Universitat Autònoma de Barcelona

Unravelling the pathobiological diversity of highly pathogenic avian influenza in birds

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UNRAVELLING THE PATHOBIOLOGICAL DIVERSITY OF HIGHLY PATHOGENIC AVIAN INFLUENZA IN BIRDS

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Unravelling the pathobiological diversity of highly pathogenic avian influenza in birds

Tesi doctoral presentada per Raúl Sánchez González per optar al grau de Doctor en Veterinària dins del programa de doctorat de Medicina i Sanitat Animals del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. Natàlia Majó i Masferrer i el Dr. Antoni Ramis Salvà.

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AI	Avian influenza
AIV(s)	Avian influenza virus (viruses)
BSL-3	Biosecurity level 3
C-ELISA	Competitive enzyme-linked immunosorbent assay
CNS	Central nervous system
CReSA	*Centre de Recerca en Sanitat Animal*
cRNA	Complementary RNA
CS	Cloacal swabs
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
Dpi	Days post-inoculation
DPPA	Densely Populated Poultry Areas
EID_{50}	50% egg infective dose
ELD_{50}	50% embryo lethal dose
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FP	Feather pulp
HA	Hemagglutinin
HE	Hematoxylin/eosin
HPAI	Highly pathogenic avian influenza
HPAIV(s)	Highly pathogenic avian influenza virus (viruses)
HPNAI	Highly pathogenic notifiable avian influenza
Hpi	Hours post-inoculation
IA	Influenza A
IAV(s)	Influenza A virus (viruses)
Ig	Immunoglobulin
IHC	Immunohistochemistry
IRTA	*Institut de Recerca i Tecnologia Agroalimentàries*
IVPI	Intravenous pathogenicity index
LPAI	Low pathogenic avian influenza
LPAIV(s)	Low pathogenic avian influenza virus (viruses)

LPM	Live poultry market
LPNAI	Low pathogenic notifiable avian influenza
MBCs	Multibasic cleavage site
MCs	Monobasic cleavage site
MDT	Mean death time
mRNA	Messenger RNA
M1	Matrix protein
M2	Membrane ion channel protein
NA	Neuraminidase
NAI	Notifiable avian influenza
NDV	Newcastle disease virus
NSL	Nuclear localization signal
NP	Nucleoprotein
NS1	Nonstructural protein 1
NS2/NEP	Nonstructural protein 2/Nuclear export proteins
OIE	World Organization for Animal Health
OS	Oropharyngeal swabs
PA	Polymerase acidic protein
PBS	Phosphate-buffered saline
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
RNP	Ribonucleoprotein
SA	Sialic aicd receptors
SEM	Standard error mean
SPF	Specific pathogen free
vRNA	Genomic viral RNA
VSV	Vesicular stomatitis virus

Avian influenza (AI) is considered one of the most important viral diseases affecting the poultry industry and a continuous threat to human population and wildlife. The majority of highly pathogenic avian influenza (HPAI) epidemics have affected land-based poultry, and classical lineages of HPAI viruses (HPAIVs) have been more sporadically isolated or rarely caused high mortality in aquatic poultry, wild birds and peridomestic avian species. However, the epidemiology and pathobiology of HPAI have radically changed since the emergence of Goose/Guangdong (Gs/GD) H5 lineage of HPAIVs. This lineage present unique biological characteristics among HPAIVs, including the capacity to infect and cause mortality in a broad range of domestic, captive and wild avian species. These demonstrate the large differences in infection outcome depending of the HPAIV isolate. Several studies show that the infection outcome is also influenced by host factors. Particularly, a broad variation in susceptibility to HPAIV infection exists among chicken breeds, suggesting that the genetic background of particular breeds confers a higher resistance to HPAIV infection. Usually, local chicken breeds have been considered more resistant to disease than commercial breeds due to lack of artificial selection towards production-related genes, which could be negatively associated with resistance to pathogens.

To date, a direct comparison of the pathobiology of classical and Gs/GD H5 HPAIVs in avian species belonging to distinct taxonomic groups is lacking. The variation in susceptibility to HPAIV infection among chicken breeds has not been studied in detail in Europe, as well as the existence of breed-related differences in susceptibility to HPAIVs in minor and peridomestic avian species. Consequently, in the present dissertation we systematically evaluated the differential pathobiological features of a HPAIV belonging to a classical lineage (H7N1 isolated in Italy in 1999) and a HPAIV of Gs/GD H5 lineage (H5N8 isolated in Spain in 2017) in different breeds of chickens (*Gallus gallus domesticus*), domestic geese (*Anser anser var. domestica*) and pigeons (*Columba livia var. domestica*).

In Study I, the Gs/GD H5N8 HPAIV isolated in Spain in 2017 was genetically characterized. The pathobiological properties of Gs/GD H5N8 HPAIV were then compared side by side with the classical H7N1 HPAIV in three experimental infections (Studies II, III and IV). We evaluated the differences in clinical presentation, gross and microscopic lesions, distribution of viral antigen in tissues (IHC techniques), viral shedding (qRT-PCR technique) and seroconversion (cELISA) between both HPAIVs after intranasal inoculation in chickens (II), geese (III) and pigeons (IV). In Study II, the genotype and allele frequency of a single nucleotide polymorphism (SNP) at position 2032 of chicken Mx gene and their association with susceptibility to HPAIVs were also determined.

In **Study I**, the Gs/GD H5N8 HPAIV that was used in the following experimental infections was characterized by means of full-genome sequencing. The phylogenetic analyses of the H5N8 isolated in a backyard goose in Spain in early 2017 indicated that the isolate belongs to clade 2.3.4.4 Group B of Gs/GD H5 lineage of HPAIVs, and presented high nucleotide identity with H5N8 HPAIVs previously isolated in Europe, Africa and Asia in 2016/2017. Based on the differential clustering of the strains, Europe was affected by at least five different H5N8 HPAIVs reassortants (CL1-5). All gene segments of the H5N8 HPAIV isolated in Spain clustered in group 2, indicating that the local H5N8 HPAIV strain does not represent a new reassortant virus. The amino acid characterization revealed several amino acid substitutions associated to adaptation, virulence and transmissibility in chickens, ducks and different mammal species, but the most common markers of human transmission and virulence were not present.

In **Study II**, the inoculation of H7N1 and H5N8 HPAIVs in chickens confirmed that both viruses are highly lethal for this species. However, the highest frequency of severe clinical signs, highest mortality ratio and shortest mean death time in those inoculated with H7N1 HPAIV demonstrated that this virus is more virulent for chickens than H5N8 HPAIV. High levels of viral shedding by the oral and cloacal routes were observed in chickens inoculated with H7N1 and H5N8 HPAIVs, but the comparatively lower number of chickens inoculated with H5N8 HPAIV presenting cloacal excretion suggests that the horizontal transmission of this virus could be affected. The microscopic examination of the tissues revealed that H5N8 HPAIV replicated in a broader spectrum of tissues in comparison with H7N1 HPAIV, demonstrating the existence of differences in cell tropism between both HPAIVs. However, the comparatively higher viral replication of H7N1 HPAIV in the brain of inoculated chickens suggest that the higher mortalities caused by H7N1 HPAIV could be associated with the higher neurotropism of this strain. We detected broad differences in the susceptibility to HPAIV infection between the chicken breeds included in this study. Three chicken breeds were comparatively more resistant to infection based on the lower frequency of clinical signs, mortality and number of birds shedding virus. However, local breeds were more susceptible to HPAIV infection than commercial breeds, demonstrating that local breeds do not necessarily present a higher resistance to HPAIVs. The analyses of the SNP at position 2032 of chicken Mx gene with infection outcome revealed that AA and AG genotypes are statistically associated with longer mean death times (MDT) than GG genotype.

In **Study III**, severe neurological signs were observed in domestic geese inoculated with H5N8 HPAIV and, by 10 dpi, all birds had succumbed to infection. This study demonstrates that Gs/GD clade 2.3.4.4 H5N8 HPAIVs circulating in Europe in 2016/2017 acquired high virulence for domestic geese. H5N8

HPAIV produced a systemic infection; however, the most severe lesions and highest detection of viral antigen were in central nervous system, followed by pancreas, liver, primary lymphoid organs (spleen and thymus) and heart, indicating that the birds likely died as a result of neurological dysfunction or multi-organ failure. The high oral and cloacal shedding in geese and the detection of AIV RNA in pool water indicate that this species could play an important role in the epidemiology of Gs/GD H5N8 HPAIV. In contrast, domestic geese inoculated with H7N1 HPAIV did not present any evident clinical sign, gross or microscopic lesions or viral antigen in tissues along the experiment. However, the detection of AIV RNA in plasma and tissues and the seroconversion demonstrate that several geese became subclinically infected. Oral shedding was detected in several geese inoculated with H7N1 HPAIV, and in some birds at high titers. Some birds were still shedding virus by the oral route at the end of the study, suggesting that domestic geese may play a role in the perpetuation and interspecies transmission of classical lineages of H7 HPAIVs. No breed-related differences were detected

In **Study IV**, H7N1 and H5N8 HPAIVs caused subclinical infections in pigeons, as demonstrated by the lack of clinical signs of disease, gross lesions, microscopic lesions, viral antigen in tissues and viral RNA in feather pulps but seroconversion and detection of viral shedding by oral and cloacal routes in several birds. One pigeon inoculated with H5N8 HPAIV presented severe nervous signs and in the further pathological examination of tissues, severe areas of necrosis associated to widespread AIV antigen were observed in the brain. Therefore, we demonstrate for the first time that Gs/GD clade 2.3.4.4 H5N8 HPAIVs could potentially cause lethal infections in pigeons by neurological dysfunction. The viral shedding in pigeons inoculated with H7N1 and H5N8 HPAIVs was inconsistent, short and generally the titers were low. However, since pigeons gather in large numbers and present a wide habitat use, the biological implications of the viral shedding detected here should be further assessed. No breed-related differences were detected.

Taken together, the present dissertation demonstrates that the clinico-pathological outcome and viral shedding after infection with HPAIVs varies largely depending on the virus and the host, highlighting the necessity to study the pathobiology of HPAIVs in different virus-host combinations. Considering the broad differences, this data also represents a start point to study viral and hosts factors associated with the observed results.

La influença aviària (IA) és considerada una de les malalties víriques més importants de la indústria avícola i una amenaça constant per als humans i les aus salvatges. La majoria d'epidèmies d'influença aviària d'alta patogenicitat (IAAP) han afectat principalment aus de producció terrestres. En canvi, els virus d'IAAP en rares ocasions han estat aillats o han causat mortalitats elevades en aus domèstiques d'espècies aquàtiques, aus salvatges i espècies d'aus peridomèstiques. Malgrat això, l'epidemiologia i patobiologia de la IAAP ha canviat radicalment des de l'aparició del llinatge dels virus d'IAAP Goose/Guangdong (Gs/GD) H5. Aquests virus presenten característiques biològiques úniques, incloent la capacitat d'infectar i causar la mort en un ampli rang d'espècies d'aus domèstiques, captives i salvatges. Aquestes dades demostren les grans diferències entre virus d'IAAP quant al resultat de la infecció. Diversos estudis demostren que el resultat de la infecció també està influenciat en gran mesura per factors de l'hoste. Particularment, existeix una àmplia variació en la susceptibilitat a la infecció per virus d'IAAP entre diferents races de pollastre, cosa que indica que la base genètica d'algunes races confereix una major resistència a la infecció per virus d'IAAP. Normalment, les races locals de pollastre es consideren més resistents que les races comercials degut a la manca de selecció artificial cap a gens relacionats amb la producció, la qual podria estar associada negativament amb la resistència a patògens.

Fins a dia d'avui, no hi ha una comparació sistemàtica de la patobiologia de virus d'IAAP clàssics i Gs/GD H5 en espècies aviars de different grups taxonòmics. La variació en la susceptibilitat de diferents races de pollastre als virus d'IAAP tampoc s'ha estudiat amb detall a Europa, així com l'existència de diferències en susceptibilitat als virus d'IAAP entres races d'aus de producció menor i peridomèstiques. Tenint en compte això, a la present tesi es va avaluar sistemàticament les característiques patobiològiques diferencials d'un virus d'IAAP pertanyent a un llinatge clàssic (H7N1 aillat a Itàlia al 1999) i d'un virus d'IAAP pertanyent al llinatge Gs/GD H5 (H5N8 aillat a Espanya al 2017) en diferents races de pollastres (Gallus gallus domesticus), oques domèstiques (Anser anser var. domestica) i coloms (Columba livia var. domestica).

A l'estudi I, es va caracteritzar el virus d'IAAP H5N8 aillat a Espanya al 2017. Les característiques patobiològiques d'aquest virus es varen comparar amb el virus d'IAAP clàssic H7N1 en tres infeccions experimentals (Estudis II, III i IV). Es van avaluar les diferències en la presentació clínica, lesions macroscòpiques i microscòpiques, distribució d'antigen víric en els teixits (tècniques d'IHC), excreció vírica (tècnica qRT-PCR) i la seroconversió (cELISA) entre els dos virus d'IAAP en pollastres (II), oques (III) i coloms. (IV). A l'estudi II també es va determinar la freqüència genotípica i al·lèlica d'un polimorfisme de nucleòtid únic (SNP) a la posició 2032 del gen Mx del pollastre i la seva associació amb la susceptibilitat als virus d'IAAP.

A **l'estudi I** es va caracteritzar el virus d'IAAP Gs/GD H5N8 que es va utilitzar en les infeccions experimentals mitjançant seqüenciació massiva de tots els segments del virus. L'anàlisi filogenètic del virus d'IAAP H5N8 aïllat en una oca domèstica a Espanya a principis del 2017 va indicar que el virus pertany al *clade* 2.3.4.4 Grup B del llinatge Gs/GD H5 dels virus d'IAAP. El virus presentava una alta identitat nucleotídica amb virus d'IAAP H5N8 aïllats a Europa, Àfrica i Àsia al 2016/2017. Tenint en compte les diferents agrupacions de les soques víriques, Europa es va veure afectada per almenys cinc virus d'IAAP H5N8 recombinants diferents (CL1-5). Tots els segments de gens del virus aïllat a Espanya s'agrupaven dins del *cluster* 2, indicant que la soca d'IAAP H5N8 local no representa un nou recombinant. La caracterització dels aminoàcids va revelar diverses substitucions d'aminoàcids associades a adaptació, virulència i transmissió en pollastres, ànecs i diferents espècies de mamífers, però els marcadors més comuns de transmissió i virulència a humans no estaven presents.

A **l'estudi II**, la inoculació dels virus d'IAAP H7N1 i H5N8 en pollastres va confirmar que ambdós virus produeixen infeccions letals en aquesta espècie. Tanmateix, la major freqüència de signes clínics greus, la major mortalitat i el temps mínim de mortalitat més curt en els inoculats amb el virus d'IAAP H7N1 demostren que aquest virus és més virulent en pollastres que el virus d'IAAP H5N8. Es van observar nivells elevats d'excreció oral i cloacal en els pollastres inoculats tant amb el virus d'IAAP H7N1 com en aquells inoculats amb el virus d'IAAP H5N8, però el nombre relativament inferior de pollastres inoculats amb H5N8 excretant per la cloaca suggereix que la transmissió horitzontal d'aquest virus podria veure's afectada. L'observació microscòpica dels teixits va indicar que el virus d'IAAP H5N8 va replicar en un rang més ampli de teixits que el virus d'IAAP H7N1, demostrant l'existència de diferències en tropisme cel·lular entre els dos virus d'IAAP. Tot i així, la major replicació del virus d'IAAP H7N1 en el cervell del pollastres suggereix que la major mortalitat causada pel virus d'IAAP H7N1 podria ser deguda al major neutropisme d'aquest virus. Es varen detectar grans diferències en la susceptibilitat a la infecció als virus d'IAAP entre les races de pollastre incloses en aquest estudi. Tres races de pollastre varen ser relativament més resistents a la infecció basat en la menor freqüència de signes clínics, la mortalitat i el nombre d'individus excretant virus. Tot i això, les races locals varen ser més susceptibles a la infecció pels virus d'IAAP que les races comercials, demostrant així que les races locals no presenten necessàriament una major resistència als virus d'IAAP. L'anàlisi del SNP a la posició 2032 del gen Mx de pollastre amb el resultat de la infecció va revelar que els genotips AA i AG estan associats estadísticament a temps mitjos de mortalitat més tardans que el genotip GG.

A **l'estudi III** es van observar signes neurològics greus en les oques domèstiques inoculades amb el virus d'IAAP H5N8 i, al dia 10 post-inoculació, totes les aus havien sucumbit a la infecció. Aquest estudi demostra que l'H5N8 que circulava a Europa al 2016/2017 va adquirir una alta virulència per les oques

domèstiques. El virus d'IAAP H5N8 va produir una infecció sistèmica; tanmateix, les lesions més greus i la major detecció d'antigen víric es varen detectar al sistema nerviós central, seguit de pàncrees, fetge, òrgans limfoides primaris (melsa i timus) i cor, indicant que les aus probablement van morir a causa de la disfunció neurològica o d'una fallida multisistèmica. L'elevada excreció oral i cloacal en les oques domèstiques i la detecció d'ARN de virus d'AI a l'aigua indiquen que aquesta espècie podria jugar un paper important en la epidemiologia del virus d'IAAP Gs/GD H5N8. En canvi, les oques domèstiques inoculades amb el virus d'IAAP H7N1 no varen presentar cap signe clínic evident, lesions macroscòpiques o microscòpiques o antigen víric en els teixits al llarg de l'experiment. Tot i això, la detecció d'ARN de virus d'IA en plasma i teixits i la seroconversió demostren que diverses oques es van infectar de forma subclínica. Es va detectar excreció oral en algunes oques inoculades amb el virus d'IAAP H7N1, i en algunes oques a nivells elevats. Algunes oques encara excretaven virus per via oral al final de l'estudi, el qual suggereix que les oques domèstiques podrien jugar un paper en la perpetuació i transmissió entre espècies de virus d'IAAP de llinatges clàssics d'H7. No es varen detectar diferències associades a la raça.

