Pathobiological features of highly pathogenic avian influenza virus (H5N8) in commercial chicken and geese

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Highly-pathogenic Avian Influenza viruses (HPAIV) pose a continuous threat to animal health. Since October 2016, the HPAI H5N8 (H5N8/HP) belonging to H5N1 A/goose/Guangdong/1/1996 lineage has been responsible of extensive outbreaks in domestic bird holdings in Europe. In the present study, we assessed the pathobiology of the novel H5N8 in commercial chicken and geese breeds and SPF chicken. Ross 308 broiler, specific-pathogen free (SPF) chicken and G35-line geese were intranasally inoculated with 10^5 ELD_{50} of A/Duck/Spain/Girona/2017 (H5N8) virus in a final volume of 50 µl and monitored daily during 10 days. Clinical signs, gross and microscopic lesions, presence of viral particles in tissues (IHC techniques) and viral shedding (qRT-PCR techniques) were evaluated. Commercial geese exhibited severe clinical signs, including prostration and ataxia. Mortality reached 100 % at day 10 post-infection. Gross and microscopic lesions included areas of hemorrhages and necrosis in internal organs, which correlated with widespread viral replication in tissues. Particularly, the highest viral positivity was detected in central nervous system, pancreas, heart and liver. Moreover, strong viral shedding was confirmed from both oral and cloacal routes in all commercial geese. In commercial chickens, clinical signs were only observed in a few animals, and included apathy and cutaneous edema. Mortality recorded in broiler and SPF chicken was 25 % and 8 %, respectively, and macroscopic findings observed were those typical of HPAIV infection. However, low number of chickens presented remarkable viral shedding in oral and cloacal routes. Our results demonstrate that commercial geese are highly susceptible to H5N8/HP. In contrast, commercial chickens present considerable higher survival rates than geese, suggesting the existence of not only viral but also species-specific host factors affecting viral replication. Commercial geese and chicken should be closely monitored during HPAIV outbreaks in order to reduce the interspecies transmission and propagation of the virus.

Los virus de influenza aviar de alta patogenicidad (IAAP) suponen una continua amenaza para la sanidad animal. Desde su aislamiento en Octubre del 2016, el virus de IAAP H5N8 (H5N8/AP) perteneciente al linaje H5N1 A/goose/Guangdong/1/1996, ha producido numerosos brotes en granjas de aves domésticas en Europa. En el presente estudio, se evaluó la patogenia del virus de IAAP H5N8 en pollos y ocas comerciales. Broilers Ross 308, pollos libres de patógenos específicos (SPF) y ocas de la línea G-35 fueron inoculados vía intranasal con 10^5 ELD_{50} del virus A/Duck/Spain/Girona/2017 (H5N8) con un volumen final de 50µl y fueron monitorizados diariamente durante 10 días. Se evaluó la sintomatología clínica, las lesiones macroscópicas y microscópicas, la presencia de partículas víricas en distintos tejidos (técnicas de IHQ) y la excreción viral (técnicas de qRT-PCR). Las ocas comerciales mostraron sintomatología clínica grave, e incluían postración y ataxia. La mortalidad alcanzó el 100 % al día 10 post-infección. Las lesiones macroscópicas y microscópicas observadas fueron áreas de necrosis y hemorragias en órganos internos, los cuales correspondían con una extensa replicación del virus en los tejidos. Concretamente, la mayor positividad al virus se detectó en sistema nervioso central, pancreas, corazón e hígado. Además, se detectó una elevada excreción viral tanto por vía oral como cloacal en todas las ocas comerciales. En los pollos comerciales, se observó sintomatología clínica en pocos animales, e incluyeron apatía y edema cutáneo. La mortalidad en broilers y

pollos SPF fué del 25 y 8 %, respectivamente, y las lesiones macroscópicas fueron las típicas de los virus de IAAP. Sin embargo, se detectó excreción viral oral y cloacal en pocos animales. Los resultados demuestran que las ocas comerciales son altamente susceptible al virus H5N8/AP. En cambio, los pollos comerciales presentan ratios de supervivencia considerablemente superiores, sugiriendo la existencia de factores virales pero también específicos de especie que afectan a la replicación viral. Las ocas y pollos comerciales deberían ser estrechamente vigilados en caso de brote infeccioso por virus de IAAP con el objetivo de reducir la propagación y transmisión del virus entre distintas especies.

