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Tissue tropism and pathology of highly pathogenic avian influenza H5N6 virus in chickens and Pekin ducks

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ABSTRACT

The highly pathogenic avian influenza (HPAI) H5N6 virus caused outbreaks on commercial poultry farms in the Netherlands in 2017–2018, holding chickens and Pekin ducks. Intravenous pathogenicity index (IVPI) tests confirmed the high pathogenicity of the virus. Tissues derived from birds from infected farms (natural infection) and IVPI tests (experimental infection) were used to compare histopathology and virus distribution in both poultry species. After natural infection in chickens, histopathologic changes were present in the respiratory tract and several internal organs in both chickens and Pekin ducks. Viral antigen expression in the tissues of chickens varied from that in ducks. Virus expression was found in epithelial, mononuclear and endothelial cells in chickens. In contrast to the major role infected endothelial cells seem to play in systemic infections of chickens, in ducks the number of infected endothelial cells was very limited. Therefore, endothelial cell infection likely does not play a major role in systemic infection and disease progression in HPAI H5N6 virus infected Pekin ducks.

During the 2017–2018 winter season, outbreaks of a novel reassortant highly pathogenic avian influenza (HPAI) subtype H5N6 virus belonging to HPAI H5 clade 2.3.4.4 group B occurred in Asia and Europe (Beerens et al., 2019; Kim et al., 2018; Poen et al., 2019). In the Netherlands, several HPAI H5N6 virus infected wild birds were detected, and the virus was introduced at one commercial chicken farm and two commercial duck farms (Beerens et al., 2019). The intravenous pathogenicity index (IVPI) test was performed for the index virus according to OIE regulations (*World Organisation for Animal Health*, 2015). This resulted in a IVPI score of 2.99, which confirms high pathogenicity of the H5N6 virus for chickens (*Gallus gallus domesticus*). A similar IVPI score (3.00) was measured for this virus in Pekin ducks (*Anas platyrhynchos domesticus*) (Beerens et al., 2019).

Infections with low pathogenic avian influenza (LPAI) virus are typically restricted to the respiratory and intestinal system in birds, whereas HPAI viruses cause systemic disease in poultry. HPAI viruses can infect endothelial cells, resulting in altered permeability of the blood vessels, viremia and subsequent systemic spread to organs. The majority of gallinaceous birds will die during this acute stage of infection. Mortality was suggested to correlate with the number of endothelial cells that are infected in gallinaceous birds (Perkins and Swayne, 2001). Interestingly, systemic virus spread has also been observed in ducks, whereas experimental studies did not detect infection of endothelial cells of the cardiovascular system (Short et al., 2014). These findings illustrate a lack of knowledge on the pathogenesis of HPAI viruses in these poultry species.

To investigate the pathogenesis of the HPAI H5N6 virus, we selected five naturally infected chickens with advanced disease signs at the infected farm in Oldekerk and five Pekin ducks at the farm in Biddinghuizen, before culling of the farms. In addition, five experimentally chickens and Pekin ducks were collected that died within 24 h after inoculation with the HPAI H5N6 virus in the previously performed IVPI experiments. Analysis of the full genome sequences of the H5N6 viruses detected at the farms (EPI_ISL_332430 and EPI_ISL_287907) and the

Abbreviations: HE, haematoxylin and eosin; HPAI, highly pathogenic avian influenza; IHC, immunohistochemistry; IVPI, intravenous pathogenicity index; LPAI, low pathogenic avian influenza; OIE, World Organisation for Animal Health; WBVR, Wageningen Bioveterinary Research.

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H5N6 virus inoculated in the IVPI experiments (EPI_ISL_287907) showed that the viruses have a similar genetic constitution with 99.6% identity at nucleotide level, and only few amino acid differences were observed (Supplementary Table S1). During necropsy, tracheal and cloacal swabs were taken and infection of all birds was confirmed by PCR analysis, as described previously (Beerens et al., 2019). Furthermore, samples of the respiratory tract (conchae, trachea, lungs and air sac), intestinal tract (ileum and cloaca), internal organs (spleen and pancreas) and brain were collected for histopathologic and immunohistochemical analysis. The tissues were fixed in 10% formalin, embedded in paraffin and sectioned at 4-µm-thickness. Slides were stained with haematoxylin and eosin (HE), and immunohistochemistry (IHC) staining was performed using an antibody to the nucleoprotein (Supplementary Materials and Methods).