A **l'estudi IV**, els virus d'IAAP H7N1 i H5N8 van produir infeccions subclíniques en els coloms, com es demostra per la falta de signes clínics, lesions macroscòpiques, lesions microscòpiques, antigen víric en els teixits i ARN viral en polpes de ploma, però es detectà seroconversió i excreció vírica tant per via oral com cloacal en diversos coloms. Un colom inoculat amb el virus d'IAAP H5N8 va presentar signes nerviosos greus i en l'examen patològic posterior dels teixits es varen observar àrees àmplies de necrosi al cervell associades a extensa presència d'antigen de virus d'IA. Així, hem demostrat per primer cop que els virus d'IAAP H5N8 del llinatge Gs/GD tenen el potencial per produir infeccions letals en coloms a causa de disfunció neurològica. L'excreció vírica detectada en els coloms inoculats amb els virus d'IAAP H7N1 i H5N8 era inconsistent, curta i en general els títols eren baixos. Tot i així, tenint en compte el comportament gregari i l'ampli ús d'hàbitats utilitzats pels coloms, la implicació biològica de la detecció vírica detectada hauria de ser avaluada amb més detall. No es varen detectar diferències associades a la raça.

En conjunt, la present tesi demostra que el resultat clínico-patològic i l'excreció vírica després de la infecció amb els virus d'IAAP varia en gran mesura del virus i de l'hoste, destacant la necessitat d'estudiar la patobiologia dels virus d'IAAP en diferents combinacions virus-hoste. Tenint en compte les grans diferències, aquestes dades també representen un punt de partida per estudiar els factors virals i de l'hoste associats als resultats observats.

PART I

General Introduction and Objectives

CHAPTER 1

GENERAL INTRODUCTION

1. AVIAN INFLUENZA INFECTION

1.1. HISTORY

Through the history there has been several reports of high flock mortality in poultry. The epizootic in United States in 1872, when a highly lethal disease in poultry progressed temporally with an equine influenza panzootic suggests that it was an early event of Avian influenza (AI) [1]. However, Perroncito was the first to document outbreaks of a poultry disease capable of causing extremely high mortality that was distinguishable of bacterial diseases (e.g. fowl cholera) in 1878 in Italy [2]. The disease, which was later named fowl plague [3], was recurrently detected from late 1800s to the 1950s within Europe, and also in Asia, Africa and America [4–8].

In 1901, the causal agent of this poultry disease was already shown to be ultra-filterable, being one of the earliest diseases discovered to be caused by a virus [9]. In 1931, the isolation of swine influenza viruses was achieved [10], and two years later the agent of human influenza was discovered [11]. However, it was not until 1955 that fowl-plague viruses were classified within Influenza A viruses (IAVs), and that their genetic relationships with mammalian IAVs were revealed [12]. Shortly after, a virus causing mild respiratory signs in chickens associated to low mortalities was also shown to be an IAV, revealing the genetic similarities between virulent and mild forms of disease [13, 14]. However, they were not officialy designated as highly pathogenic AIVs (HPAIV) and low pathogenic AIVs (LPAIV) until 1981 [15] and 2002 [16], respectively.

Until the mid-1950s, all HPAIVs belonged to the H7-subtype, but in 1959 the first outbreak in chickens caused by a HPAIV of the H5 subtype was reported [17]. Only two years later, another HPAIV of the H5 subtype was isolated in common terns (*Sterna hirundo*), which was also the first AIV isolated in wild birds [18, 19]. Despite further data evidenced the circulation of AIVs in wild birds, it was not until the systematic investigations carried out in mid-1970s that it was demonstrated the role of waterfowl and shorebirds in perpetuating enormous pools of AIVs [20].

At that time, three human influenza pandemics occurred: in 1918 (H1N1), in 1957 (H2N2) and in 1968 (H3N2). H1N1 IAV, which caused deadliest event in the history of humanity, seems to be a fully avian-like virus that adapted to humans [21]. In contrast, H2N2 and H3N2 were human IAVs that acquired segments from AIVs [22, 23]. Since then, several AIVs subtypes (H5, H6, H7, H9, H10) have crossed the species barrier directly from avian species and produced infections in humans [24–27]. Despite AIV

infections in humans range from asymptomatic to fatal, none of them have acquired a sustained human-to-human transmission.

AIVs outbreaks have been recurrently reported in poultry in the last decades. The outbreaks of HPAIVs increased specially after the industrialization in the 1970s, when the poultry population grew worlwide, and the poultry production suffered a radical change from small flocks to large integrated farms with intense poultry trade [28]. This industrial development also resulted in the formation of Densely Populated Poultry Areas (DPPAs), where HPAIVs have a more visible impact [28]. Poultry production has continue to grow: 120 and 81 million tonnes of meat and eggs, respectively, were produced worldwide by 2016 [29], and the expectation is that demand will continue to grow. Therefore, new HPAIVs outbreaks are expected to ocurr.

Most HPAI epizootics have been geographically restricted, have affected a low number of farms and/or have been eradicated often within a year [30–34]. In contrast, some HPAIVs have become widespread and/or have persisted over long periods of time before its successful erradication, thus causing devastating economic losses in the economy of the poultry industry (millions of birds dead or culled) [35–42] **(Table 1).**

HPAIV strain	Subtype	Number of infected farms (birds)	Reference
A/chicken/Pennsylvania/1370/83	H5N2	356 (> 17,000,000)	[35]
A/chicken/Mexico/8623-607/94	H5N2	Many (na)	[36]
A/chicken/Pakistan/447/94	H7N3	Many (> 6,000,000)	[37]
A/turkey/Italy/99	H7N1	413 (16,000,000)	[38]
A/chicken/Netherlands/2003	H7N7	255 (> 28,000,000)	[39]
A**/chicken/Canada-BC/2004**	H7N3	53 (> 19,000,000)	[40]
Achicken/Jalisco/12283/2012	H7N3	Na (>22,000,000)	[41]
AH/13-lineage/2016	H7N9	On-going (na, millions?)	[42]

Table 1. Examples of major epidemics reported in the last decades (excluding the Asian-origin H5N1). Information partially obtained from reference [28].

The majority of HPAI epidemics have affected the chicken species the most, which represents the 91 percent of the world's poultry population [43]. Outbreaks associated to high mortalities in other gallinaceous species such as turkeys, partridges and pheasants have also occurred, as well as in ostriches [44]. In contrast, ducks, geese and other species have been more sporadically affected, and generally

lacked or presented low mortalities [28]. However, the first demonstration that poultry species infected subclinically suppose potential sources of infection due to the lack of evident signs of disease ocurred in 1983, when a HPAIV was detected in a commercial duck farm located between two turkey farms where the virus had caused the first outbreaks [45].

The higher frequency of HPAI epidemics in the last decades is a concern; however, any has reached the magnitude and geographic extension of that caused by the Goose/Guangdong (Gs/GD) H5 lineage of HPAIVs, which is considered a panzootic [46]. Over 70 different countries have reported outbreaks of Gs/GD H5 HPAIVs and the lineage is endemic in at least 8 countries [47]. Gs/GD H5 HPAIVs have evolved rapidly and to date, 10 distinct phylogenetic clades (0-9) and up to fourth-order sub-clades (e.g. 2.3.4.4) are characterized [48]. This lineage of HPAIVs has caused the death of millions of land-based and aquatic poultry of numerous species by either infection or culling. In addition, Gs/GD H5 HPAIVs have caused lethal infections in zoo and wild birds, including migratory aquatic birds, synanthropic birds, predators and scavengers [49]. Importantly, these viruses have recurrently crossed the species barrier and caused infection in mammals, including at least 462 deaths in humans by June 2019 (fatality rate of about 53%) [50].

The ancestor of all Gs/GD H5 HPAIVs is A/goose/Guangdong/1/1996 H5N1, which was firstly isolated in domestic geese in 1996 causing unexpected high mortalities for this species (up to 40%) [51]. In 1997, the virus spilled to Hong Kong poultry, where caused several outbreaks until all poultry was depopulated [52]. Despite the stamping out program was succesful, the virus reappeared and in the following years, several genotypes were identified in domestic bird populations (mainly in ducks and geese) [53–55]. Another sign that the pathobiology of HPAIVs was changing was in late 2002, when H5N1 caused the death of wild migratory birds in two Hong Kong Parks [56]. From 2003 to 2004, H5N1 HPAIVs spread to mainland China and to other countries in Southeast and East Asia, where they caused numerous outbreaks in poultry and were isolated in wild bird populations. In addition, multiple sublineages of these viruses established in poultry in different geographical regions [57].

In April 2005, the Qinghai strain (clade 2.2) caused a massive died-off of several wild bird species at Qinghai Lake, China. More than 6.000 wild birds died, being the 90% of them bar-headed geese *(Anser indicus)* [58]. The detection of H5N1 HPAIV in Qinghai Lake preceded the detection of the virus in poultry and wild bird species in Central and East Asia in mid-2005, and in late 2005 and 2006, these viruses disseminated for the first time westwards into south-western and central Europe, Russia, Middle East and North and West Africa [59]. Multiple clades continued to emerge and circulate in Asia, and the

H5N1 HPAIVs belonging to clade 2.3.2.1c were responsible of two new intercontinental waves in 2009 and 2014 [59].

The rapid evolution of this lineage of HPAIVs have resulted in the continous detection of novel variants containing diverse internal gene constellations. However, until 2008 all strains belonged to the H5N1 subtype. Since 2008 onwards, Gs/GD H5 HPAIVs of different subtypes (referred to as H5Nx) have been identified, increasing the complexity of this lineage [60]. These viruses are classified within subclade 2.3.4.4, and include the subtypes H5N1, H5N2, H5N5, H5N6 and H5N8 HPAIVs that were first isolated in China [61–64], and H5N3 that originated in Taiwan [65]. Provisionally, they have been further separated into four distinct groups (A to D), each containing different subypes **(Figure 1)**. The four groups have caused numerous outbreaks in wild and poultry species in different Asian countries. In addition, Group A and Group B spread out causing two new intercontinental waves in 2014 and 2016, respectively, where reassorted with local LPAIVs generating a complex viral population of subtypes and genotypes [66]. For the first time in this lineage, the HPAIVs of clade 2.3.4.4 also reached North America (Group A) [67] and Southern Africa (Group B) [68]. By 2015, 2.3.4.4 HPAIVs represented the dominant clade of the Gs/GD H5 lineage in different countries. The number of outbreaks have decreased in 2018 and 2019, but outbreaks are still reported in several countries in either domesticated or wild birds [60, 69].

Europe has been affected by all the transcontinental Gs/GD H5 lineage epizootics (clade 2.2 in 2005, 2.3.2.1c in 2009 and 2014, clade 2.3.4.4 Group A in 2014, and 2.3.4.4 Group B in 2016) [59]. However, the caused by Gs/GD H5N8 clade 2.3.4.4 Group B in 2016/2017 was the largest in severity (reported poultry outbreaks and deaths), geographic spread, speed of events, and diversity of wild bird species infected [70, 71]. A novel intercontinental wave of Gs/GD H5 lineage could occur as novel variants with different biological properties continue to emerge.

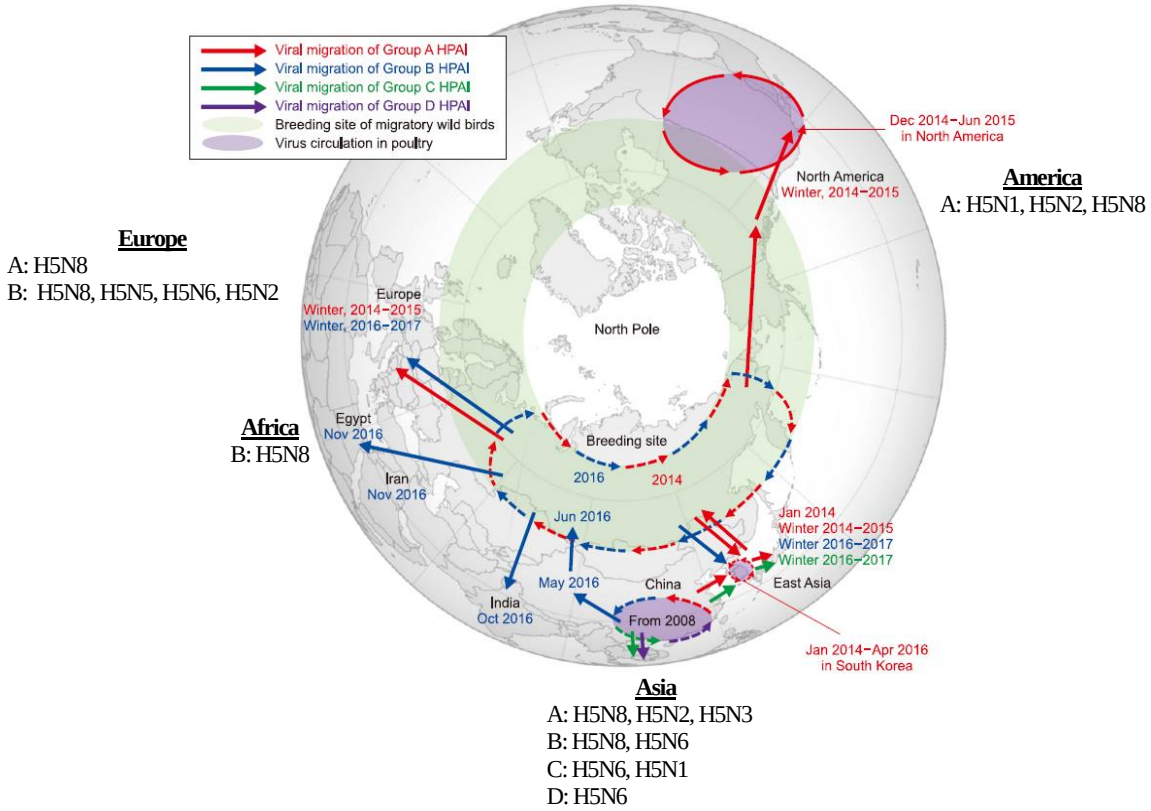


Figure 1. Movement patterns of Gs/GD clade 2.3.4.4 HPAIVs (groups A to D) and H5Nx subtypes identified at the different continents. H5N8 HPAIVs belonging to Group A (Buan-like) and B (Gochang-like) were isolated in South Korea in 2014. H5N8 A HPAIV spread in 2014 to other parts of Asia, Europe and North America. In North America and Taiwan, H5N8 A HPAIV reassorted with local LPAIVs generating different HPAIV subtypes. H5N8 B HPAIV spread worlwide in 2016/2017, and was detected in numerous countries from Europe, Middle East, Asia, and Africa, with evidence for genetic echange with LPAIVs and generation of multiple HPAIVs subtypes. HPAIV reassortants from Europe were transmitted back into several Asian countries in 2017. H5N6 Group C was detected in China in 2013 and then became established in Laos and Vietnam in 2014, were generated other HPAIV subtypes. In 2016/2017, they caused outbreaks in South Korea and Japan. H5N6 HPAIVs of Group D have been isolated in China and Vietnam. Information summarized from references [60, 66]. Image modified from reference [66].

1.2. ETIOLOGY

1.2.1. CLASSIFICATION AND NOMENCLATURE

AIVs belong to the Orthomyxoviridae family, which includes seven genus: *Influenzavirus A* (Type A), *Influenzavirus B* (Type B), *Influenzavirus C* (Type C), *Influenzavirus D* (Type D), *Thogotovirus, Isavirus and*

Quaranfilvirus [72]. The genus are classified based on antigenic differences in the viral nucleoprotein (NP) and matrix (M1) proteins. AIVs belong to *Influenzavirus* A, which is the genus that presents the higher genetic variability and the broadest host range among the seven, and is the only known to cause natural infections in birds [72].

IAVs are further classified into subtypes based on the antigenicity of the glycoproteins hemagglutinin (HA) and neuraminidase (NA) present in the surface of the virion. To date, 18 and 11 different subtypes of HA and NA, respectively, are described. Among them, 16 HA and 9 NA subtypes in virtually all possible combinations have been isolated from birds [73]. AIVs can also be divided into lineages, which are usually categorized on a geographical basis (e.g. Eurasian versus North American), and into genetic groups based on their internal gene constellation [74].

A standarized nomenclature system is used for all Influenza viruses. The viral strain is named using the follow components: the antigenic type (A, B,C or D), the host where the virus was isolated (e.g. chicken, swine, equine), the geographical origin (either a city, state, province or country), the identification number of the strain and the year of isolation. The subtype is shown in brackets For example, A/chicken/Italy/5093/99 (H7N1). If the virus is isolated from a human being, host is not included [75].

1.2.2. VIRAL STRUCTURE AND PROTEIN FUNCTIONS

IAVs are pleomorfic viruses (spherical of 80 to 120 nm in diameter to filamentous that reach up to 20 μm in length) enveloped by a lipid membrane derived from the host cell that contain segmented, negative-sense, single-stranded ribonucleic acid (RNA) (-ss vRNA) genomes [72, 76]. IAVs present 8 gene segments that range in size between 890–2341 nucleotides length each and, in total, its genome contains approximately 13.600 nucleotides [77, 78].