Keywords: Avian influenza; Commercial birds; Pathobiology

Introduction

Avian Influenza (AI) is a main disease affecting numerous domestic and wild bird species as well as an important threat to human health (Capua & Alexander, 2006). To date, 16 haemagglutinin and 9 neuraminidase subtypes of AI viruses have been reported in avian population. Despite AI viruses are further divided into low-pathogenic (LPAI) or high-pathogenic (HPAI) depending on virulence motifs present in viral genome and the mortalities that cause in poultry, HPAI infections have been limited to those carrying H5 and H7 haemagglutinin (OIE, 2015).

In gallinaceous species, HPAI strains produce high morbidity and mortality, causing important economic losses in avian-food producing industry (Capua & Alexander, 2009). In contrast, birds of the Order Charadriformes and Anseriformes, including waterfowl, are considered the natural reservoirs of AI viruses so far (Webster et al., 1992). In these species, LPAI viruses are usually isolated without presence of evident clinical signs, and are generally resistant to natural infection with classical HPAI strains (Pantin-Jackwood & Swayne, 2009). However, the genetic reassortments between distinct AI strains in a phenomena known as genetic shift continually generates variants with potentially-distinct pathobiological features and host-specificity properties (i.e. HPAI H5N3 and Eurasian-African HPAI H5N1 lineage).

In May 2016, HPAI H5N8 belonging to Asian-origin H5N1 A/goose/Guangdong/1/1996 (Gs/GD) lineage emerged in the Uvs-Nuur Lake, which is located at the Mongolia–Russia Federation border. Despite the quick alarm raised by the Food and Agriculture Organization of the United Nations (FAO), closely related viruses were detected in India and Europe in October 2016 and continued spreading during following seasons, affecting a total of 48 countries by the end of June 2017 (FAO, 2018). HPAI H5N8 also reached Southern Africa, including Zimbabwe and South Africa. In the European Union, a total of 1190 outbreaks in domestic birds, 3051 in wild birds and 62 in captive birds were reported by December 2017, being France and Hungary the most severely-affected in production terms (Napp et al., 2018). In 2018, new outbreaks caused by HPAI H5N8 are still reported, mainly in Italy, South Africa and Middle East. Nowadays, HPAI H5N8 is the largest epidemic produced by a HPAI virus reported in Europe.

In Spain, HPAI H5N8 was firstly isolated in February 2017 in a white stork (*Ciconia ciconia*) in a wetland located in the north-east region of Catalonia (Aiguamolls de l'Empordà, Girona), thanks to the surveillance programs in wild birds carried out by Centre de Recerca en Sanitat Animal (Programa de Sanitat Animal, IRTA) and Departament d'Agricultura, Ramaderia, Pesca i Alimentació (DARP). During the following weeks, H5N8 was isolated in a total of 10 duck farms. The laboratory assays conducted at CESAC (Centre de Sanitat Avícola de Catalunya i Aragó) evidenced that the virus was widely distributed in those farms. However, the rapid establishment of control measures by DARP and CESAC resulted in the prevention of HPAI H5N8-dissemination among the territory (Napp et al., 2017).

Epidemiological data evidences that 80% and 10% of domestic outbreaks caused by HPAI H5N8 in European countries were in waterfowl (ducks and geese) and chicken holdings, respectively (Napp et al., 2018). This data suggests that HPAI H5N8 may have an increased avidity towards Anseriformes species than classical HPAI strains. Despite chicken farming is the leading producer in poultry meat sector, with almost 80 % of European Union global production, rearing minor avian species such as

domestic waterfowl are also of economic interest in several countries (EUROSTAT, 2015). Specifically, geese are raised mainly in specialized farms for meat, fatty liver and feather production, but also as guarding animals (FAO, 2012). Moreover, it is also frequent to rear geese in outdoor production systems mixed with poultry, with facilitates the interspecies transmission and propagation of AI viruses.

Despite the global spread of HPAI H5N8 belonging to Gs/GD lineage in domestic waterfowl and chicken, the pathobiological features of HPAI H5N8 in commercial species as well as their role in the transmission and propagation of the virus remain unclear. In the present study, we evaluated the infectivity, the pathogenicity and the viral shedding after infection with HPAI H5N8 in commercial geese, commercial chicken and SPF chicken.

