figure E.** Proventriculus, Dalmatian pelican. Mononuclear proliferation (arrow). H&E staining. Scale bar = 20µm for all figures.



nously (Swayne, 2007), via peripheral nerves (Shinya et al., 2011) and via the olfactory route (Schrauwen et al., 2012). In our study, the most affected parts in the brain were the cerebral hemispheres. These observations can serve as the foundation for the hypothesis that main route of AIV infection of the brain in dalmatian pelicans is through transport of AIV via the olfactory nerve, following fecal ingestion (fecal-oral route) from infected birds.

Histopathological lesions were associated with mild inflammatory reactions. The character and distribution of lesions in dalmatian pelicans suggest an acute course of disease. Due to the massive infection of brain a pronounced neurotropism of H5N1 HP AIV may have led to nervous disturbances. Virological investigations of the brain revealed a high viral load

in this tissue, which can explain the mild to moderate meninges and brain congestion during the viremic spread of HP AIV. The lethal outcome is attributed to the systemic viral infection.

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## CONFLICT OF INTEREST

There is no conflict of interest. ■

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