Each segment encodes at least one viral protein, and virions contain a minimum of ten different polypeptides that are grouped into three different categories: surface proteins (HA, NA, M2), internal proteins (PB2, PB1, PA, NP, M1), and nonstructural proteins (NS1, NS2) [73]. The transmembrane glycoproteins HA and NA represents approximately the 80% and 17%, respectively, of the proteins present in the viral envelope [79]. HA is a trimeric protein that protrudes from the viral surface in a rod-like shape, whereas NA forms a tetrameric structure that protudes in mushroom-like shape [80]. The

viral envelope also contains a small portion of the tetrameric protein M2. M1 protein is located in the inner surface of the viral envelope. In the viral core, the eight vRNA segments are coated within the NP forming double-helical hairpin structures [81]. This formation also carries the RNA-dependent RNA polymerase heterotrimer (RdRp) formed by PB1, PB2 and PA proteins. Altogether, these structures are named ribonucleoprotein particles/complex (vRNPs) [81] **(Figure 2).**

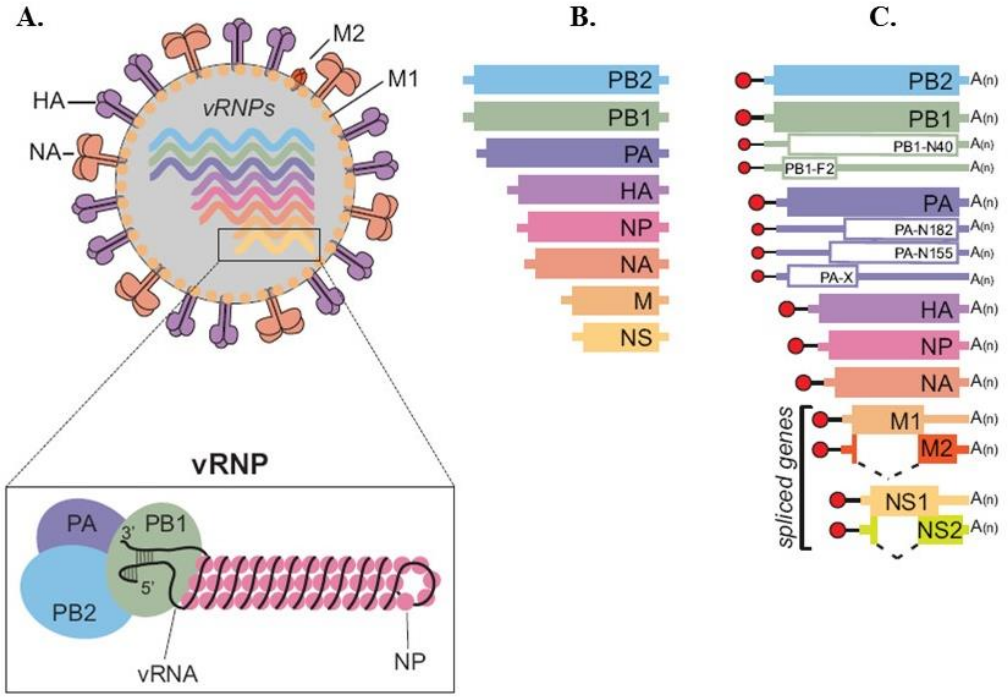


Figure 2. Structure of IAVs. A. Morphology of the virion and schematic representation of the vRNP. The vRNA (B) and messenger RNA (mRNA) (C) segments are represented in order of decreasing size. Adapted from reference [82].

Novel proteins in IAVs have been identified, which are the result of translation from alternative reading frames, gene splicing or ribosomal frameshift. These proteins include the most studied PB1-F2 [83] and PA-X [84], and the more recently discovered PB2-S1, PB1-N40, PA-N155, PA-N182, M3, M4, M42 and NS3 [85–89]. However, these proteins are not detected in all IAVs subtypes. The main functions of several of these newly identified viral proteins have been reported, while in other cases the functions and amounts of molecules in the virions remain unknown. It has been demonstrated that virions also contain cytoplasmic and membrane-bound cellular host proteins [90]. The main functions of the viral proteins are presented in **Table 2.**

Segment	Size	Protein (mol/virion)	Main functions
1 (PB2)	2341	PB2 (30-60)	Cap recognition and binding RIG-I-mediated IFN-expression
		PB2-S1 (nd)	Inhibition of RIG-I-dependent interferon signaling pathway Interfere RdRp activity
2 (PB1)	2341	PB1 (30-60)	Prime viral mRNA transcription Transcribes vRNA into cRNA Initiates vRNA synthesis
		PB1-F2 (nd)	Induces apoptosis Modulates host interferon response Modulates susceptibility to secondary bacterial infection
		PB1-N40 (nd)	May balance expression of PB1 and PB1-F2
3 (PA)	2233	PA (30-60)	RNA endonuclease activity: cleave capped RNA for viral mRNA synthesis
		PA-X (nd)	Modulation of the host response and viral virulence
		PA-N155 (nd)	Promote viral replication and virulence
		PA-N182 (nd)	Promote viral replication and virulence
4 (HA)	1778	HA (500)	Sialic acid-receptor binding Membrane fusion Major antigen
5 (NP)	1565	NP (1000)	vRNA binding, protection and synthesis Nuclear import of vRNP
6 (NA)	1413	NA (100)	Sialidase activity: release of progeny virions Facilitate the virion to penetrate the mucus barrier and infect new cells.
7 (M)	1027	M1 (3000)	Controls the morphology of the virion Viral assembly andbudding Nuclear import and export of vRNPs
		M2 (20-60)	Ion channel activity Uncoating process
		M3 (nd)	not known
		M4 (nd)	not known
		M42 (nd)	Complements M2 function
8 (NS)	890	NS1 (nd)	vRNP entry by hijacking importin-α Viral mRNA splicing, maturation and translation

	Counters cellular antiviral responses, including interferons Inhibits cellular mRNA maturation and translation
NS2/NEP (130-200)	vRNP nuclear export Regulation of vRNA transcription and replication
NS3 (0)	not known

Table 2. vRNA segments and length, encoded proteins and their functions. Information obtained from reference [91]. nd: not determined.

1.2.3. VIRUS REPLICATION CYCLE

The replication of IAVs can be divided into different steps **(Figure 3)**. After entry into the host, the initial stage of host cell infection is mediated through interaction of the viral HA protein with cell surface sialic acid receptors (SA) present on cellular glycoprotein or glycolipids [92]. The binding of HA to SA triggers the endocytosis of the virion, which is mainly receptor-mediated by clatrhrin-dependent mechanisms [93] or via macropinocytosis [94]. The endocytosis results in a virion-endosome structure that is exposed to gradual lower pH through the process of maturation inside the cell. The acidification of the endosome causes a conformational change in the HA, which leads to the fusion of the viral and endosomal membrane [95]. The entry of protons and potassium ions through M2 protein further acidify the virion, causing the dissociation of the vRNP from the M1 protein and the subsequent release of the contents of the viral core into the cytoplasm of the host cell [96].

The vRNP is translocated into the host nucleus by nuclear localization signals present in the NP [96]. In the nucleus, the replication and transcription of vRNA occurs [82]. vRNAs are copied into different types of RNA: complementary RNA ([+]cRNA), used as a template by RdRp for generation of more copies of vRNA that will be coated within the new virions [97]; [+]mRNA, which are used for protein translation; small viral RNAs that control the switch from transcription to genome replication [98]; and mini viral RNAs produced as a result of aberrant replication of the vRNA genome that act as innate immune agonists [99]. These processes are carried out predominantly by the vRNP, but multiple nuclear and cytoplasmic host proteins act as cofactors at different stages [100–102].

IAVs vRNA segments lack a 5' cap sequence that is necessary for transcription. Therefore, RdRp performs a process known as "cap-snatching", where the 5'-cap structure of host pre-mRNAs are recognized and cleaved by PB2 and PA, respectively, obtaining primers that allow the transcription of vRNA segments [103]. mRNA transcripts are then polyadenylated [104]. 5'capped,3'polyadenylated viral mRNAs are released into the cytoplasm by NS1 protein and then translocated into the host ribosomes, where they are translated using the host machinery [105]. The newly-synthesized HA, NA and M2 viral proteins are translocated into the lumen of the rough endoplasmic reticulum and Golgi apparatus for post-translational modifications. Afterwards, they are transported to the cell membrane, where they attach to and remain in the lipid bilayer [106–108]. In contrast, PB2, PB1, PA, NP, M1, NS1 and NS2 proteins are imported back into the nucleus. There, PB2, PB1, PA and NP proteins are involved in the secondary cycle of transcription and in the replication of vRNA, or bind to copied vRNA to assemble new vRNPs [82, 109]

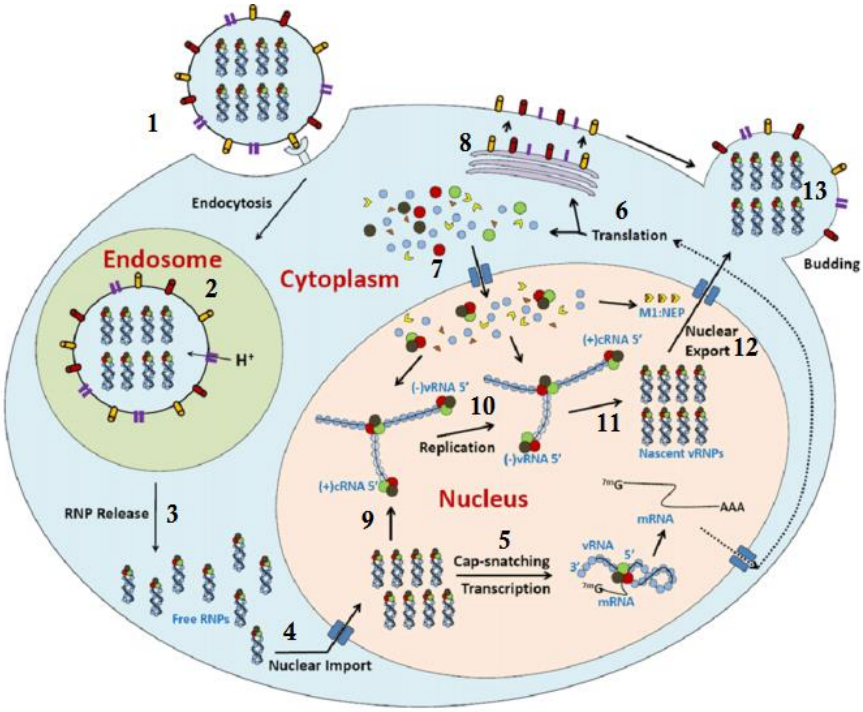


Figure 3. Schematic representation of IAV replication cycle. 1. Virion binds to SA in the surface of host cells. **2.** Endocytosis of the virion and acidification of the endosome. **3.** Fusion of viral and endomosal membranes, and release of vRNPs into the cytoplasm. **4.** vRNPs are transported into the nucleus. **5.** RdRp performs transcription of vRNA segments. **6.** Export of viral mRNAs and translation. **7.** Viral PB1, PB2, PA, NP, M and NS proteins are transported back into the nucleus. **8.** HA, NA and M2 are post-translationally modificated and transported into the cell membrane. **9,10.** RdRp replicates vRNA into cRNAs, which act as templates for the production of vRNAs. **11.** Assembly of vRNPs. **12.** Progeny vRNPs, M and NS are transported to the cell membrane. **13.** Assembly of progeny virions in the membrane and release. Adapted from reference [110].

Once completed the replication, transcription and translation of viral genome, vRNPs are exported from the nucleus and translocated into the host cell membrane by M1 [111] and NS2 [112] proteins using recycling endosomes [113] and tubules within a modified endoplasmic reticulum [114]. There, the assembly of viral components and budding of progeny virions occur. HA, NA and M1 proteins sequentially cause the modification of the membrane curvature, and M2 further release budding viruses by membrane scission [115]. NA protein then cleaves SA moieties present on the host cell surface and from decoy receptors (e.g. mucus), which avoid the self-binding of the proteins and aggregation of progeny viruses and enable their spread to bystander cells [116]. Despite all vRNA segments contain highly conserved regions that act as packaging signals, the replication of IAVs is considered highly inefficient, and more than 90% of the viral particles can be non-infectious due to inadequate packaging of vRNA segments [117].

1.2.4. ANTIGENIC EVOLUTION

The continous evolution of IAVs enables the production of novel virus variants with different genetic, antigenic and biological characteristics. That evolution is driven by two phenomenoms that produces either minor (antigenic drift) or major (antigenic shift) changes [118].

Antigenic drift is based on the accumulation of point mutations in vRNA segments that consequently alter the amino acid sequence of the proteins. The vRNA replication of IAVs is highly prone to errors due to the lack of proofreading activity in the polymerase complex, resulting in high mutation rates (2-3 mutations in each replicated genome) [119]. Thus, IAVs can be considered as a complex population of heterogeneous, albeit related genetic variants based on the quasispecies concept [120]. Some of the mutations can be detrimental (eg. reduced polymerase activity), whereas the viruses carrying mutations that are beneficial are likely positively selected. The phenomenom of antigenic drift occurs in all gene segments, leading to rapid evolutionary dynamics [121]. However, it is more frequent in HA and NA glycoproteins, particularly in the antibody-binding sites which are under high selection pressures. In HA, the nucleotide substitution rate can be as high as 10.15×10^{-3} substitutions/site/year [122]. Despite antigenic epitopes have been detected in serveral proteins, the protection against IAVs is mediated mainly by specific antibodies targetting the HA protein, and to a lesser extent the NA. The high mutation rate in these segments result in the emergence of viral populations with potentially diverse antigenic properties [123–125], and some may acquire the capacity to escape the host immune responses. For that reason, antigenic drift is the major responsible of reduced vaccine eficacy in the human

population and the main cause of IAV seasonal epidemics [126]. Similarly, commercial vaccines used in poultry may not elicit complete protection againsts different genetic variants of AIVs [127–129]. Therefore, the analysis of the antigenic characteristics of the new emergent viruses for updating and selecting the most suitable vaccines is needed.

Antigenic shift/re-assortment is based on the genetic exchange of whole vRNA segments between IAVs in a single co-infected cell. Since the genome of IAVs is segmented, the simultaneous assembly of different IAVs can result in the generation of progeny viruses that contain a combination of segments different from all the parental strains. In theory, co-infection with two IAVs could led to 254 possible recombinants, plus the two parental strains. However, the frequency of reassortments and biological efficiency of recombinant progeny vary largely depending on many factors, including compatibility among RNA packaging signals, compatibility of polymerase subunits and HA/NA functional balance [130]. In humans, antigenic shift have been the cause of devastating IAV human pandemics in the 20th century, when IAVs carrying a novel HA or HA/NA subtype combination to which most of the people were immunologically naïve were introduced into the population [131]. In birds, novel AIV reassortants are isolated from different wild and domestic avian species during surveillance and clinical diagnosis [132, 133]. In addition, experimental incoulation of two AIVs in birds can lead to a high frequency of reassortment events and in the generation of reassortants with higher virulence than the parental strains [134]. Antigenic shift can also refer to the direct transmission of IAVs to naïve hosts once crossed the species barrier (e.g. from birds to humans), as well as the re-emergence of a previously circulating strain [135].

1.2.5.VIRAL PATHOTYPES

AIVs are classified into LPAIVs or HPAIVs pathotypes in the basis of their virulence in chickens and the amino acid motif at the HA proteolytic cleavage site. HPAIVs are those that present an intravenous pathogenicity index (IVPI) greater than 1.2 (mean clinical score obtained as follows: 0=normal, 1 =sick, 2 =severely sick, 3 =dead) in six-week-old chickens within 10 days or, alternatively, cause at least 75% mortality in four-to eight-week-old chickens, following intravenous inoculation with 0.2 ml of a 1/10 dilution of infective allantoic fluid. H5 and H7 AIVs that have an IVPI <1.2 or cause less than 75% mortality are further sequenced to determine the amount of basic amino acids (arginine –R- and lysine –K-) present at the cleavage site of the HA [136]. LPAIVs possess a mono-basic cleavage site (MCs), thus, contain a single basic amino acid in the critical position -1 of the cleavage site (e.g.

PEKQT**R**/GLF). In contrast, HPAIVs contain a multi-basic cleavage site (MBCs). These viruses present several basic amino acids in the cleavage site -1 and in the direct previous sequence (e.g. PQRES**RRKK**/GLF) [137]. If the amino acid motif of the virus is similar to that observed in other HPAIVs, the virus is classified as HPAIV. All AIVs that do not meet the criteria for HPAIVs are then classified as LPAIVs [136].

LPAIVs are the direct ancestors of HPAIVs [138]. For unknown reasons and with few exceptions, HPAIVs have been restricted to H5 and H7 subtypes. From 1959 onwards, there have been at least 39 independent H5 and H7 LPAIV to HPAIV conversion events [139]. Since LPAIVs of H5 and H7 subtypes are prone to mutation to HPAIVs, all H5 and H7 AIVs detected in poultry or wild birds are of mandatory notification to the World Organization for Animal Health (OIE) as notifiable avian influenza (NAI), either as highly pathogenic notifiable avian influenza (HPNAI) or low pathogenic notifiable avian influenza (LPNAI). Therefore, LPAI usually refers to LPAIV subtypes that are not included within the classification of NAI [140].

Thes shift from LPAIV to HPAIV is the result of the acquisition of multiple basic amino acids at the HA cleavage site, and there are evidences that this occurs after circulation of the virus in gallinaceous poultry, including chickens and turkeys [141–143]. The period until the emergence of the HPAIV variant is highly variable, but the probability is expected to be higher if the precursor circulates extensively in the population [144]. This process is mediated by several mechanisms. For most H5 AIVs, spontaneous duplication of purine triplets due to a transcription error by the polymerase complex and substitution of non-basic with basic amino acids appear to be the main mechanism of acquisition of basic amino acids. In contrast, non-homologus recombination with other gene sequences (either with another gene segment or host genome) and accumulation of single nucleotide insertions without repeating nucleotides are the predominant for H7 AIVs [145].