Materials and methods

Virus. The virus used in this study was A/Duck/Spain/Girona/2017 (H5N8) (H5N8/HP), which was isolated in March 2017 from a domestic duck farm located in the north-east region of Catalonia.

Virus stocks were produced in 10 days-old SPF embryonated eggs. The allantoic fluid was obtained at 24 hours post-inoculation, filtered and aliquoted at -75ºC until use. Serial ten-fold dilutions of the filtered virus in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean egg lethal dose (ELD_{50}) was determined by Reed and Muench method (Reed & Muench, 1938).

Animals, facilities and experimental infection. Ross 308 broiler, specific-pathogen free (SPF) chicken (*Gallus domesticus*) and G35-line geese (*Anser anser* var.domestica) were tested in this study. 20 three-week old broiler and SPF chicken and 7 commercial geese of approximately 3 months of age were included. At arrival, the animals were individually identified and placed in separated negative-pressured HEPA-filtered isolators (chicken) and boxes (geese) present in Biosecurity Level 3 (NBS-3) facilities in Centre de Recerca en Sanitat Animal (Programa de Sanitat Animal, IRTA). In order to achieve animal welfare standards, pools of 1 meter of diameter were set up in geese installations.

Prior to infection, serum samples were obtained from all animals to ensure that they were seronegative to Influenza A virus by an ELISA competition test (ID-VET, Montpellier, France). Furthermore, oropharyngeal (OS) and cloacal swabs (CS) were collected from 5 random animals in each group and confirmed to be negative to AIV by one-step quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

After 5 days of acclimation, 15 broiler, 15 SPF chicken and 5 commercial geese were intranasally challenged with H5N8/HP diluted in PBS in order to inoculate 10^5 ELD_{50} in a final volume of 0.05 mL (0.025 ml inoculated in each nostril). 5 broiler, 5 SPF chicken and 2 commercial geese remained as negative control animals. During the experimental procedures, food and water were provided ad libitum. The experimental design were approved by the ethical commission of Institut de Recerca i Tecnologia Agroalimentària (IRTA). Experimental groups are summarized in Table 1.

Table 1. Experimental design of the experiment. ELD_{50}; Egg Letal Dose 50.

Group	Inoculum	Titer (volume)	No. animals
Ross 308 Broiler	H5N8/HP	10^5 ELD_{50} (50 µl)	15
Ross 308 Broiler	-	-	5
SPF Chicken	H5N8/HP	10^5 ELD_{50} (50 µl)	15
SPF Chicken	-	-	5
G-35 line geese	H5N8/HP	10^5 ELD_{50} (50 µl)	5
G-35 line geese	-	-	2

Clinical signs. All birds were monitored daily for clinical signs until 10 days post-inoculation (dpi). A standardized OIE clinical scoring system with minor modifications was used (OIE, 2005). Animals with absence of clinical signs were classified as 0. Birds presenting one of the following clinical signs were considered as sick (1) and those showing more than one were considered as severely sick (2): respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs. Animals found dead were scored as 3.

Moribund chickens were euthanized with intravenous pentobarbital for ethical reasons and scored as dead. The severity of clinical signs according to OIE scoring, percentage of mortality and mean death time (MDT) were calculated in each experimental group.

Pathologic examination and immunohistochemical testing. Three broiler and SPF chicken and one commercial geese inoculated with H5N8/HP were sacrificed at 3 (chicken) or 4 (geese) dpi for pathological studies. Animals found dead or euthanised for ethical reasons throught the study were also included.

Standardized necropsies were performed in order to detect gross lesions. Tissue samples were collected, immersed in 10% formalin for fixation during 72 hours, and embedded in paraffin wax. Samples included skin, thymus, pectoral muscle, nasal cavity, trachea, lung, central nervous system, heart, spleen, liver, kidney, proventriculus, gizzard, pancreas, duodenum, cecum, colon and bursa of Fabricius.