Histopathological changes were observed in the tissues of infected chickens and Pekin ducks (Fig 1, Table 1 and Table S2). Lesions were observed in the respiratory tract of both chickens and ducks, but appeared more severe in naturally infected birds compared to experimentally infected birds. Histopathological changes in conchae, trachea and air sac (Fig 1A and B) of all birds were characterized by multifocal degeneration and necrosis of epithelial cells with infiltrates of heterophils and fewer macrophages and lymphocytes in underlying connective tissue. Histopathological changes in the conchae were particularly prominent in the nasal mucosal epithelium of the naturally infected chickens. Histopathological changes in the lung of ducks (Fig 1D) appeared more severe than the changes observed for chickens (Fig 1C). The congested lungs displayed heterophilic and fibrinonecrotic interstitial pneumonia with edema. However, histopathological changes in the upper respiratory tract were mostly milder in ducks compared to the chickens. Severe histopathological changes were also found in the spleen and pancreas of infected chickens and ducks, the changes in the spleen appeared more severe in experimentally infected birds. Depletion of the white pulp in the spleen, characterized by multifocal lymphocytic necrosis, was observed in both chickens and ducks. Histopathological changes in the pancreas were most pronounced in naturally infected birds, particularly in chickens, and were characterized with areas of acinar degeneration and necrosis most prominent in the peripheral parts of the organ (Fig 1E and F). Histopathological changes were also observed in the intestinal tract in ileum and cloaca, and in the brain, but were mainly mild to moderate with no clear difference between the bird species.

The localisation of the HPAI H5N6 virus was studied in different organs using IHC (Fig 1, Table 1 and Table S3). In all infected birds, viral antigen expression was detected in the conchae and trachea, which represent the primary sites of viral entry and shedding during natural avian influenza infection. Chickens showed overall a higher virus expression in the upper respiratory tract compared to ducks. All infected birds showed virus expression in the air sac (Fig 1G and H) and lung (Fig 1I and J) which was most prominent in experimental infected chickens. In the lung, viral antigen was mainly concentrated in the epithelium lining the air capillaries of the parabronchus, whereas no staining of the squamous epithelium of the parabronchus was observed. Viral antigen was mainly expressed in epithelial cells of the respiratory tract, but also in mononuclear cells and endothelial cells in chickens (Fig 1G and I). In Pekin ducks the expression in endothelial cells was very limited (Fig 1H and J). The viral antigen was highly expressed in the spleen of all experimentally infected birds. As the spleen and lung are highly vascularised tissues, strong viral antigen expression in these organs may be related to direct virus injection into the blood stream during experimental infection. Interestingly, this high viral antigen expression in