It is important to highlight that the definition of LPAIV or HPAIV based on the pathogenicity in chickens and the proteolytic cleavage site of the HA does not necessarily correspond with their virulence in other species of birds, or in mammals [146].

1.3. EPIDEMIOLOGY

1.3.1. HOST RANGE

IAVs can infect a broad diversity of hosts belonging to different taxonomic groups; however, almost all antigenic variants have been isolated in the class Aves. Over 105 species belonging to 26 different families and 12 Orders of birds have been infected with IAVs [147]. Despite there is a general consensus that all orders of birds are susceptible to IAVs, wild birds belonging to Anatidae in the Order *Anseriformes* (ducks, geese, swans), followed by Scolopacidae (shorebirds/waders) and laridae (gulls, terns) in the order *Charadriiformes* constitute the reservoirs of IAVs [73]. In these species, 16 HA and 9 NA different subtypes of IAVs in almost all the possible combinations naturally circulate in an apparent conserved status (low evolutionary rates) and subclinical way [148]. The remaining two HA (17-18) and NA (10-11) IAVs subtypes have been identified in several species of bats [149, 150]. A potential new subtype was recently isolated in Egyptian bats [151].

Phylogenetic studies demonstrate that IAVs from waterbirds represent the ancestors of IAVs isolated in other avian species, including poultry, and mammals **(Figure 4)**. The transmission of IAVs between different species is mainly determined by the receptor binding properties of the virus, but numerous viral and host factors are involved [152, 153]. In general, IAVs from the wild reservoir present poor binding affinity and/or replication fitness in other hosts and must undergo changes in order to efficiently infect a new species. This process may require multiple mutations or reassortment of viral segments, and may even need passage in intermediate hosts (e.g. quail, swine) in order to overcome the species-barriers [154]. The interspecies transmssion of IAVs is more common between wid birds and poultry and taking into account the genetic similarities, domestic waterfowl become more readily infected with wild bird-origin AIVs than most land-based poultry species [155]. IAVs can acquire a high degree of adaptation to land-based poultry, resulting in IAVs that can infect and be transmited efficiently in the population [156]. Some IAVs have also adapted to non-avian species, resulting in the formation of major lineages of IAVs in humans [157], swine [158], horses [159] and dogs [160]. As a result of adaptation in a new species, these IAVs usually lose the capacity to replicate easily in the wild reservoir [161]. In other species, the isolation of IAVs is generally more sporadic and there is no clear evidence of stable lineages. This group include cats, ferrets, whales and marine mammals [162, 163]. Other mammal species such as mice and guinea pigs have are susceptible to infection and therefore, like ferrets, they are broadly used as animal models [164]. Antibodies againsts influenza viruses or influenza virus-like RNA have been

detected in amphibians and reptiles, and even in hagfish and spiny ells, but their genus remains undefined in most cases [165, 166].

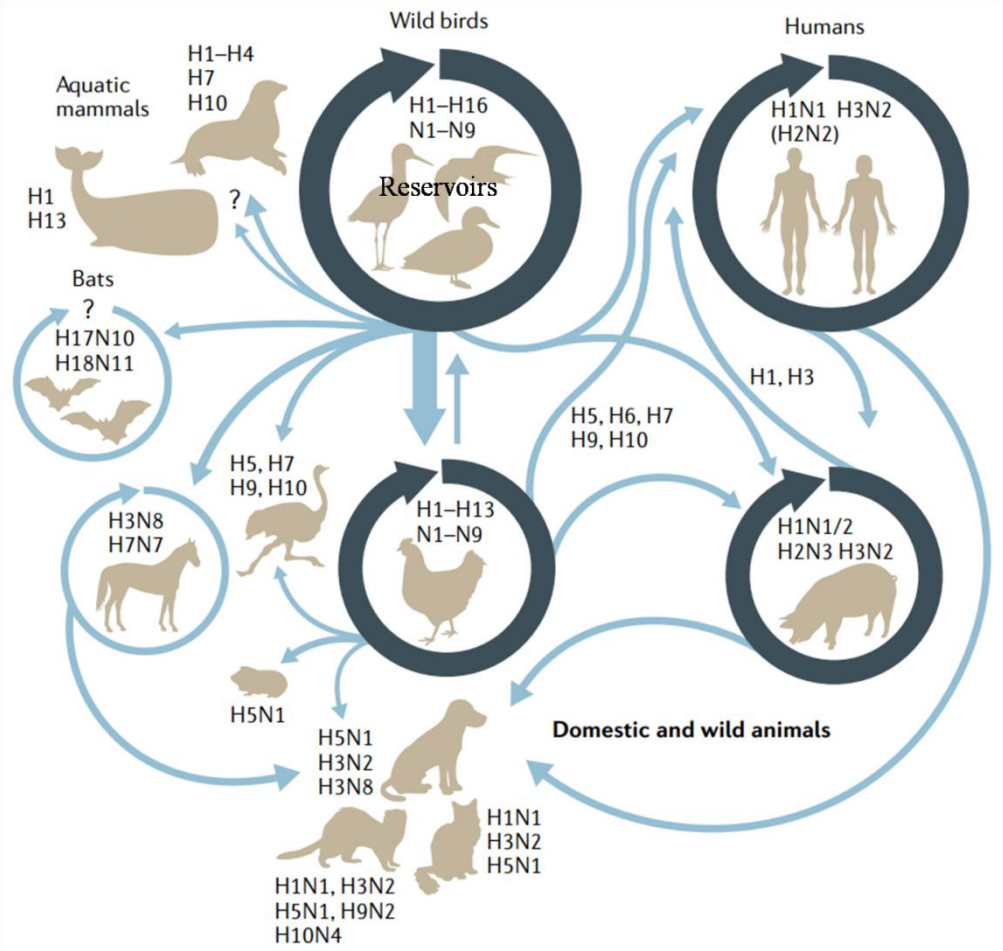


Figure 4. Host range of IAVs. Diagrammatic representation of the host range and transmission of IAVs between different hosts, and the most common subtypes isolated in each species. Obtained from reference [152].

For most IAVs subtypes and without taking into account the wild reservoirs, the host range is generally restricted to one species, or two in particular strains (e.g. H3N8 in dogs and horses) [167]. A small number of IAVs have infected three or more non-reservoir species (e.g. H3N2 in turkeys, pigs and humans) [168]. The exception are the HPAIVs belonging to Gs/GD H5 lineage, which are unique regarding their host range. These viruses have been detected in more than 172 species, including members of the avian orders of *Galliformes, Anseriformes, Charadriiformes, Columbiformes, Psittaciformes, Struthioniformes, Ciconiiformes, Falconiformes, Gruigormes, Passeriformes, Pelecaniformes, Strigiformes, Podicipediformes, Phoenicopteriformes,* and the mammalian orders of *Artiodactyla, Carnivora, Lagomorpha, Primates, Perissodactyla* and *Rodentia* [49].

1.3.2. RESERVOIR

Wild Anseriforms and Charadriiforms represents the **reservoirs** of AIVs, where they circulate as LPAIVs [73]. The prevalence and diversity of AIVs are usually higher in waterfowl, particularly in dabbling ducks of the Anatinae sub-family and Anseriformes species, including mallards, teals and pintails. Among them, the largest amounts and variety of AIVs subtypes are generally isolated from wild mallard ducks (*Anas platyrhynchos*), especially in young individuals (up to 30% isolation rate) [147, 169, 170]. However, waterfowl do not represent the reservoir of all AIVs subtypes (e.g. H13 and H16) and high prevalences have been detected in other waterbird species, including gulls [171–173].

The perpetuation of AIVs in wild waterfowl populations is associated with the high influx of adults and immunologically naïve juveniles of different avian species in breeding areas in spring and summer. These conditions facilitate largely the interspecies transmission of LPAIVs and just before the fall migration, the prevalence of infection usually reaches the highest values [147]. The overlap of different migratory species facilites the cross-flyway of AIVs and even there are numerous evidences of intercontinental exchange by migratory birds [174–177]. The prevalence declines troughout the fall migration because of the acquisition of immunity in the juveniles and the distribution of birds among the different migratory routes. How wild waterfowl acquire AIVs again in the spring is still under debate: some waterfowl species may perpetuate the viruses through all the migration period [178], other waterbird species (e.g. shorebirds) may carry the viruses back into waterfowl breeding areas [179], or infectious particles may persist through the winter period in the frozen waters and reinfect the birds in the spring [180]. In the southern hemisphere the migration patterns of wild birds appear to be more complex, and therefore the annual cycle of AIVs is less predictable. Domestic waterfowl can also act as reservoirs of AIVs. High prevalences and variety of LPAIV subtypes have been detected in domestic ducks, particularly in breeds raised in free-range husbandry [181–183]. However, since they are raised as poultry, their role in the epidemiology of AIVs is different.

In ducks, LPAIVs replicate predominantly in the epithelial cells of the intestinal tract, despite virus replication in the respiratory tract has also been shown. High quantitities of infectious particles are shed by the cloaca within the faeces (up to 10^8 mean egg infectious doses /gram of feces), and the viruses can be shed up to 30 days after infection [184, 185]. The contamination of the environtment preceeds the fecal-oral route of transmission (ingestion) of the viruses by either dabbling in contaminated water or preening on feathers contaminated with feces [186]. The fecal-cloacal route by the mechanism known as "cloacal-drinking" represents an additional route of infection [186]. In some waterfowl species, viral

shedding via the oropharynx may also ocur during relatively long periods of time [187], and the transmission of LPAIVs via the respiratory route (oral-oral) has been speculated to play a role in waterfowl species that mainly forage in landfills [188].

Apart from those belonging to Gs/GD H5 lineage, HPAIVs have not caused widespread infections in free-living waterbirds populations [189], with the exception of H5N3 detected in terns in South Africa in 1961 [18]. Similary, HPAIVs have been more sporadically detected in domestic waterbirds and, in case of infection, it was usually associated greograhically and chronologically with active outbreaks in land-based poultry [28, 190]. Since HPAIVs appear to be adapted to gallinaceous species and therefore less likely to infect efficienty waterbirds [161], these species have not been historically considered reservoirs of HPAIVs [191]. However, the high detection of HPAIVs in domestic ducks in particular outbreaks [192] and the demonstration that classical lineages of HPAIVs can be transmitted in different ducks species by direct contact [161, 193–195] suggest that the comparatively low isolation of HPAIVs in waterfowl populations in some cases could be due to insufficient exposure rather than to poor biological compatibility.

The exception are the HPAIVs belonging to Gs/GD H5 lineage. Gs/GD H5 HPAIVs have been recurrently isolated in a broad variety of migratory waterbird species and is kwown that they have been pivotal in their dispersal among broad geographic regions, even between continents [196–198]. Despite particular clades and/or genotypes of Gs/GD H5 lineage of HPAIVs seems to be in process of adaptation to wild waterbirds, these viruses have been often isolated from dead or sick individuals [199]. Mallards, Eurasian wigeons and Norhern pintails are three of the few species able to shed high quantities of Gs/GD H5 HPAIVs in relatively long periods of time without exhibiting any evident clinical sign of disease, and even big differences between isolates exists [200–202]. Therefore, if this lineage can perpetuate stably in the wild population or if it mirrors a continuous spill back from infected poultry is still debatable [199]. In contrast, evidences indicate that the Gs/GD H5 lineage of HPAIVs have become established in domestic waterfowl populations. Gs/GD H5 HPAIVs are detected at high rates in domestic waterfowl infected subclinically, especially in domestic ducks [203–206]. After experimental inoculation, domestic ducks can present viral shedding during long periods of time (up to two weeks) in the absence of clinical signs [207–209]. Therefore, domestic waterfowl populations appear to play a main role in the perpetuation of Gs/GD H5 HPAIVs in endemic countries. A special role in the epidemiology of Gs/GD H5 HPAIVs has been attributed to free-grazing ducks. This type of production is based on the movement of ducks among harvested rice paddy fields and waterlands, where ducks make direct contact and share the same environtment with other duck flocks and with

wild birds, facilitating then the maintenance of Gs/GD H5 HPAIVs in the wild-domestic interface [210, 211].

In contraposition with the predominant cloacal shedding of LPAIVs, HPAIVs have acquired the capacity to be predominantly shed by the respiratory route rather than by the cloaca [209], and excretion via the conjunctiva has also been documented [212]. However, the mean virus titer shed is generally lower and the median infectious period shorter for HPAIVs than for LPAIVs in both wild and domestic waterfowl species, which results in lower environmental contamination [213].

The **environment** could act as an abiotic reservoir of AIVs. Several AIVs subtypes that circulate in the wild reservoir have been detected in water, including rivers, lakes and ponds [214]. The maintenance of the virus in the environment is largely influenced by numerous factors, including the relative humidity, temperature, pH, salinity, content of organic material and ammonia, contamination status, UV radiation, freezing/thawing cycles and the viral strain [215–217]. In general, is considered that clean filtered freshwater maintained at a low temperature (below 17ºC), low salinity (<20.000 parts per million), and neutral to slightly basic pH (7.4–8.2) represent the ideal conditions [217]. Under the appropriate environment, the virus may maintain its infectivity for more than a year [215]. However, the molecular basis for environmental stability of AIVs remains unknown. AIVs can also persist long periods of time in infected tissue samples, but the survival largely varies dependent of the tissue type and the temperature [218, 219].

1.3.3. INTRODUCTION AND TRANSMISSION

The **introduction** and **spread** of AIVs in poultry is complex and is influenced by numerous factors, including the strain and virulence of the viral isolate, the ecological characteristics of the area, the concentration of poultry premises and the level of biosecurity. In general, AIVs, either as LPAIVs or HPAIVs, may enter into a poultry flock by the following routes: 1) direct contact with waterbirds, especially waterfowl [220]; 2) use of unpurified, raw surface water sources for drinking or food contaminated with infective droppings [220]; 3) mechanical transmission via movement of people, vehicles, fomites, food or water between farms or flocks [221, 222]; 4) movement and trade (legal or illegal) of live infected poultry, poultry products and captive wild birds [223–225]; 5) contact with peri-domestic avian and mammal species and flying insects that act either as biological vectors or carry the

virus mechanically (e.g. in feet and feathers) [226–228]; 6) introduction via non-avian intermediate hosts (e.g. swine) [229]; and 7) airborne by dust or water droplets in case of proximity to infected flocks or contaminated manure [230, 231].

There are strong evidences indicating that direct or indirect contact with migratory waterbirds represent the main source of **primary introduction** of AIVs to poultry. Generally, the frequency of isolation of LPAIVs in poultry is higher in farms located within wild bird migratory routes and in outdoor production systems [232]. Moreover, AIVs from poultry present high nucleotide identity with LPAIVs previously isolated from wild birds in the same territory [233–236]. However, the direct transmission of a HPAIV from wild to domestic birds was not demonstrated until the emergence of the Gs/GD H5 lineage. The temporal and geographical correlation between poultry outbreaks with annual wild waterbird migratory movements, particularly of waterfowl [237–240], and the genetic relationship between Gs/GD H5 HPAIVs isolated from poultry and wild birds [241] indicate that direct spillover of HPAIVs between wild-domestic populations may also occur in this lineage. Domestic ducks are also considered to play an important role in the introduction of wild bird-origin AIVs to land-based poultry, including the HPAIVs of Gs/GD H5 lineage [242–244]. In addition, international trade of poultry seems to be responsible for the initial introduction of AIVs in different countries, for example during the expansion of the Gs/GD H5 lineage of HPAIVs in South-East Asia in 2004 [245].

The main sources of AIVs **spread** after the index case is reported apear to be related to human activities. Movements of farm personnel and live poultry between holdings represent important sources of virus dispersal, especially in poultry flocks raised under low biosecurity standards and in farms included in large contact networks [246, 247]. In the appropiate conditions (continous contact between infected and susceptible birds), AIVs can be perpetuated in the domestic bird population.

A key source of AIVs infections to poultry has been attributed to live bird markets (LBM). This marketing system allows the contact of numerous species of birds of variable origin, and therefore respresent a hotspot for interspecies transmission, perpetuation and genetic evolution of AIVs [248–250]. In some studies, the prevalence of AIVs in LBMs can reach higher levels than in farms [251], and given the contact network among them, LBMs represent a potential source of infection for poultry flocks. Several studies also indicate that LBMs are playing a pivotal role in the epidemiology of Gs/GD H5 HPAIVs [252–254]

In chickens and other gallinaceous species, AIVs are shed from both the oral and cloacal routes; however, slightly higher quantities are generally detected in the oropharynx. Despite the marked

differences among isolates, LPAIVs and HPAIVs can be detected in respiratory secretions and faeces as early as 1-2 day post-inoculation (dpi) under experimental conditions [255]. In addition to the fecal/oral route, the high shedding of AIVs via the oropharynx in this species suggest the potential role of oral/oral transmission. Experimental studies reveal that chickens are susceptible to aerosolized AIVs [256] and can be transmitted by this route, but usually within short distances [257, 258]. In general terms, the transmission to contact birds appear to be easier for HPAIVs than for LPAIVs, especially driven by the higher viral shedding reported in HPAIV-infected birds [259, 260]. However, the mean infectious period is shorter for HPAIVs as a result of the rapid death in infected individuals, which leads to similar or lower basic reproductive ratios (mean number of secondary cases from an infected individual) than the reported for LPAIVs [261, 262]. Although the experience at the field level demonstrates that HPAIVs can spread rapidly beween farms, some risk assessments also conclude that LPAIVs are also more likely to be introduced into other farms due to the lower probability of detecting the virus in a timely fashion [263].

HPAIVs have also been detected in poultry products such as meat, eggs and feathers of different species, and sometimes in the early stages of infection (1-3 dpi) [264]. Therefore, they suppose additional sources of environmental contamination and viral transmission [265]. Infected carcasses may contribute to the interspecies transmission of HPAIVs by predation, as evidenced by the death of different birds of prey [266] and mammalian carnivores [267–269].