Cut sections of 3 µm (Leica RM2255, Nussloch, Germany) from formalin-fixed, paraffin-embedded tissues were processed, stained with haematoxylin and eosin and examined under light microscopy. An immunohistochemical (IHC) technique for detecting viral antigen was performed as previously described (Bertran et al., 2011). Briefly, a mouse-derived monoclonal commercial antibody against nucleoprotein (NP) of influenza A virus (ATCC, HB-65, H16L-10-4R5) was used as a primary antibody. The samples were then incubated with biotinylated goat anti-mouse IgG secondary antibody (Dako, immunoglobulins As, Denmark).The antigen–antibody reaction was visualized using the chromogen 3,3'-diaminobenzidine tetrahydrichloride. Samples were classified as follows: negative (-), single positive cells (+), presence of groups of positive cells (++) and widespread positiveness (+++).

Viral RNA detection and quantification in swabs and water. Oral and cloacal swabs were collected from 9 chicken and 5 comercial geese inoculated with H5N8/HP and from negative control animals at 1, 3, 6 and 10 dpi and placed in 0.5 ml of sterile PBS with 6% antibiotics (Penicillin-Streptomycin-Nystatin. Water was also collected from the pools present in geese installations at the same days post-infection. All samples were conserved at -75ºC until further use.

Viral RNA was extracted using Nucleospin RNA virus kit (Macherey-Nagel, Düren, Germany), following manufacturer's intructions. A highly conserved region of AIV Matrix gene (M1) was detected by one-step Taqman RT-PCR in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA), using the primers and probe as well as conditions of amplification previously described (Spackman et al., 2002). To extrapolate the copies genome equivalents (CGE) present in the samples, a standard curve obtained by amplification of a highly-conserved region of AIV M1 gene (99 bp) was used. Briefly, the amplified region was ligated in pGEM-T vector (Promega, USA). The ligation product was purified using MinElute Reaction Cleanup Kit and transfected into electrocompetent E.coli cells by electroporation. Then, the recombinant plasmid was purified from transformed colonies using NucleoSpin Plasmid (Macherey-Nagel) and quantified in Biodrop. RNA copy numbers were calculated using DNA Copy Number Calculation (ThermoFisher Scientific). Serial ten-fold dilutions, ranging from 10^6 to 10^1 CGE, were used to obtain the standard curve.

Results and Discussion

Clinical signs typical of HPAI, which have been described elsewhere (Chaves et al., 2011), were observed in all H5N8/HP-inoculated groups. However, those were more severe and frequent in H5N8/HP-inoculated geese than in broilers and SPF chicken. Clinical signs started at 6 and 3 day post-infection in geese and chicken, respectively.

Commercial geese exhibited severe clinical signs, including prostration and nervous signs (i.e. ataxia, head shaking and torticolis); however one goose was found without presenting previous evident clinical signs. In contrast, only ruffled feathers and mild to moderate apathy were observed in sick H5N8/HP-inoculated broiler and SPF chicken; nevertheless, few animals presented severe apathy or were found dead. Furthermore, one SPF chicken presented severe congestion and edema of the head and comb.

Infection with H5N8/HP resulted in a 100 %, 25 % and 8 % mortality in commercial geese, broiler and SPF, respectively. The mean death time in commercial geese, broiler and SPF was 7, 3,7 and 6 days post-infection, respectively. Clinical signs scoring according to OIE standards and mortality

ratios (Table 2) confirm that H5N8/HP isolated in 2017 is highly virulent to geese, which are typically resistant to other HPAI viruses (Capua & Alexander, 2009). In chicken, the comparatively low mortality and severity of clinical signs suggests that this virus is less pathogenic to gallinaceous species than classical HPAI strains as has been described (Bertran et al., 2013, Chaves et al., 2011). The high susceptibility of geese and the comparatively resistance of chicken observed in this study correlate with the natural outbreaks reported in domestic waterfowl and poultry holdings during HPAI H5N8 epdemics in Europe (Napp et al., 2018).

Table 2. Clinical parameters observed in H5N8/HP-inoculated geese, broiler and SPF chicken. MDT; Mean Death Time.