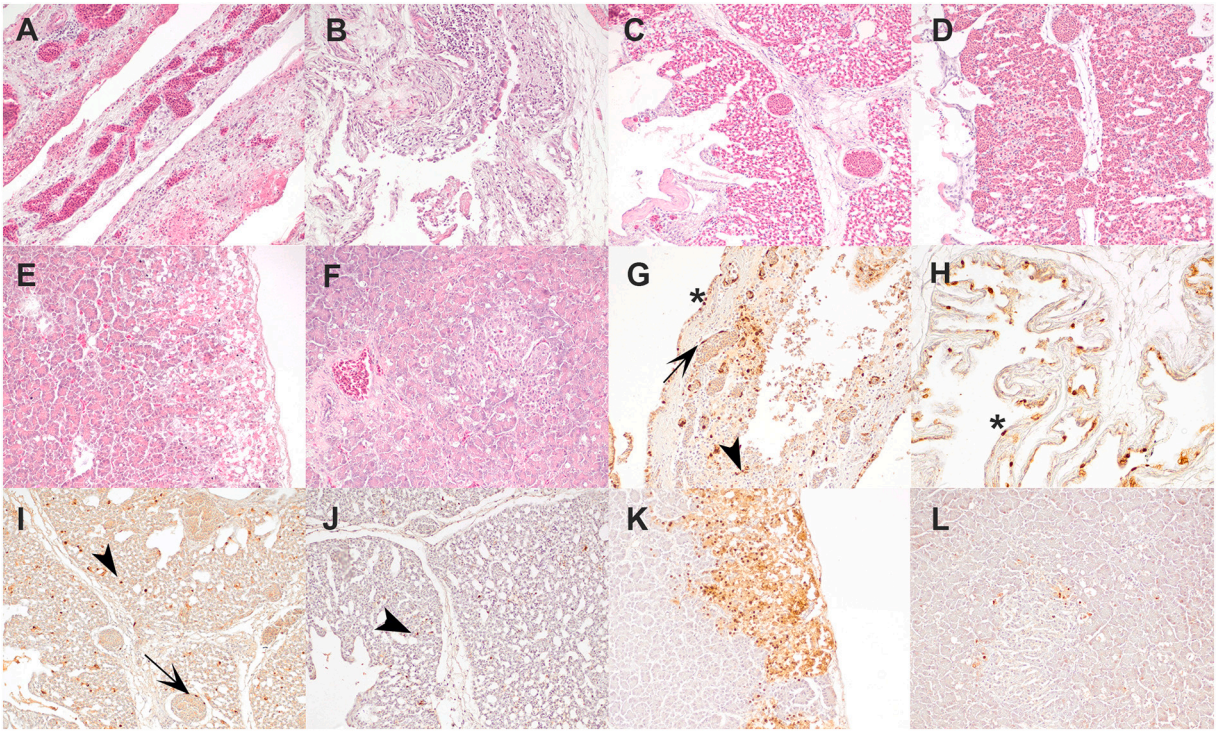

Fig. 1. Histopathological changes and virus expression in tissues of chickens and Pekin ducks after natural infection with HPAI H5N6 virus. Air sac of chicken (A) and Pekin duck (B) with heterophilic air sacculitis, more prominent in chicken; lung chicken (C) and Pekin duck (D) with fibrinous to fibrinonecrotic pneumonia with edema, more severe in the Pekin duck; pancreas of chicken (E) with areas of acinar degeneration and necrosis most prominent in the peripheral parts and pancreas of Pekin duck (F) with lymphohistiocytic pancreatitis with acinar degeneration and necrosis, A-F haematoxylin and eosin (HE) stain, objective 20×; air sac of chicken (G) with abundant virus expression in epithelial cells (asterisk), mononuclear (arrowhead) and endothelial cells (arrow) and air sac of Pekin duck with virus abundant expression in only epithelial cells (asterisk) (H); lung of chicken (I) with moderate virus expression in epithelial/mononuclear cells (arrowhead) and endothelial cells (arrow) and lung of Pekin duck (J) with moderate virus expression in epithelial /mononuclear cells (arrowhead); pancreas of chicken (K) and Pekin duck (L) with virus expression in epithelial and mononuclear cells; G-L immunohistochemistry Influenza virus A nucleoprotein, objective 20×.

Table 1
Overview of histopathological changes and virus expression in tissues of chickens and Pekin ducks after natural and experimental infection with HPAI H5N6 virus.

Species	Chicken				Pekin Duck			
Infection	Natural		Experimental		Natural		Experimental	
	HE	*IHC*	*HE*	*IHC*	*HE*	*IHC*	*HE*	*IHC*
Brain	+	+	+	+/++	−/+	−/+	+	−/+
Conchae	+++	+++	+/++	++/+++	++	−/+	++	+/++
Trachea	+++	++	++	+++	++	+/++	na	na
Lung	++	+/++	++/+++	++++	++/+++	++	+++	+++
Air sac	++/+++	++/+++	+/++	++/+++	++	+/++	++/+++	++
Ileum	+	−/+	−/+	+++	−/+	−/+	+	+
Cloaca	++	++/+++	++	+++	+	−/+	+	+
Spleen	+/++	+/++	+++	++++	++	+/++	+++	+++
Pancreas	++/+++	+++	+	+	++	−/+	+	–

Haematoxylin and eosin (HE) slides were scored for severity of histopathological changes: none (−), mild (+), moderate (++), severe (+++) and for expression of influenza virus A nucleoprotein by immunohistochemistry (IHC): negative (−), sparse (+), moderate (++), abundant (+++), excessive (++++). The mean scores of each group are presented ($n = 5$); na is not analyzed.

endothelial cells of the spleen was observed in both naturally and experimentally infected chickens, whereas this endothelial expression was very limited in ducks. Only in chickens there was strong viral expression in the pancreas (Fig 1K), which was predominantly restricted to acinar epithelial cells and locally abundant in the peripheral parts of the organ, suggesting extension per continuitatem from the air sacs. Peking ducks showed minimal or no viral expression in the pancreas (Fig 1L). The ileum, cloaca and brain of experimental infected chickens showed a moderate to high viral expression, whereas only mild or no expression was observed in Pekin ducks.