1.4. PATHOBIOLOGY

The outcome after AIV infection in birds is the consequence of the gene constellation of the virus and the susceptibility of the host. It ranges from lack of infection, to asymptomatic infection, to mild disease involving decrease in activity and respiratory and reproductive signs, to severe, systemic disease with mortalities that can reach the 100% [270]. Other variables such as dose [271] and route of infection [272], previous exposure to homologous or heterologous subtypes of AIVs [273] and other viral agents [274], and concomitant infections [275–277], have also been demonstrated to influence largely the infection outcome, demonstrating the complex pathobiology of AIVs.

1.4.1. PATHOGENESIS

In gallinaceous species, the chains of events that occur after AIV infection are well described. **LPAIVs** replicate mainly in the nasal epithelium, with further spread to the epithelial cells in other parts of the respiratory tract and into the intestinal tract [278–280]. Some LPAIVs can spread systemically and infect epithelial cells of kidney and oviduct [280]. Rarely, LPAIVs have been detected in the pancreatic acinar epithelium, hepatocytes, lymphoid organs, skeletal muscles and bone marrow [281, 282]. In Anseriformes species, LPAIVs replicates mainly in the epithelial cells of the intestinal tract and in Bursa of Fabricius [279], but replication in respiratory organs, including lung and air sacs, has also been detected [283, 284]. Less information is recorded in other taxonomic groups of birds, but in general terms the replication of LPAIVs seems to be mostly restricted to the respiratory and intestinal tracts [270].

Similar to LPAIVs, **HPAIVs** in gallinaceous species replicate first in the epithelial cells of the nasal cavity but within 24 hours, the virus can be already detected in capillary endothelium and inflammatory cells, including heterophils and macrophages. The replication of HPAIVs in these cells precedes the virus spread through the vascular (viremia) and lymphatic systems, which allows their dissemination into numerous organs. HPAIVs can be detected at 24 hours post-infection (hpi) in a large variety of cells in different organs, including parenchymal, endothelial and inflammatory cells, and the peak of viral titers can be reached as soon as 48 hpi. The organs most commonly affected are brain, pancreas, heart, adrenal gland, kidney and lung, but large differences are present among strains and species [270]. In non-gallinaceous species, the different stages of HPAIV infection other than those of Gs/GD H5 lineage have been comparatively less studied. In some studies, HPAIVs were not reisolated from internal organs in the inoculated birds [285]. However, other HPAIVs have been recovered from several organs in different species, including ducks, demonstrating the potential capacity of classical lineages of HPAIVs to disseminate systemically in other taxonomic groups of birds [193, 279, 286].

The HPAIVs belonging to Gs/GD H5 lineage have acquired unusual pathological characteristics, including increased infectivity and tissue tropism in a huge range of domestic, captive and wild avian species. In gallinaceous species, the pathogenesis is similar to that reported for other lineages of HPAIVs, with high virus replication detected in vascular endothelial cells and inflammatory cells early after infection and later on in parenchymal cells of visceral organs, brain and skin [270]. In non-galliformes species, the infection process varies largely depending on the species and the viral strain, but in general terms four situations may occur: 1) lack of evident virus replication; 2) virus replicates but at low titers and is locally restricted; 3) high viral replication is detected in two to three vital organs (usually

involving brain); and 4) systemic infection with high viral titers in numerous organs [270, 287]. Since Gs/GD H5 HPAIVs have continually evolved into different clades and genetic groups, the pathogenesis can diverge greatly within the same species. For example, early Gs/GD H5N1 HPAIVs isolates replicated to a very limited extent in ducks [288], whereas strains isolated from 2002 onwards acquired the capacity to produce a systemic infection in this species [289].

Despite the wide variation in the pathogenesis of AIVs depending on the viral pathotype and the species and current gaps in knowledge, the damage caused by AIVs has been associated with three **pathophysiological mechanisms**: direct replication of the virus in cells, indirect effects from production of innate immune response mediators, and endothelial cell dysfunction [270].

Necrosis and apoptosis play a main pathogenic role in AIV infections. The importance of necrosis and apoptosis is demonstrated by the correlation of high levels of AIV antigen/RNA in tissues and compatible cellular, histological and biochemical alterations of necrosis and apoptosis in affected organs [290, 291]. High levels of cell death in critical organs can disturb the maintenance of organ function and lead to single or multi-organ failure. However, the molecular bases of cell death during AIV infection in birds remain poorly understood. For apoptosis, activation of Fas-/FasL-mediated apoptosis pathway [292] and acceleration of extracellular Ca2+ influx that leads to mitochondrial dysfunction [293] have been proposed. Alternatively, some data suggests that apoptosis could play a protective role during AIV infections. Ducks infected with Gs/GD H5N1 HPAIV induced a rapid apoptosis of infected cells in comparison with chickens, with associated lower viral titers in tissues [294]. This limited early replication may have an important effect in the their reduced susceptibility to HPAIVs.

The role of **vascular dysfunction** as a pathogenic factor of HPAIVs infections is known so far, but is species-specific [295]. Chicken endothelial cells are particularly susceptible to HPAIVs. In this species, the high endothelial cell tropism of HPAIVs produces an increase in vascular permeability, thermoregulation impariment, profuse recruitment of inflammatory cells and coagulopathies. These is associated to edema, hemorrhages and micro-thrombosis that ultimately leads to ischemia in tissues and multiple organ failure [296–299]. In other gallinaceous species, the endothelial cell tropism of HPAIVs appears to be less intense [295]. AIV antigen is not usually detected in the endothelial cells of waterbirds infected with HPAIVs. Sporadic infection of endothelial cells has been recorded in mute swans, whopper swans, tufted ducks, wood ducks, call ducks, commercial ducks, Canada geese and laughing gulls, but the detection is restricted to few birds and/or low proportion of endothelial cells [295, 300], indicating that HPAIVs also present a limited tropism for the endothelial cells in waterbirds species. It

has also been demonstrated *in vitro* that duck endothelial cells are much less susceptible to HPAIVs than chicken endothelial cells [301]. The exception are black swans, where infection with Gs/GD H5N1 HPAIV has been associated with widespread detection in endothelial cells [302].

Another mechanism of pathogenicity in AIV infections is the **immune-mediated damage**. AIVs infection triggers the production of antiviral proteins, chemokines and pro-inflammatory cytokines in an attempt of the host to limit the viral replication. Whereas a proper innate immune response is key to limit viral spread, an aberrant immune response can result in immunopathologies [303]. The replication of HPAIVs in chickens is usually associated with the dysregulation of inflammatory responses and exacerbated production of cellular mediators such as pro-inflammatory cytokines (hypercytokinemia) [304–307], which can lead to capillary leakage and tissue damage [308]. In ducks, the tolerance to most HPAIVs have been associated with either a mild inflamatory response [309] or an early inflammatory response and robust production of type I interferon and other antiviral genes [310], but this species appear to limit the duration of the pro-inflammatory cytokine expression [311]. Therefore, early innate immune responses appear to play a major role in the differences in susceptibility between chickens and waterfowl species. Interestingly, some Gs/GD H5 HPAIVs acquired the capacity to overcome and destroy early host immune responses or induce a sustained and/or excessive expression of pro-inflammatory cytokines in ducks, which lead to increased disease severity [309, 312, 313]. In pigeons, the reduced pathogenicity caused by HPAIVs is also accompanied by a moderate host cytokine expression of the host [314].

1.4.2. CLINICAL PRESENTATION

The limited tropism of **LPAIVs** in gallinaceous species generally results in the absence of clinical signs in infected birds [315, 316]. If present, the signs are mild and non-specific, including ruffled feathers, decreased activity, decreased feed and water consumption that lead to mild weight loss, upper respiratory signs, including coughing, sneezing, rales and excessive ocular discharge, and diarrhea [317, 318]. In hens, drop in both production and quality of eggs (misshapen, fragile and lack of pigment) have been also documented [319]. The morbidity caused by LPAIVs can be high (>50%). However, mortality rates are generally low (<5%). Mortalities can be considerably higher in juvenile birds, in case of concomitant infections, or in unfavorable environmental conditions (e.g. excess ammonia, improper

room temperature) [320]. The susceptibility varies between species; turkeys generally present higher mortalities than chickens [193, 315].

In waterbirds, LPAIVs infections are asymptomatic [283, 284, 315]. However, some studies indicate that LPAIVs infection may have detrimental effects on these species. Mallards may exhibit a transient decrease in body weight and egg production [284, 321], whereas swans can present a delayed migratory timing as a consequence of an impaired digestive system that increment the rate of stop-overs [322]. Pekin ducks inoculated with particular LPAIVs presented mild conjunctivits and nasal discharge [315], and slight weakness and loss of appetite have been reported in goslings [323]. In ratites, LPAIVs causes respiratory signs such as ocular and/or nasal discharge, and in particular cases, diarrhoea may be observed [270]. In Columbiformes, LPAIVs cause subclinical infections [316, 324, 325], whereas clinical signs in passeriformes [326] and psittaciformes have been sporadically reported [327].

HPAIVs generally cause high morbidities and mortalities (up to 100%) in gallinaceous birds, but the MDT varies depending on the viral strain and the species. In HPAIVs that present a strong replication in endothelial cells, the presentation can be peracute (1-2 dpi) and the birds are found dead without prior clinical signs or only few birds exhibit apathy or prostration [193, 317, 328, 329]. HPAIVs can also cause an acute or subacute presentation (3 to 10 days) associated with the replication of the viruses in parenchymal cells in different organs. In this stage, the birds can present a broad variety of clinical signs; however, none of them is pathognomonic. Infected birds ususally exhibit lower activity that progresses to severe apathy and prostration, and decrease in feed and water consumption leading to weight loss and dehydration. Several birds may also present evident nervous signs, such as tremors of head and neck, torticollis, opisthotonus, nystagmus, repetitive movements (e.g. rolling, circling, pedaling), ataxia, paresis and/or paralysis. Diarrhoea may also be present, but respiratory signs are generally less frequent than in LPAIV infections. In hens, the birds stop the production of eggs or they present severe deformations [317, 329–333]. Similar as in other lineages of HPAIVs, those belonging to Gs/GD H5 lineage cause high morbidities and mortalities in chickens and other gallinaceous species. However, marked differences are found among clades and subtypes: early Gs/GD H5N1 HPAIVs generally caused death in chickens in a short period of time and in some cases without previous evident signs of disease [297], whereas several Gs/GD clade 2.3.4.4 H5Nx HPAIVs reassortants have caused comparatively lower mortalities and/or extended MDTs [334–338].

HPAIVs produce no or mild clinical signs in ducks and geese, such as transient decrease in activity and body weight [193, 279, 285, 286, 330, 331, 339–343]. Neither morbiditity nor motablity have been recorded in gulls [344]. However, particular H7 HPAIVs subtypes have caused severe clinical signs, including apathy,

diarrhea and nervous signs (circling, rolling, incoordination, steady gait and/or opisthotonus), in domestic ducks. The mortalities range from 20 to 80 % depending on the virus and route of inoculation [329, 345, 346], demonstrating the potential virulence of classical lineages of HPAIVs for other taxonomics groups of birds. HPAIVs can also cause severe disease and mortality in ostriches, being severe apathy and nervous signs the most common signs [270]. Pigeons rarely show clinical signs after HPAIV experimental infection, and in case of mortalities it is limited to a small percentage out of the total inoculated [333, 341, 347–351]. HPAIVs of Gs/GD lineage have generally acquired a higher virulence for non-galliformes species in comparison with classical strains. After experimental inoculation, the infection ranges from assymptomatic to severe associated to high mortalities, largely varying on the species and the isolate. Moreover, there are large differences in the severity of infection even within the same species. In susceptible birds, severe clinical signs, generally involving severe apathy and progressive neurologic disorders, may be observed prior to death. This group includes domestic and wild waterbirds such as geese [288, 302, 352–361], ducks [289, 336, 357, 362–369] and storks [370], emus and ostriches [355, 371], birds of prey [363, 372], songbirds [373–375] and columbiformes [375–379], among others. The MDT in these species is generally longer than in galliforms, but it can be similar (3 to 7 dpi). In other cases, infection may also result in mild or lack of evident signs of disease. The most evident examples of this group include domestic and wild ducks [200, 201, 288, 334–336, 355, 362, 366, 368, 380–387], pigeons [354, 355, 388–392] and to a lesser extent, geese [367, 393].

Alltogether, existing literature evidences the complex pathobiology of HPAIVs and the variety in clinical presentation among avian hosts, even between closely related isolates and species. In **Table 3 to 5** are summarized the clinico-pathological outcomes after intranasal inoculation with classical lineages of HPAIVs and Gs/GD 2.3.4.4 H5 HPAIVs in chickens (Table 2), domestic geese (Table 3), and pigeons (Table 4).

A. Virus	Breed (age in weeks)	Mortality in % (dose)	MDT/range/last death in days	AIV antigen	Transmission (Y/N)	Ref
Classical HPAIVs						
A/turkey/England/50-92/91 (H5N1)	SPF WL (6)	80 ($10^{4.2}$), 100 ($10^{5.2}$), 80 ($10^{6.2}$)	3.5 ($10^{4.2}$), 2.6 ($10^{5.2}$), 3.5 ($10^{6.2}$)	CNS[1]	nd	[394]
	SPF light breed (3)	100 ($10^{7.3}$)	nd	Systemic	nd	[279]
	SPF WL (6)	100 ($10^{7.2}$)	1-5	Systemic	nd	[395]
A/chicken/Scotland/59 (H5N1)	SPF WL (2)	50 ($10^{6.2}$)	4.6	nd	nd	[331]
A/chicken/Pennsylvania/1370/83 (H5N2)	SPF WL (2)	90 ($10^{6.2}$)	5.2	nd	nd	[331]
	WL (5 to 6)	100 (10^{4}), 100 (10^{5})	5-9 (10^{4}), 4-7 (10^{5})	nd	Y	[396]
	Laying hens (>6 months)	100 (10^{4}), 100 (10^{5})	4-7 (10^{4}), by 4 (10^{5})	Systemic	Y	[396]
	SPF WL (6)	100 (10^{6})	6.2/7.4 (replicates)	nd	Y	[259]
A/chicken/Pennsylvania/SERPL-PA/83 (H5N2)	na (1-3 d)	100 (na)	2.2 to 2.8	nd	nd	[332]
	na (1 to 2)	100 (na)	4.6 to 5.9	nd	nd	[332]
	na (3 to 4)	83 to 100 (na)	7.1 to 8.6	nd	nd	[332]
	na (5)	50 to 67 (na)	8 to 8.5	nd	nd	[332]
	na (Adult)	92 (na)	6.5	nd	nd	[332]
A/chicken/Taiwan/0502/2012 (H5N2)	na (4)	100 (10^{6})	By 4	Systemic	nd	[285]
A/chicken/Queretaro/114588-19/95 (H5N2)	na (4)	100 (na)	2.8 to 4.9	nd	nd	[397]
	SPF WL (3 to 4)	100 (10^{7})	nd	Systemic	nd	[398]
A/tern /South Africa/61 (H5N3)	SPF WL (2)	100 ($10^{6.2}$)	5.3	nd	nd	[331]
	SPF WL (6)	80 ($10^{4.2}$), 80 ($10^{5.2}$), 80 (10^{6})	4 ($10^{4.2}$), 3.5 ($10^{5.2}$), 3.25 (10^{6})	CNS[1]	nd	[394]
A/chicken/Chile/184240-1/02 (H5N3)	WL (4)	100 (10^{6})	2.3	Systemic	nd	[328]
A/turkey/Ireland/1378/83 (H5N8)	SPF WL (2)	90 ($10^{5.8}$)	4.3	nd	nd	[331]
A/duck/Ireland/113/84 (H5N8)	SPF WL (2)	100 ($10^{5.2}$)	2.7	nd	nd	[331]
	SPF light breed (3)	100 ($10^{7.5}$)	nd	Systemic	nd	[279]
A/turkey/Ontario/7732/66 (H5N9)	SPF WL (2)	20 ($10^{5.6}$)	5	nd	nd	[331]
	na (6)	50 (10^{2}), 50 (10^{3}), 100 (10^{5}), 100 (10^{8})	5.5 (10^{2}), 6 (10^{3}), 5.8 (10^{5}), 5.5 (10^{8})	nd	nd	[333]
A/chicken/Italy/13474/99 (H7N1)	Broiler A (7-10)	100 ($10^{3\pm0.25}$)	2-7	nd	nd	[399]
	Broiler B (7-10)	27 ($10^{3\pm0.25}$)	4-8	nd	nd	[399]
	Broiler C (7-10)	90 ($10^{3\pm0.25}$)	2-6	nd	nd	[399]
	WL D (7-10)	100 ($10^{3\pm0.25}$)	3-6	nd	nd	[399]
	NH E (7-10)	65 ($10^{3\pm0.25}$)	2-7	nd	nd	[399]