Group	OIE Clinical signs scoring (0-3)	Mortality (%)	MDT (dpi)
Commercial geese	1,9	100 %	7
Broiler	0,6	25 %	3,7
SPF	0,4	8 %	6

Gross examination revealed the presence of typical lesions of HPAI in H5N8/HP-inoculated geese, broiler and SPF chicken described in the bibliography (Bertran et al., 2013, Chaves et al., 2011). In geese, the lesions included multifocal areas of haemorrhages and necrosis in pancreas, whitish foci compatible with necrosis in heart and liver, haemorrhages in gizzard mucosa, moderate congestion in central nervous system, cardiomegaly and esplenomegaly (Image 1-C).

Less prevalent but similar lesions were observed among H5N8/HP-inoculated broilers and SPF chickens. Diffuse congestion in internal organs as well as multifocal areas of haemorrhages and necrosis in proventriculus and pancreas were the lesions most comonly observed. Moreover, multifocal hemorrhages in thymus, severe edema and cyanosis of the head and comb, as well as diffuse haemorrhages in leg skin were observed in one chicken (Image 1D-F).

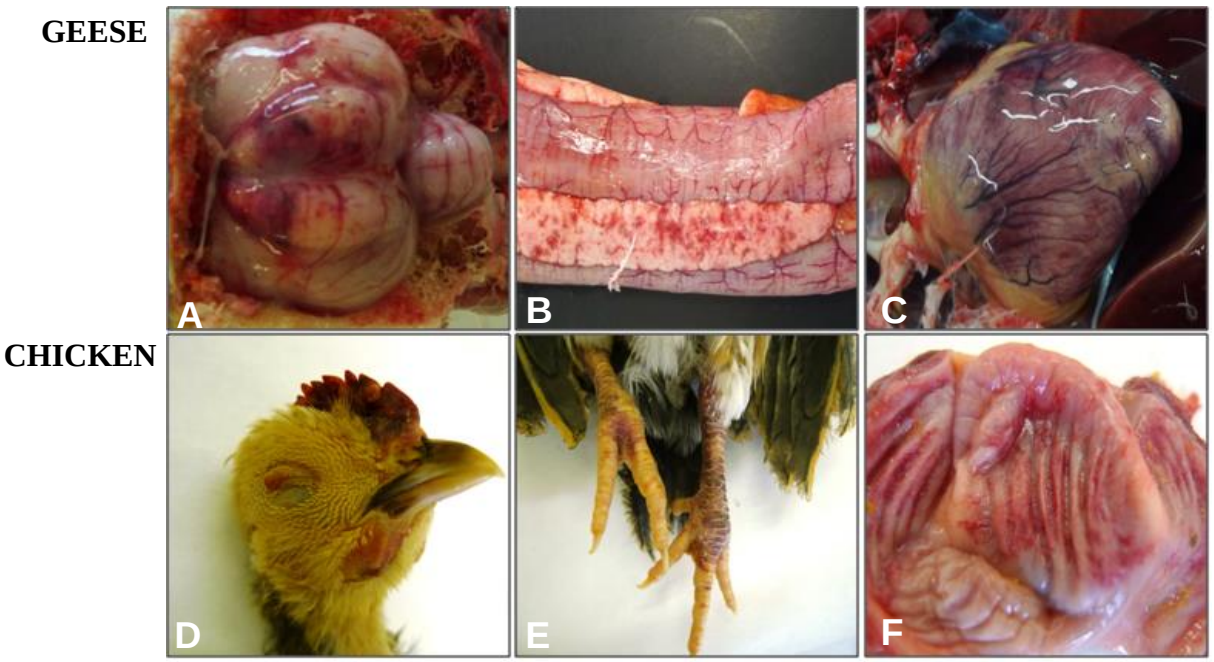

Figure 1. Gross lesions observed in commercial geese (A-C) and chicken (D-F) experimentally inoculated with H5N8/HP. A. Multifocal hemorrhages in central nervous system. B. Diffuse hemorrhages in pancreas. C. Cardyomegalia and focal areas compatible with necrosis in heart. D. Congestion and edema of the head and comb. E. Diffuse hemorrhages in leg skin. F. Multifocal hemorrhages in gizzard mucosa.

Microscopic observation of tissues revealed evident lesions in several H5N8/HP-inoculated geese tissues. The most severely affected organs were central nervous system, pancreas, heart, liver and spleen. The main microscopic findings were multifocal to diffuse areas of necrosis and hemorrhages

associated with inflammatory cell infiltration of variable intensity (Images 2A-C). Widespread AIV-positive cells in H5N8/HP-inoculated geese were observed in mostly all collected tissues and correlated well with pathological findings, indicating a systemic disemination of the virus (Images 2D-F).