This study describes for the first time the histopathology of a natural HPAI H5N6 virus infection in two different commercial poultry species during the 2017–2018 epizootic in the Netherlands. In chickens viral antigen was detected in most organs analyzed, whereas in Pekin ducks there was mainly viral expression in the respiratory tract and spleen. These results demonstrate systemic spread of the HPAI H5N6 virus in both poultry species, although virus distribution appeared more restricted in ducks compared to chickens. Viral antigen expression was often associated with histopathology characterized by necrosis and inflammation, which is consistent with the acute stage of HPAI virus infection (Short et al., 2014). Analysis of birds that died during the IVPI experiments mostly showed more viral antigen and more histopathological lesions compared to naturally infected birds, which may be due to intravenous injection of virus, a higher virus dose and/or a later infection stage compared to naturally infected birds.

Limited virus expression was observed in the ileum and cloaca of ducks, but virus excretion was detected by PCR in cloacal swabs (Table S4).This suggest that virus replication may occur in unsampled parts of the intestine, or organs that drain into the cloaca (e.g. kidney, pancreas). Previous experimental studies in ducks also showed that viral antigen was mainly detected in the respiratory tract after infection with HPAI H5 viruses of the GsGd lineage, but the amount of viral antigen detected in other tissues varied between studies (Kim et al., 2014; Kim et al., 2018; Pantin-Jackwood et al., 2017; Pantin-Jackwood and Swayne, 2009). These differences may be due to differences in duck breed or species, virus strain and/or infection stage. In our study, Pekin ducks developed severe fibrinonecrotic changes in air capillaries and parabronchi of the lungs. These changes probably have compromised oxygen exchange, thereby contributing to death of the infected ducks. Whether the respiratory tropism of the HPAI H5N6 virus is also present in other species of the *Anatidae* family remains to be investigated.

In this study, we observed differences in endothelial tropism of the HPAI H5N6 virus in chickens and Pekin ducks. In chickens, besides epithelial and mononuclear cells, endothelial cells were abundantly infected. However, in ducks the number of infected endothelial cells was very limited, and visible infection was absent in many tissues. Previous studies suggest that replication of HPAI viruses in chicken endothelial cells may be associated with the apoptotic death of these cells (Ito et al., 2002), resulting in edema, haemorrhage (Perkins and Swayne, 2001; van Riel et al., 2009),loss of blood coagulation, but also formation of microthrombi and disseminated intravascular coagulopathy in the end stage of disease (Swayne, 2007). It has also been suggested that the replication of HPAI viruses in endothelial cells could disrupt the innate immune response (Suzuki et al., 2009) and facilitate the systemic spread of the virus (Pantin-Jackwood and Swayne, 2009). Together, these features may be responsible for rapid disease progression and mortality observed for HPAI viruses in chickens and other *Galliformes* species after natural infection. The limited endothelial infection may contribute to the milder disease progression observed in Pekin ducks and Eurasian wigeons experimentally infected with HPAI H5N6 virus compared to chickens (Beerens et al., 2021). Previous studies also showed limited endothelial tropism of several HPAI viruses in wild ducks of the *Anatidae* family (Short et al., 2014; van den Brand et al., 2018). However, in black swans endothelial cell infection and rapid disease progression were observed after infection with HPAI H5N1 virus (Brown et al., 2008). The mechanism underlying the endothelial tropism of the HPAI H5N6 virus in chickens remains to be studied. However, it appears clear that endothelial cell infection is unlikely to play a major role in systemic infection and pathogenesis in Pekin ducks.

Ethical statement

The animal experiment and associated procedures were conducted in accordance with the national regulations on animal experimentation under the approval of the Dutch Central Authority for Scientific Procedures on Animals (CCD) (permit number AVD4010020172824; experiment number 2017.D-0054.001). The experiment was part of another study published previously (1). The experiment was performed conform the guidelines from the European Union directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes. The experiment was performed in biosafety level 3 facilities at Wageningen Bioveterinary Research (WBVR, Lelystad, the Netherlands).

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.03.010.

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