A/chicken/Italy/5093/99 (H7N1)	SPF (15d)	0 ($10^{1.5}$), 0 ($10^{3.5}$), 75 ($10^{5.5}$)	4.8 ($10^{5.5}$)	Systemic	nd	[400]
A/fowl/Germany/34 (H7N1)	na (2)	100 (na)	1.9	nd	Y	[329]
A/chicken/Jalisco/CPA-37905/2015 (H7N3)	SPF WL (3)	100 (10^6)	3.2	nd	nd	[401]
A/chicken/Jalisco/CPA1/2012 (H7N3)	SPF White Rock (10)	100 (10^6)	nd	Systemic	nd	[41]
	SPF WL (5)	100 (10^6)	nd	Systemic	nd	[41]
A/fowl/Victoria/75 (H7N7)	na (2)	100 (na)	7.2	nd	Y	[329]
A/chicken/Victoria/76 (H7N7)	Broilers (6)	100 (na)	By 7	nd	nd	[330]
A/chicken/Victoria/1/85 (H7N7)	SPF WL (6)	60 (10^3), 80 (10^4), 100 (10^5)	5 (10^3), 3.75 (10^4), 3.4 (10^5)	CNS[1]	nd	[394]
	SPF WL (6)	100 (10^6)	2-5	Systemic	nd	[395]
	Broilers (6)	100 (na)	By 6	nd	nd	[330]
	Broilers (14)	100 (na)	6.9	nd	Y	[330]
	SPF (6 to 8)	100 (na)	By 3	Systemic	nd	[402]
	SPF light breed (3)	100 ($10^{6.7}$)	nd	Systemic	nd	[279]
A/chicken/Netherlands/621557/03 (H7N7)	SPF WL (6)	100 (10^6)	4	nd	Y	[403]
A/turkey/Indiana/16-001403-1/2016 (H7N8)	SPF WL (3)	0 (10^2), 80 (10^4), 94 (10^6)	2.5 (10^4), 2.75 (10^6)	Systemic	N	[193]
A/chicken/Tennessee/17-007147-2/2017 (H7N9)	SPF WL (4)	60 (10^2), 100 (10^4), 100 (10^6),	2.3 (10^2), 2.4 (10^4), 2.2 (10^6),	Systemic	Y	[260]
A/Chicken/Heyuan/16876/2016 (H7N9)	SPF (6)	100 (10^5)	By 3	Systemic	Y	[404]
A/Chicken/Huizhou/HZ-3/2016 (H7N9)	SPF (6)	100 (10^5)	By 3	Systemic	Y	[404]
A/Chicken/Guangdong/SD008/2017 (H7N9)	SPF (6)	100 (10^6)	By 4	Systemic	Y	[405]
Gs/GD clade 2.3.4.4 H5Nx HPAIVs						
A/chicken/China/0603/2008 (H5N1)	SPF (6)	100 (10^6)	By 2	Systemic	nd	[334]
A/peregrine falcon/Hong Kong/810/2009 (H5N1)	na (4)	100 (10^6)	By 4	Systemic	nd	[285]
DK/ECL0230/10 (H5N2)	SPF (6)	100 (10^6)	4-5	Systemic	nd	[334]
DK/EC/L0131/14 (H5N2)	SPF (6)	100 (10^6)	4-5	Systemic	nd	[334]
A/northern pintail/Washington/40964/2014 (H5N2)	SPF WL (4)	0 (10^2), 0 (10^4), 60 (10^6)	3	Systemic	N	[337]
A/turkey/Minnesota/12582/2015 (H5N2)	SPF WL (4)	0 (10^2), 60 (10^4), 100 (10^6)	2.3 (10^4), 2 (10^6)	Systemic	Y	[336]
	Broiler (5)	0 (10^2), 0 (10^4), 100 (10^6)	4.8	Systemic	Y	[406]
	Broiler (8)	0 (10^2), 0 (10^4), 100 (10^6)	3.2	Systemic	Y	[406]
	B.Breeder (>30)	0 (10^2), 0 (10^4), 100 (10^6)	3.2	Systemic	Y	[406]
	SPF WL (4)	0 (10^2), 60 (10^4), 100 (10^6)	2.3 (10^4), 2 (10^6)	Systemic	Y	[406]
A/turkey/South Dakota/12511/2015 (H5N2)	SPF WL (4)	0 (10^2), 80 (10^4), 100 (10^6)	4 (10^4), 2.2 (10^6)	Systemic	N	[336]

A/chicken/Iowa/13388/2015 (H5N2)	SPF WL (4)	20 (10^2), 60 (10^4), 100 (10^6)	2 (10^2), 2.3 (10^4), 2.4 (10^6)	Systemic	N	[336]
A/turkey/Arkansas/7791/2015 (H5N2)	SPF WL (4)	0 (10^2), 0 (10^4), 89 (10^6)	2.1	Systemic	N	[336]
A/goose/Eastern China/ 1106/2016 (H5N2)	SPF (5)	10 (10^3), 100 (10^6)	3 (10^3), 3-5 (10^6)	Systemic	Y	[381]
A/duck/Eastern China/YD1516/2016 (H5N2)	SPF (5)	10 (10^3), 100 (10^6)	6 (10^3), 4-7 (10^6)	Systemic	Y	[381]
GS/EC/S0513/13 (H5N6)	SPF (6)	100 (10^6)	4-5	Systemic	nd	[334]
DK/EC/S0711/14 (H5N6)	SPF (6)	100 (10^6)	4-5	Systemic	nd	[334]
A/duck/Laos/XBY004/2014 (H5N6)	SPF (5)	100 (10^6)	1.75	Systemic	nd	[407]
A/duck/Guangzhou/018/2014 (H5N6)	SPF WL (6)	100	By 6	Systemic	Y	[408]
A/duck/Guangzhou/021/2014 (H5N6)	SPF WL (6)	100	By 7	Systemic	Y	[408]
A/duck/Quang Ngai/AI334/2014 (H5N6)	SPF WL (4)	0 (10^2), 40 (10^4), 100 (10^6)	3 (10^4), 3.2 (10^6)	nd	nd	[366]
A/oriental magpie-robin/Guangdong/SW8/2014 (H5N6)	SPF WL (6)	100 (10^6)	2.5	Systemic	Y	[383]
	SPF (6)	100 (10^5)	4	Systemic	Y	[409]
A/common moorhen/Guangdong/GZ174/2014 (H5N6)	SPF WL (6)	100 (10^6)	2.2	Systemic	Y	[383]
A/Pallas's sandgrouse/Guangdong/ZH283/2015 (H5N6)	SPF WL (6)	100 (10^6)	2.2	Systemic	Y	[383]
	SPF (6)	100 (10^5)	3.3	Systemic	Y	[409]
A/chicken/Japan/AQ-HE144/2015 (H5N6)	SPF WL (4)	0 (10^2), 0 (10^4), 100 (10^6)	2 (10^6)	nd	nd	[366]
A/Goose/Guangdong/GS144/2015 (H5N6)	SPF WL (5)	100 (10^4)	3.63	Systemic	Y	[410]
A/Goose/Guangdong/GS148/2016 (H5N6)	SPF WL (5)	0 (10^4)	-	Systemic	Y	[410]
A/Goose/Guangdong/QY01/2016(H5N6)	SPF (6)	100 (10^6)	By 4	Systemic	Y	[354]
A/Environment/Korea/W541/2016 (H5N6)	SPF WL (5)	100 (10^6)	By 2	Systemic	Y	[368]
A/Waterfowl/Korea/S57/2016 (H5N6)	WL (3)	100 (10^5)	By 5	Lung[1]	N	[411]
A/duck/Korea/ES2/2016 (H5N6)	SPF WL (na)	0 (10^3), 75 (10^4), 100 (10^5), 100 (10^6)	7.9 (10^4), 6.3 (10^5), 2.6 (10^6)	Systemic	Y	[412]
	Broiler (na)	0 (10^3), 60 (10^4), 100 (10^5), 100 (10^6)	4.9 (10^4), 2.8 (10^5), 2.1 (10^6)	Systemic	Y	[412]
	KNC (na)	40 (10^4), 80 (10^5), 100 (10^6), 100 (10^7)	6 (10^4), 6.3 (10^5), 3.1 (10^6), 3.1 (10^7)	Systemic	Y	[412]
A/Chicken/Guangdong/CK46/2016 (H5N6)	SPF WL (4)	100 (10^5)	3	Systemic	Y	[413]
A/Goose/Guangdong/GS74/2016 (H5N6)	SPF WL (4)	100 (10^5),	3.38	Systemic	Y	[413]
A/chicken/Anhui/MZ33/2016 (H5N6)	SPF (4)	100 (10^3)	3	Systemic	Y	[384]
A/chicken/Anhui/MZ34/2016 (H5N6)	SPF (4)	100 (10^3)	2.6	Systemic	Y	[384]
A/chicken/Henan/YB0597/2016 (H5N6)	SPF (4)	100 (10^3)	4	Systemic	Y	[384]
A/chicken/Niigata/1-1T/2016 (H5N6)	SPF WL (4)	0 (10^2), 80 (10^4), 100 (10^6)	2.75 (10^4), 2.4 (10^6)	nd	nd	[366]
A/muscovy duck/Aomori/1-3T/2016 (H5N6)	SPF WL (4)	0 (10^2), 0 (10^4), 80 (10^6)	2.75 (10^6)	nd	nd	[366]
A/duck/Hyogo/1/2016 (H5N6)	SPF WL (4)	0 (10^2), 0 (10^4), 100 (10^6)	2.2 (10^6)	nd	nd	[366]

A/chicken/Gifu/1-1T/2017 (H5N6)	SPF WL (4)	0 (10^2), 0 (10^4), 100 (10^6)	2 (10^6)	nd	nd	[366]
A/chicken/Kumamoto/1-2C/2016 (H5N6)	SPF WL (4)	0 (10^2), 0 (10^4), 100 (10^6)	2 (10^6)	nd	nd	[366]
A/mute swan/Kyoto/1T/2016 (H5N6)	SPF WL (4)	0 (10^2), 100 (10^4), 100 (10^6)	3.4 (10^4), 2.2 (10^6)	nd	nd	[366]
A/chicken/Miyazaki/2-2C/2017 (H5N6)	SPF WL (4)	0 (10^2), 20 (10^4), 100 (10^6)	2 (10^4), 2.2 (10^6)	nd	nd	[366]
A/black swan/Akita/1/2016 (H5N6)	na (7)	100 (10^6)	3	Systemic	nd	[382]
A/AP/Korea/W612/2017 (H5N6)	SPF (na)	100 (10^6)	By 4	Systemic	Y	[385]
A/chicken/Kagawa/1T-1/2018 (H5N6)	SPF WL L-M-6 strain (4)	0 (10^2), 0 (10^4), 80 (10^5), 100 (10^6)	By 4 (10^5), by 3 (10^6)	Systemic	Y	[414]
A/Northern Goshawk/Tokyo/1301B003T/2018 (H5N6)	SPF WL L-M-6 strain (4)	0 (10^2), 20 (10^4), 100 (10^5), 100 (10^6)	3 (10^4), by 5 (10^5), by 3 (10^6)	Systemic	nd	[414]
A/Jungle crow/Hyogo/2803E023C/2018 (H5N6)	SPF WL L-M-6 strain (4)	0 (10^2), 20 (10^4), 40 (10^5), 100 (10^6)	5 (10^4), 4.5 (10^5), by 4 (10^6)	Systemic	nd	[414]
A/Waterfowl/S005/Korea/2014 (H5N8)	WL (3)	100 (10^5)	By 4	Lung[1]	N	[411]
A/breeder duck/ korea/Gochang1/2014 (H5N8)	SPF (5)	100 ($10^{6.5}$)	2.5	Systemic	Y	[415]
A/broiler duck/ korea/Buan2/2014 (H5N8)	SPF (5)	100 ($10^{6.5}$)	4.5	Systemic	Y	[415]
	SPF WL	0 ($10^{3.5}$), 0 ($10^{4.5}$), 60 ($10^{5.5}$), 100 ($10^{6.5}$)	4.6 ($10^{5.5}$), 3.2 ($10^{6.5}$)	Systemic	nd	[416]
	KNC	0 ($10^{5.5}$), 40 ($10^{6.5}$), 100 ($10^{7.5}$), 100 ($10^{8.5}$)	8 ($10^{6.5}$), 5 ($10^{7.5}$), 4.4 ($10^{8.5}$)	Systemic	nd	[416]
GS/EC/L1204 (H5N8)	SPF (6)	100 (10^6)	4-5	Systemic	nd	[334]
DK/EC/S1109/14 (H5N8)	SPF (6)	100 (10^6)	4-5	Systemic	nd	[334]
A/gyrfalcon/Washington/40188-6/2014 (H5N8)	SPF WL (4)	0 (10^2), 40 (10^4), 100 (10^6)	4 (10^4), 4.1 (10^6)	Systemic	N	[337]
A/Baikal teal/Korea/K14-E016/2014 (H5N8)	Layers (3)	100 (10^6)	4.8	nd	Y	[338]
	KNC (10)	60 (10^6)	nd	nd	Y	[338]
A/chicken/Kumamoto/1-7/2014 (H5N8)	WL (5)	0 (10^2), 0 (10^4), 100 (10^6)	4.7	Systemic	nd	[335]
	WL (10)	0 (10^2), 0 (10^4), 100 (10^6)	7.3	Systemic	nd	[335]
	na (4)	75 (10^6)	By 4 dpi	Systemic	nd	[285]
A/chicken/Miyazaki/7/2014 (H5N8)	SPF WL (4)	0 (10^3), 25 (10^4), 75 (10^5), 100 (10^6), 100 (10^7)	8 (10^4), 8.3 (10^5), 5 (10^6), 3.8 (10^7)	nd	nd	[417]
A/duck/Chiba/26-372-48/2014 (H5N8)	SPF WL (4)	0 (10^3), 100 (10^4), 100 (10^5), 100 (10^6), 100 (10^7)	7 (10^4), 8 (10^5), 4 (10^6), 4.5(10^7)	nd	nd	[417]
A/Common Teal/Korea/W555/2017 (H5N8)	SPF WL (5)	100 (10^6)	By 5	Systemic	Y	[368]

Table 3. Clinico-pathological outcome after intranasal inoculation of selected HPAIVs belonging to classical lineages and Gs/GD clade 2.3.4.4 H5 lineage in chickens. Y: yes; N: no; Na: not available; nd: not determined. [1]Other organs not evaluated.

Virus	Breed , age in weeks	Mortality in % (dose)	MDT/range/last death in days	AIV antigen	Transmission (Y/N)	Ref
Classical HPAIVs						
A/turkey/Ontario/7732/66 (H5N9)	na (6-month, 2 years-old)	0 (10^8)	-	nd	N	[333]
A/chicken/Leipzig/79 (H7N7)	White Chinese geese (5)	0 ($10^{7.5}$)	-	nd	nd	[339]
A/Goose/Leipzig/137-8/1979 (H7N7)	White Chinese geese (5)	0 ($10^{7.5}$)	-	nd	nd	[339]
A/Goose/Leipzig/187-7/1979 (H7N7)	White Chinese geese (5)	0 ($10^{7.5}$)	-	nd	nd	[339]
A/Goose/Leipzig/192-7/1979 (H7N7)	White Chinese geese (5)	0 ($10^{7.5}$)	-	nd	nd	[339]
Gs/GD clade 2.3.4.4 H5Nx HPAIVs						
A/chicken/BC/FAV-002/2015 (H5N1)	Chinese geese (Adults)	22	5	Systemic	Y	[358]
A/Northern Pintail/Washington/40964/2014 (H5N2)	White Chinese geese (2)	0 (10^2), 0 (10^4), 25 (10^6)	8	Systemic	Y	[359]
A/Goose/Guangdong/QY01/2016 (H5N6)	na (3)	33 (10^6)	5	Systemic	Y	[354]
A/gyrfalcon/Washington/40188-6/2014 (H5N8)	White Chinese geese (2)	0 (10^2), 0 (10^4), 0 (10^6)	-	Systemic	Y	[359]
A/turkey/Germany-MV/R2472/2014 (H5N8)	Pommeranian land (Adults)	0 (10^6 $TCID_{50}$)	-	Systemic	Y	[367]

Table 4. Clinico-pathological outcome after intranasal inoculation of selected HPAIVs belonging to classical lineages and Gs/GD clade 2.3.4.4 H5 lineage in domestic geese. Y: yes; N: no; na: not available; nd: not determined.

Virus	Breed , age in weeks	Mortality in % (dose)	MDT/range/last death in days	AIV antigen	Transmission (Y/N)	Ref
Classical HPAIVs						
A/chicken/Penn/1370/83(H5N2)	na (8-32 months)	0 (10^5)	-	nd	nd	[348]
A/turkey/Ontario/7732/66 (H5N9)	na (6 months, 2 years)	0 (10^8)	-	nd	N	[333]
	na (2-3 years)	5 (na)	nd	nd	nd	[341]
A/FPV/Rostock/34 (H7N1)	na (Adults)	0 (5% suspension of EMT[1])	-	nd	nd	[349]
A/Carduelis/Germany/72 (H7N1)	na(Adults)	0 ($10^{8.1}$)	-	nd	N	[350]
	Racing ,fancy and show (adults)	9 ($10^{2.1}$), 0 ($10^{8.1}$)	9	nd	nd	[347]
A/Chicken/Australia/32972/85 (H7N7)	na (8-32 months)	0 (10^5)	-	nd	nd	[348]
2003 outbreak strain, the Netherlands (H7N7)	na (na)	0 (10^7)	-	-	N	[351]
Gs/GD clade 2.3.4.4 H5Nx HPAIVs						
A/Northern pintail/Washington/40964/2014 (H5N2)	Rock pigeons (na)	0 (10^5 PFU)	-	nd	nd	[391]
A/turkey/Minnesota/9845-4/2015 (H5N2)	Rock pigeons (na)	13 (10^5 PFU)[2]	7	nd	nd	[391]
A/Goose/Guangdong/QY01/2016 (H5N6)	na (6)	0 (10^6)	-	Systemic	N	[354]
A/gyrfalcon/Washington/41088-6/2014 (H5N8)	Rock pigeons (ns)	0 (10^5 PFU)	-	nd	nd	[391]
A/baikal teal/Korea/2406/2014 (H5N8)	Domestic (ns)	0 (10^7/ml 0.2 ml)	-	Systemic (weak)	N	[386]
A/Mallard/Korea/KU3-2/2015 (H5N8)	Domestic (ns)	0 (10^7/ml 0.2 ml)	-	Systemic (weak)	N	[386]
A/Speckled pigeon/South Africa/08-004B/2017 (H5N8)	Racing (11)	0 (10^3), 0 ($10^{4.5}$), 0 (10^6)	-	nd	N	[392]

Table 5. Clinico-pathological outcome after intranasal inoculation of selected HPAIVs belonging to classical lineages and Gs/GD clade 2.3.4.4 H5 lineage in pigeons. Y: yes; N: no; na: not available; nd: not determined. [1]Embryo membrane tissue; [2]Cause of death not established.