Multifocal areas of necrosis and hemorrhages with inflammatory cell infiltration as well as diffuse congestion in several organs were also observed in few commercial chicken (Images 2G-I). Numerous AIV-positive cells were detected in skin, pectoral muscle, heart, lung, proventriculus, gizzard, spleen and liver (Images 2J-L). However, lower positivity was detected in central nervous system and pancreas, which are hallmarks of HPAI pathogenesis in galliformes species (Chaves et al., 2011).

In both geese and chicken, AIV-positive staining was detected in epithelial cells, inflammatory cells and endothelial cells.

No evident clinical signs, gross and microscopic lesions or AIV-antigen positive cells were observed in negative control geese and chicken trough the study.

GEESE

CHICKEN

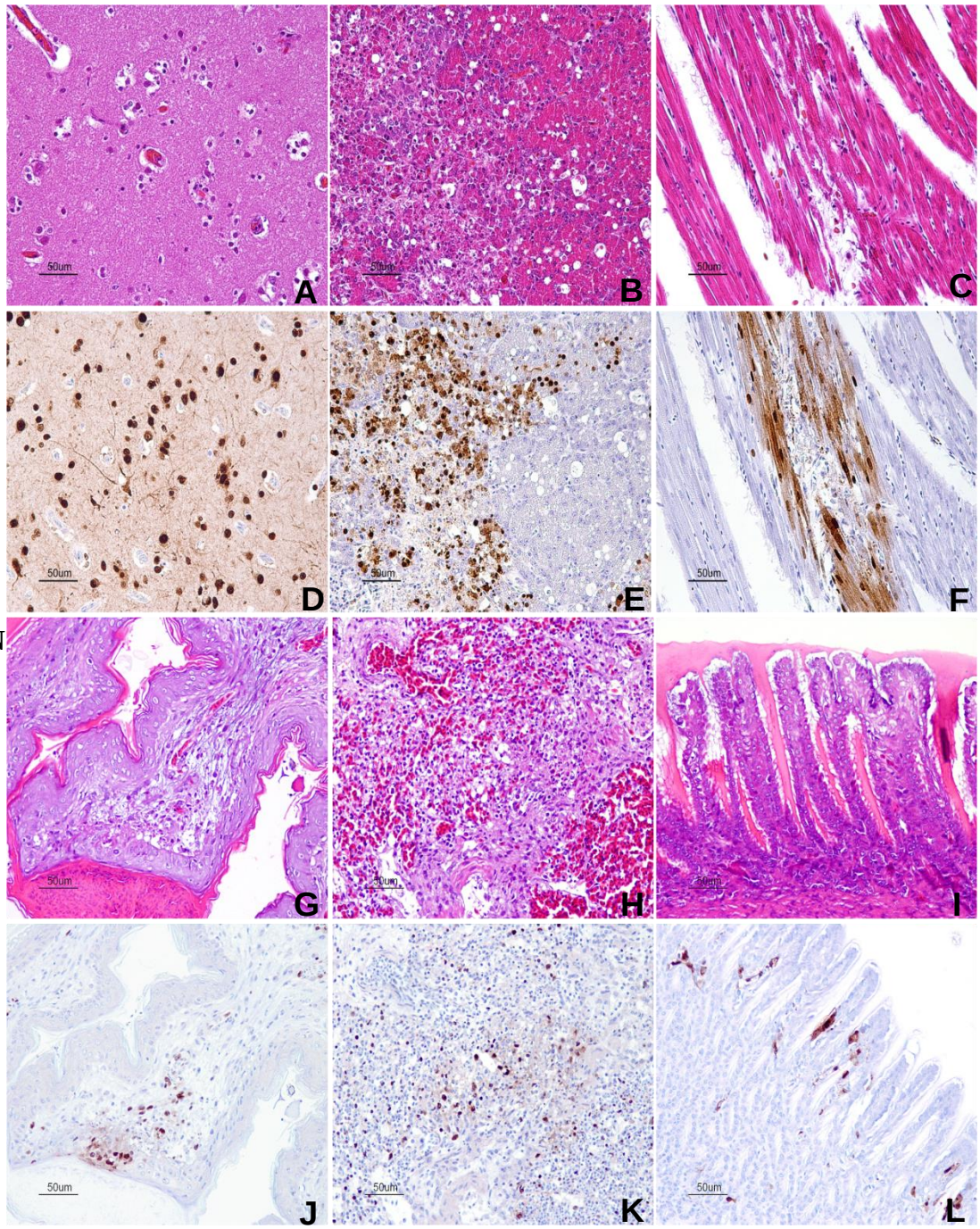

Image 2. Microscopic lesions and AIV-IHC staining in commercial geese (A-F) and chicken (G-L) experimentally inoculated with H5N8/HP. A. CNS: focal gliosis. B. Pancreas: diffuse necrosis of pancreatic acinar cells. C. Heart: focal necrosis of muscle fibers. D. CNS: AIV-detection in neurons and glial cells. E. Pancreas: AIV detection in pancreatic acinar cells. F. Heart: AIV detection in muscle fibers. G. Skin: diffuse edema and heterophilic inflammatory infiltrate in dermis H. Lung: diffuse congestion. I. Gizzard: focal heterophlilic inflammatory infiltrate in mucosa. J. Skin: AIV-detection in keratinocytes, inflammatory cells and endothelial cells. K. Lung: AIV-detection in interstitial inflammatory cells. L. Gizzard: AIV-detection in epithelial and inflammatory cells.

The immunohistochemical testing revealed that HPAI H5N8 can produce a systemic disease associated to widespread viral replication in a broad range of tissues in both geese and chicken. However, the differences detected in the number of animals presenting AIV-positivity as well as amounts of viral replication among the different groups of birds (geese, broilers and SPF) indicate that commercial geese are considerably more susceptible to HPAI H5N8 infection and dissemination than broiler and SPF chicken.