1.4.3. GROSS AND MICROSCOPIC LESIONS

The most common gross lesions reported in **LPAIV**-infected gallinaceous species include congestion, edema and occasionally hemorrhages in organs of the upper respiratory tract, including nasal cavity, sinuses, conjunctiva and trachea. In some cases, inflammation of intestine and hemorrhages in cecal tonsils are also observed. Egg yolk peritonitis and swollen oviducts have been reported in hens, and rarely the kidneys may be enlarged. In case of secondary infection by bacteria, more severe lesions such as fibrino-purulent bronchopneumonia, air sacculitis and coelomitis are described [317]. Microscopic findings consist in inflammatory lesions in respiratory tissues, including rhinitis, sinusitis and tracheitis [418]. Salpingitis, oophoritis and interstitial nephritis can also be observed in some cases [319]. Other microscopic lesions that may be present include lymphoid depletion (without evident signs of necrosis) in bursa of Fabricius, thymus and spleen [317]. Infected domestic ducks and geese generally lack gross and microscopic lesions, but conjunctivitis, sinusitis, tracheitis and pneumonia have been reported in some cases [283, 284, 418]. Inflammatory lesions in respiratory and digestive organs can also be observed in other taxonomic groups of birds, such as in ratites [270].

The gross lesions reported in **HPAIV** infections in gallinaceous species vary largely dependent of the clinical presentation. In peracute deaths gross lesions are not generally observed, whereas if the birds succumbed in the acute or subacute stage they are more frequent [317]. The most common lesions include edema of the face (predominantly in periorbital area, including conjunctiva and eyelid), comb, wattles, snood, upper neck, leg shanks and feet, which may be accompanied by subcutaneous haemorrhages (petechias and/or ecchymosis), and cyanosis of the wattles, combs and snoods, and hyperaemia of the conjunctiva and eyelids [317, 328]. Areas compatible with necrosis and haemorrhages of variable intensity may be observed in multiple internal organs, usually in pancreas, heart, mucosa of proventriculus and gizzard, brain, lung, liver, kidney, pectoral muscles, cecal tonsil, Peyer's patches and spleen. Several birds can also present haemorrages in fat tissues such as coronary and pad fat. Other lesions that may appear include edema, congestion and enlargement in several organs, including primary lymphoid organs [317, 328, 402, 419]. Affected hens may also present free yolks in the coelomic cavity [317]. Similar as in the macroscopic examination, birds dying during the peracute stage generally lack microscopic lesions. In the acute and peracute stage, the microscopic lesions are usually more evident. Areas of necrosis and inflammation of variable intensity frequently associated to haemorrhages and oedema can be observed in multiple organs. Usually, the more severe microscopic lesions are detected in the brain, heart, pancreas, skin, lungs, adrenal glands, and primary and secondary lymphoid organs, but other organs may also be affected dependent of the tropism of each virus [395, 419, 420]. In general,

the histological finding of inflammation tend to predominate in comparison with necrosis in those birds that survive a longer period of time [317].

Few reports have described the gross and microscopic lesions caused by classical lineages of HPAIVs in non-gallinaceos species. Infected birds generally lack gross lesions, but mild interstitial pneumonia and pancreatic mottling have been observed in rare ocasions [344]. In contrast, there are numerous studies reporting the gross and microscopic lesions caused by Gs/GD H5 HPAIVs in a broad range of avian species belonging to different taxonomic groups. Despite the broad variety of gross lesions, they are generally similar to those reported in gallinaceous species, including the presence of necrotic areas and hemorrhages in pancreas, gizzard, heart and central nervous system, among other organs. However, edema and hemorrhages in head and legs are rare in non-galliformes species [360, 364, 370, 421, 422]. Microscopic necrotico-inflammatory lesions can be observed in different organs, but the main organs affected may vary between species [360, 364, 370, 371, 421, 422]. Particularly, Gs/GD H5 HPAIVs seems to have acquired a strong tropism towards the central nervous system, and non-suppurative encephalitis associated to severe areas of necrosis has been reported in a broad range of species [365, 367, 372–374, 376, 423–426]

1.4.4. DETERMINANTS OF INFECTION

1.4.4.1. VIRAL FACTORS

The HA is the main viral determinant of species susceptibility, tissue tropism and pathogenicity in IAVs infections. In order to initiate infection, IAVs requires the binding to SA receptors present in the surface of host cells. Avian-adapted IAVs preferentially bind to N-acetylneuraminic acid (Neu5Ac) receptors linked to the penultimate galactose residue of cell surface glycans in an α-2,3 configuration, whereas mammalian-adapted IAVs presents higher affinity towards those linked to galactose in an α-2,6 configuration [427]. The type and distribution of SA in tissues varies between species, and is considered to determine largely the host range and tissue tropism of AIVs [428–430]. However, mutations in the HA can affect the receptor binding of IAVs and therefore, their host tropism and virulence [431–433]. In addition, other substructures and modifications such as sulfation, acetylation, glycosilation, fucosylation and internal sialylation of glycans can impact the binding affinity of the HA in different hosts [434].

The binding of IAVs to host cell receptors and the merging of the viral envelope with the endosomal membrane require the post-translational cleavage of the HA, which is initially produced as a single polypeptide precursor (HA0), into HA1 and HA2 subunits [435]. The MCs present in LPAIVs is only cleavable by few extracellular host proteases such as trypsin and trypsin-like enzymes (e.g. plasmin, blood clotting factor-like proteases) that are present in the respiratory and intestinal tract of the birds [436]. In contrast, MBCs present in HPAIVs are susceptible to several common intracellular proteases, including the ubiquitous proprotein-processing endoproteases furin and PC6 [437]. HA protein is then cleaved during the assembly of virions, and are already infectious when are released from infected cells. Therefore, HPAIVs possess the potential to replicate in a broader range of tissues. The fusion of the viral envelope with the cellular endosomal membrane is also activated in a specific range of pH. Particular mutations can change the acid stability of the HA, enabling fusion at a higher/lower endosomal pH and therefore alter the pathogenicity of AIVs [438].

The reversion to a MCs abolishes the virulence of HPAIVs in chickens [439]. However, data evidences the influence of other viral factors beyond the HA proteolytic cleavage site in the pathogenicity of AIVs. Experimental engineering of a MBCs in a LPAIVs does not necessarily result in a virulent variant [440] and some AIVs that lack MBCs are highly virulent to chickens [441]. In addition, AIVs that posses a MBCs that did not cause extensive mortality in chickens have been identified [442]. Finally, the sequence analysis of LPAIV and HPAIV isolates reveal the presence of mutations in other positions beyond the cleavage site [443, 444] and in other gene segments [145]. Pathogenesis studies in chickens and ducks demonstrate that PB2, PB1, PB1-F2, PA, PA-X, NP, NA, M and NS proteins are all important determinants of adaptation, virulence, tropism and transmissibility, either by particular substitutions [445, 446] or stalks in the amino acid sequence [447–449]. However, the residues involved may differ in each species. Several experimental studies also demonstrate that the functional balance between HA and NA proteins impacts the fitness and virulence of HPAIVs, which drives in large part the higher presence of particular HxNx combinations in nature [450–452].

After the transduction of HA, NA and M2 proteins, they are moved into the endoplasmatic reticulum for translational modifications, which include the addition of oligosaccharides to the consensus N-X-S/T (where X is any amino acid except proline) glycosilation motif [453]. N-linked glycosylation sites (NLG) in HA and NA proteins are important modulators of the structure and function of these proteins. Modifications in either the number or location of NLG sites in the HA protein have demonstrated to impact the biological activity of the virus, including host range (receptor binding), virulence (cleavability of HA0) and antigenicity (recognizition and neutralization by antibodies) [454–456].

NLGs in NA protein have also been associated with interspecies transmission of IAVs; however, their biological role has not been so extensively studied [456].

1.4.4.2. HOST FACTORS

Despite the species is the main factor determining the differential susceptibility to AIVs among hosts, other factors have an impact in the infection outcome. Differences associated to the age of birds have been documented, but the effect appears to be highly dependent on the species. A study found that age is not considered a determinant factor in the susceptibility of chickens to HPAIVs, at least to the strain tested [406]. In contrast, important differences in susceptibility to HPAIVs associated to the age of the birds exists in ducks [457–459], geese [300] and turkeys [460]. In these species, younger birds present more severe clinical signs, higher mortalities and/or longer MDTs in association to higher viral titers in organs and viral shedding.

The susceptibility to AIVs differs not only between species but also within the same host species. A high degree of variation in susceptibility to HPAIV infection, including the Italian H7N1 HPAIV [399, 461] and the Gs/GD H5N1 [462–465], H5N6 [412] and H5N8 [338, 416] HPAIVs, has been described between breeds/lines of chickens, despite none is completely resistant. Generally, brown-feathered chicken breeds are more resistant to AIVs than white type breeds [399, 462, 466]. In addition, there is a generally believe that local breeds are more resistant to disease than commercial breeds [467]. Whereas local breeds have been subjected to natural selection by endemic diseases, climate conditions, availability of nutrients and other stresses, commercial breeds have been artificially selected for high production efficiency, which may lead to undesirable side effects [468]. Some studies indicated that local chicken breeds present a higher natural resistance to AIVs in comparison with commercial breeds [464]; however, other results do not support the theory of increased susceptibility to infection in high-performance breeds [462, 465]. In ducks, the differences in susceptibility between breeds seems to be much slighter [469, 470].

During AIVs infection, pathogen associated molecular patterns present in the virion are recognized by host pathogen recognition receptors present in host cells, including retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and toll-like receptors (TLR) [471]. This triggers the activation of innate signaling patways, leading to the production of pro-inflammatory cytokines, antiviral proteins and chemokines that play an important role in the innate immune response and in the further activation of adaptive

immune responses. It has been demonstrated that the activation of RIG-I plays an important role in the early induction of type I interferon responses and supression of virus replication [311]. Whereas ducks [472], geese [473] and other species such as pigeons [474] present RIG-I activity, this protein is absent in chickens. Thus, some signaling patways may be affected and contribute to their increased susceptibility to disease [475]. Ducks induce an early and robust antibody response against AIVs after intravenous inoculation in comparison with chickens, which correlated with lack of detection of the virus in internal organs [476]. Ducks also appear to produce persistent cellular immune responses againsts AIVs in comparison with chickens [477]. All these factors could be important in the species-related differences observed.

The differences in susceptibility between hosts have prompted the search of alleles in immune-associated genes that may be associated with resistance to AIV infection. These could be useful for the identification of new targets for selective breeding and even genetic modification [478] and for the prediction of AIV disease outcome in infected birds [479]. Interferon-induced GTP-binding protein Mx1 (Mx) is an antiviral protein induced by type 1 interferon that interfere in the early stages of viral replication by inhibiting viral polymerases in the nucleus [480]. A single nucleotide polymorphism (SNP) at exon 14, position 2032 of chicken Mx gene produces the amino acid substitution serine to asparagine at position 631 (S631N) in Mx protein [481]. This position is located within the C-terminal GTPase effector domain, and the presence of an asparagine appears to confer a higher antiviral response against different avian infectious agents *in vitro*, including AIVs [482, 483]. However, other studies did not found an evident inhibitory effect [484, 485]. In addition, different groups found conflicting results regarding the effects of that particular amino acid substitution against AIVs in chickens *in vivo*: whereas one study indicated that this substitution conferred a higher resistance to H5N2 HPAIV infection [486], most studies did not found significant differences [399, 461, 487]. The higher survival of chickens carrying the B21 haplotype in the Major Histocompatibility Complex (MHC) class II after a natural outbreak of Gs/GD H5N1 HPAIV suggested that this allele could also play a protective role, whereas MHC-B13 haplotype was associated with an increased susceptibility [488]. However, other studies could not associate survival and polymorphisms at MHC [461]. Several studies associate genomic regions on different chromosomes [489, 490] and expression of particular genes [491] with survivability of chickens inoculated with HPAIV, but their exact role remains unknown. Therefore, the genetic basis for resistance to AIVs is a complex, polygenic trait that still remains undeciphered.

1.5. PREVENTION, DIAGNOSIS AND CONTROL

Given the wide circulation of AIVs in the wild bird population, the high transmissibility in poultry and their long persistence in the environment in the appropiate conditions, the implementation of proper **biosecurity and hygiene** measures are the first critical pillar to avoid the introduction into poultry premises [492]. There is no a standard set of measures of mandatory compliance; instead, each country should analyse which are the major potential sources of AIV infection taking into account their ecological characteristics and type of poultry production. However, the following general measures have shown to be effective in reducing the risk of infection: avoid the entry of birds of unknown sanitary status, separate land-base poultry from domestic waterfowl, limit contact with wild birds and peridomestic species and do not keep elements that may atract them (e.g. poultry feed), maintain strict control over access by vehicles, people and fomites, clean and disinfect appropriately poultry houses and equipment, dispose properly farm waste and poultry carcasses, and in case of signs of disease compatible wih AIV infection, report rapidly to the competent authority [493].

The compliance and applicability of the biosecurity measures reported above varies largely between poultry production systems [494]. In general, is more limited in small-scale commercial farms [495, 496], backyards and in scavenging flocks [497], which intrinsically bear a higher risk for AIV introduction [498]. Household poultry are known to play an important role in the transmission and perpetuation of AIVs [499], but small backyard flocks raising local breeds of different species and ages are still predominant in developing countries and in most cases represent the main source of income (approximately 80 percent of rural households raise poultry [29]). In addition, the consumption of meat and eggs originated from poultry produced in extensive systems is prefered in certain developed countries [500], and particular products require to keep the birds outdoor at some point (e.g. during breeding stage in the production of foie gras) [501]. Therefore, there is a need to recognize risk factors and manage the biosecurity measures in these systems. Biosecurity measures should also be implemented in LBM. These include the separation of different poultry species, proper cleaning and disinfection protocols and conduct regular market closures [502]. In all cases, there is a need to educate and train the farmers, owners and/or animal handlers to early detect clinical signs compatible with AIV infection.

A proper **surveillance** is the second key pillar of the control of AIVs. Surveillance programs are useful to evaluate the circulation of AIVs in the wild bird population and to rapidly detect their introduction into poultry; however, the exact protocols can vary between countries. In European Union, a risk-based surveillance or based on a representative sampling coupled to serological diagnosis are the common

approaches to evaluate the circulation of AIVs in poultry holdings (including waterfowl flocks) [503]. In case of positive samples, futher sampling is required for testing by virological methods. In general, the serological approach is less useful in case of HPAIV infection since most poultry species usually die in a short period of time and before producing detectable antibodies. Because of that, active surveillance is complemented by a passive surveillance through notification of compatible clinical signs/mortality in the flock. In these cases, oral swabs (OS), cloacal swabs (CS) and tissues are collected for virological testing [503].. Given the central role of LBMs in the epidemiology of AI in numerous countries, these markets have also been targeted by surveillance programms. Generally, OS and CS are collected from domestic species along with environmental samples (e.g. swabs from poultry cages, feed and water tray), which are then tested by different laboratory assays in order to evaluate the presence of the virus [504].

The surveillance in wild birds have been intensified since the intercontinental spread of Gs/GD H5N1 HPAIV in 2005 [505, 506]. In the European Union, the strategy consists in targetting those species that are at a higher risk of becoming infected, named "target species". These include in particular migratory waterbirds [503]. Areas near to waterbodies should be also particularly targeted, especially if they are in proximity to poultry holdings [503]. Active surveillance in wild birds requires to trap the birds, which can be made by different techniques (e.g. baitep traps, cannon nets, mist netting), with further collection of both OS and CS. The collection of fresh feces has been shown to be a good alternative to CS [507]. Passive surveillance is also effective for the early detection of HPAIVs in wild birds. As in poultry, the collection of OS, CS and tissues in wild birds found dead are recommended [508]. These samples are used for molecular detection and/or virus isolation. Overall, a proper surveillance in wild birds allows the identification of asymptomatic carriers and sentinel species [509].

There are some practical considerations that must be taken into account when obtaining samples for surveillance in poultry and wild birds. Generally, OS and CS are placed separately. However, some authors demonstrate that swabs obtained from poultry of the same flock or premise can be pooled with succesful results at the same time that save costs [510]. Even though, a study conducted in broilers reported that this practice can reduce the probability of detecting the virus in case of high-flock prevalences [511]. In wild birds, the collection of OS and CS from the same bird in the same tube can increase the frequency of positive samples in comparison with OS or CS alone [512]. Is important that the correct sampling is coupled to accurate transport and storage conditions. In general, swabs should be maintained in a buffered, salt-balanced medium with protein at approximately 4ºC, and arrive to the laboratory in few days (ideally within the 24 hours of collection) [513].

In dead birds, the tissues of choice vary dependent of the species and nature of the isolate. For LPAIVs, tissues are not considered optimal samples, but intestine and bursa of Fabricius, and trachea and lung could be the recommended in waterfowl and land-based poultry, respectively [514]. Given the systemic dissemination of HPAIVs, numerous tissues are suitable for their detection, including brain, lung, air sac, pancreas, liver, spleen and kidney. However, tissues should not be pooled with swabs or tissues from other birds. Tissues should be maintained in refrigeration until the arrival at the laboratory [514].