High levels of AI-viral RNA were present in oral and cloacal swabs from H5N8/HP-inoculated geese from 3 to 6 day post-infection (Table 3). Moreover, viral RNA could be detected in the water collected from the pools present in geese installations at day 6 and 10 post-infection (data not shown). In contrast, none of the broilers and low number of SPF chicken presented viral shedding in oral and cloacal routes, in concordance with the milder clinical presentation and viral replication observed in this species (Table 3).

In several countries, domestic waterfowl are raised in outdoor production systems mixed with poultry. The strong viral shedding detected in this study suggest that geese could be a main source of infection of HPAI H5N8 to other avian species by both the oral-oral and fecal-oral routes, as well as shared contaminated water.

Table 3. Oral and cloacal shedding detected in commercial geese, broiler and SPF chicken by qRT-PCR techniques. Dpi; day post-infection.

Group	Sample	Average Log GEC/sample (nº positive/total)			
		1 dpi	3 dpi	6 dpi	10 dpi
Commercial geese	Oral swab	2,6 (2/5)	4,7 (4/5)	6,6 (4/4)	0 (0/1)
	Cloacal swab	0 (0/5)	5,5 (3/5)	5,0 (4/4)	0 (0/1)
Broiler	Oral swab	0 (0/9)	0 (0/9)	0 (0/6)	0 (0/6)
	Cloacal swab	0 (0/9)	0 (0/9)	0 (0/6)	0 (0/6)
SPF	Oral swab	2,4 (1/9)	4,3 (2/9)	8,2 (1/6)	0 (0/5)
	Cloacal swab	0 (0/9)	5,3 (1/9)	6,1 (1/6)	0 (0/5)

In conclusion, our results show that commercial geese, broilers and SPF chicken are susceptible to HPAI H5N8 infection, thus potentially producing important economic losses in avian-food industry. However, commercial geese are considerably more susceptible to infection than chicken by means of clinical signs, mortality rates, gross and microscopic lesions and viral replication in internal organs. This suggests the existence of species-specific host factors affecting viral replication.

Moreover, the presence of high amounts of viral RNA in oral and cloacal swabs collected from geese indicate that domestic waterfowl could facilitiate the interspecies transmission and propagation of the virus. Since geese are frequently raised in backyard or extensive production systems mixed with poultry, these animals should be closely monitored during HPAI outbreaks.

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