Since infected birds may lack signs of disease and if present they are not pathognomonic, accurate laboratory methods are always needed to **detect the causative agent**. Different serological and virological techniques have been developed and used succesfully in field surveillance and clinical diagnosis of IAVs, but their sensitivity, specificity, cost and the time needed until the obtention of results largely varies between them **(Table 6)**.

Assay	Target	Sensitivity	Specificity	Cost	Time
Virus isolation	Infectious virus	Very high	Moderate	High	1-2 weeks
Antigen detection immunoassays	IAV protein	Low	High	Moderate	15 minutes
Real-time RT-PCR	IAV RNA	Very high	Very high	Moderate	3 hours
AGID	1. IAV NP and M proteins 2. Ab to IAV NP and M proteins	Moderate	Moderate	Moderate	48 hours
ELISA	Ab to IAV	Moderate	Moderate	Low	2-3 hours
HA inhibition	1. HA subtyping 2. Ab to particular HA subtypes	High	Moderate -high	Moderate -high	2 hours
NA inhibition	1. NA subtyping 2. Ab to particular NA subtypes	Moderate	Moderate -high	Moderate	3 hours

Table 6. Characteristics of common IAV laboratory assays. Table obtained from reference [514].

The serological techniques most used to detect antibodies againsts AIVs include HA inhibition and NA inhibition tests, agar gel immunodiffusion, virus neutralization, complement fixation, enzyme and enzyme-linked immunoassays (ELISA) and indirect immunofluorescence [515]. For the direct detection of the virus, the isolation in embryonated chicken eggs remains as the gold standard due to its high sensitivity and the capacity to obtain more volume of virus for future laboratory analyzes. Therefore, it is still used for the diagnosis of the index case during a possible AIV outbreak [516]. Isolation is usually followed by other classic techniques that allow the detection of the virus, including agar gel immunodiffusion, haemagglutination assay and virus neutralization tests. The HA and NA of the AIV

are futher subtyped by HI and NI tests, respectively, using a battery of hyperimmune sera. Then, the virulence of the virus can be tested by an *in vivo* pathogenicity test [517].

The techniques included in the classical pathway are time-consuming and require high level of biosecurity, which is contributing to their gradual replacement by molecular techniques that directly target the genome of IAVs. Genetic detection techniques are considerably faster than classical methods while maintaining high sensitivity. These techniques, particularly reverse-transcriptase PCR (RT-PCR) and real-time RT-PCR (quantitative RT-PCR), have been implemented with succesful results for rapid diagnosis in clinical samples and for large-scale surveillance [518]. The first step is the detection of IAV RNA using universal primers that target highly conserved regions in their genome, generally within NP or M segments [519, 520]. Positive samples can be further subtyped by means of HA- and NA-specific primers, usually towards H5 and H7 subtypes in clinical samples [521, 522]. In addition, quantitative RT-PCR detects the fluorescence at a dose-dependent manner, allowing to quantify the viral loads in the sample [523]. Primers sets that enables the detection and sequencing of the proteolytic cleavage site of HA have also been developed [524], which are useful to rapidly assess the pathotype of the isolate without an *in vivo* pathogenicity test.

Other genetic detection techniques, including loop-mediated isothermal amplification (LAMP) [525, 526], nucleic acid sequence-based amplification (NASBA) [527, 528] and next-generation sequencing (NGS) [529], have also been useful and despite are still not widely used at the present time, they are contributing more and more to the diagnosis, subtyping and characterization of AIVs. There have been also recent developments in several antigen-capture immunoassays techniques. Antigen-capture ELISAs [530, 531] and immunochromatography protocols [532, 533] that allow the rapid detection of conserved epitopes in the NP of all IAVs or target particular HA subtypes have been described. However, they are often used as screening tests since they present low sensitivity in comparison to other techniques [516]. Detection of the virus (either proteins or RNA) in tissues by immunohistochemistry (IHC), immunofluorescense and in situ hibridization are not generally applied as primary diagnostic techniques, but instead have been widely used to study the pathogenesis of AIVs [400, 421, 534].

Once AIVs are detected in a poultry flock, different **control measures** are applied to avoid secondary transmission and eradicate the virus. General requirements include the culling of all birds within the farm, proper destruction of all potentially contaminated material and poultry waste, cleaning and disinfection of farm installations, control on movements between farms and maintain a strict quarantine before restocking. Protection (3 kms) and surveillance (10 kms) zones around the infected holding are also established. Within these zones, reinforced eradication and control measures are applied [535, 536].

In developed countries, the traditional stamping out and movement regulation policies have been successful, resolving most AIV outbreaks in a period of few months to a year. If the virus become widespread or in countries facing an endemic status, poultry can be vaccinated in order to minimize the need for culling. However, suboptimal vaccination strategies and high vaccine pressure from broad and/or long-term vaccination of poultry appears to facilitate the genetic and antigenic evolution of AIVs [537–539]. In Egypt, the mass vaccination of all commercial flocks and backyard poultry is thought to have played an important role in the emergence of the subclades 2.2.1.1 and 2.2.1.2 of Gs/GD H5 lineage of HPAIVs [540]. Moreover, vaccines may protect against clinical disease but not against virus transmission, enabling the silent spread of HPAIVs [127, 541]. Some vaccines may also interfere in the differentiation between wild-type and vaccine strains. In general terms, the vaccination of European poultry flocks is prohibited. However, the Directive gives the possibility to implement emergency vaccination programs as a short-term measure [542]. In addition, birds can be preventively vaccinated if areas or flocks at a particular high risk for virus introduction are identified. Vaccination of zoo and high valuable birds is also contemplated [542]. Vaccination plans in poultry, either preventive or as an emergence, were implemented in Italy in 2000 [543] and in Portugal in 2007 [544] against LPAIVs, and in France [545] and the Netherlands [546] in 2006 in response to the intercontinental spread of the Gs/GD H5N1 HPAIV.

Different types of AIV vaccines with variable efficacy and security under experimental conditions have been described. However, only inactivated vaccines and live recombinant vaccines are licensed for use in the field. Inactivated vaccines can be monovalent (including either H5 or H7 strains) or bivalent (including both), and may also contain neuraminidase subtypes. The use of combined vaccines with other antigens should be also considered in case of prolonged vaccination programs [547].

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

The outcome after infection with HPAIVs in birds is highly dependent of numerous viral and host factors. The subtype, clade and genetic group of the HPAIV and even specific mutations in viral genes produce significant variations in the infection outcome. Since HPAIVs evolve continuously, they can acquire unusual biological characteristics in particular avian species of different orders in the process. Host factors, including the order, species, breed, age at infection and immune status of the bird also influence the infection dynamics. Particularly, a variation in susceptibility to HPAIVs between breeds with different genetic background has been described. Moreover, there is the general believe that local breeds are more resistant to disease as a result of the natural selection by autochthonous pathogens and minor artificial selection towards productive-associated genes.

To date, studies comparing side by side the pathobiology of classical and Gs/GD H5 HPAIV lineages in avian species of different taxonomic groups are lacking. Consequently, the first general objective of the present dissertation was to systematically evaluate the differential pathobiology of two distinct HPAIVs in a range of avian species belonging to different orders. The H7N1 isolated in 1999 in Italy corresponding with a classical strain of HPAIV, and the H5N8 isolated in Spain during the 2016/2017 European epidemics that corresponds with a recent HPAIV of the Gs/GD H5 lineage were used. The second general objective was to determine the variation in susceptibility to HPAIVs in several breeds of chickens, geese and pigeons with different genetic background.

Specific objectives of this thesis are:

- To genetically characterize the H5N8 HPAIV isolated in Spain during the 2016/2017 European epidemics.

- To study the differential pathobiological features of H7N1 and H5N8 HPAIVs in a broad range of local and commercial chicken breeds, and to discern the role of viral and host factors in the infection outcome.

- To evaluate the pathobiology of H7N1 and H5N8 HPAIVs in backyard and commercial domestic geese and the role of this species in the wild-domestic interface of HPAI.

- To determine the pathobiology of H7N1 and H5N8 HPAIVs in local and urban pigeons and the role of this peridomestic and urban species as a host of HPAIVs.

PART II

STUDIES

CHAPTER 3

STUDY I: Genetic characterization of the highly pathogenic avian influenza A H5N8 (clade 2.3.4.4B) isolated from domestic waterfowl in Catalonia, Spain, during the 2016-2017 European epidemics

3.1. INTRODUCTION

Since its emergence, the HA of the Gs/GD H5 lineage of HPAIVs has continually diversified into multiple genetic clades (0-9) and subclades [48]. Particularly, the clade 2.3.4.4 has reassorted with distinct NA subtypes of AIVs circulating in domestic and wild birds, including N2, N3, N5, N6 and N8 [60]. The H5N8 HPAIV belonging to clade 2.3.4.4 of Gs/GD lineage was first isolated in domestic poultry in China in 2010 [64]. In 2013, reassortants of H5N8 with LPAIVs were isolated in healthy domestic and wild ducks [548–550]. In 2014, the virus caused several outbreaks in South Korea [551, 552] and further spread into Japan, Russia, Europe and North America. Despite two distinct genetic groups of H5N8 HPAIV were isolated (A and B), the intercontinental spread was caused by H5N8 A (Buan-like) HPAIVs [66]. In 2016, a novel reassortant H5N8 B (Gochang-like) HPAIV was simultaneously detected in dead wild birds in UVs-Nuur Lake in the Republic of Tyva (Siberia) [553] and in Qinghai Lake (China) [554]. Subsequently, the virus spread again into Europe [555, 556], and also into Russia [557], Middle East [558–560] and Africa [561]. Between October 2016 and December 2017, H5N8 B HPAIVs caused unprecedented outbreaks in numerous European countries in both domestic and wild birds, with evidence for local virus amplification [562] and gene exchange with LPAIVs [563]. Up to August 2017, this virus caused 1,112 outbreaks in domestic and 955 in wild birds in 30 European countries [71]. To date, the 2016-2017 H5N8 B HPAIV is the responsible of the largest epidemic by a HPAIV ever reported in the continent [70].

In early 2017, the H5N8 HPAIV reached Spain. In January and February 2017, two greylag geese and a white stork were found dead in Central (Castile and León) and Northeast (Catalonia) regions of Spain, respectively [564]. The samples were positive to H5N8 by specific qRT-PCR. Following the detection in the white stork, the H5N8 was detected in a commercial duck farm located in the same province. The farm presented a notorious increase in mortality and several ducks showed clinical signs typical of HPAIV infection, including coughing and torticollis. Nine additional commercial duck farms (7 farms epidemiologically linked with the primary focus and 2 holdings on the 3 km perimeter) and several backyard domestic flocks (located in the same province) tested positive to H5N8 HPAIV.

In order to study the genetic characteristics of the H5N8 virus that caused the outbreaks in domestic waterfowl in Spain in 2017, and to better elucidate its relationships with the H5N8 detected in Europe, Asia and Africa during 2016/2017 epidemics, we performed whole-genome characterization of the isolate.

3.2. MATERIALS AND METHODS

Virus isolation

The A/Goose/Spain/IA17CR02699/2017 (H5N8) isolated in a tracheal swab from a backyard goose (*Anser anser var. domesticus*) during the 2017 epidemic in Spain was propagated in 10 days-old SPF embryonated eggs. The allantoic fluid was harvested at 24 hpi. RNA was extracted from virus-containing allantoic fluid using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. A one-step Taqman RT-qPCR that targets a highly conserved region of AIV M1 segment, using the primers and probe as well as conditions of amplification previously described [521, 565], was performed in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). High viral loads in the sample were confirmed (Ct~21).

Whole-genome sequencing

The whole-genome sequencing was performed using an Ilumina Miseq platform. Briefly, a RNAseq library (Illumina, San Diego, CA, US) was constructed and checked using LabChip. A 250 Nano run of Miseq was performed (Illumina, San Diego, CA, US). Sample reads yielding a QC score >20 were accepted for further filtering. Reads were mapped against a reference genome of H5N8 AIV, and a consensus sequence for every segment was assembled using a tailor-made script. The consensus full genome sequences corresponding to the eight segments of H5N8 were deposited in Genbank under accession numbers MK494920- MK494927.

Phylogenetic and molecular genetic analysis

The obtained sequences were subjected to BLAST analyses in Global Initiative on Sharing All Influenza Data (GISAID) database [566]. The closest strains updated to GISAID until the isolation of A/Goose/Spain/IA17CR02699/2017 (H5N8) (2017.03.02) were annotated. Sequences available in GISAID until 2018.12.31 were downloaded and used for multiple sequence alignment in BioEdit 7.0. The nucleotide homologies between Spanish isolate and sequences available in databases were evaluated by the ClustalW method in BioEdit 7.0. The phylogenetic trees for each gene of AIVs (based on the nucleotides of the coding sequence) were constructed in MEGA X, using the Neighbor-joining algorithm, the Tamura-Nei model and 1000 bootstrap replicates to evaluate the confidence of the internal branches of the tree [567–570]. The presence of specific amino acids in particular positions in

AIV proteins previously associated with host tropism, transmissibility and/or virulence of HPAIVs in mammals and avian species were evaluated using BioEdit 7.0.

3.3. RESULTS

Homology and phylogenetic analyses

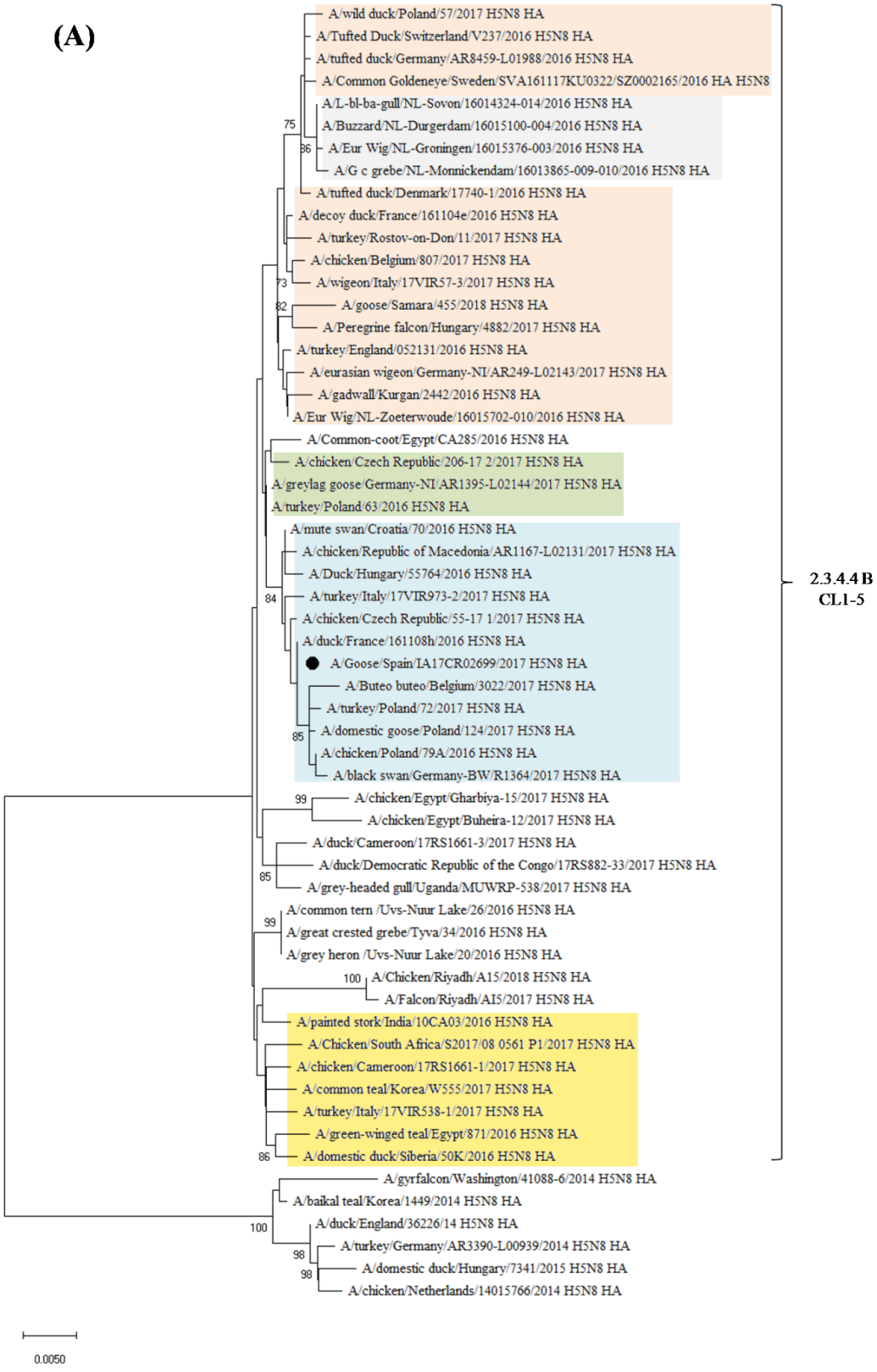
The complete genome of the positive sample was fully sequenced. The size in base pairs (bp) of the complete segments was as follows: 2337 (PB2), 2331 (PB1), 2219 (PA), 1746 (HA), 1558 (NP), 1419 (NA), 1023 (MP), 883 (NS). All genome segments of the Spanish isolate presented high nucleotide identity (99.5-99-9%) to H5N8 HPAIVs previously isolated in Europe and Asia during 2016/2017 **(Table 7).**

Genome segment	Closest strain (complete segment)	Nucleotide Id (%)	Isolation date
HA	A/goose/Hungary/55128/2016 (A/H5N8)	99,8%	16.11.11
NA	A/Indian Runner Duck/Czech Republic/749-17/2017 (H5N8)	99,6%	17.01.16
PB2	A/Anas platyrhynchos/Belgium/1899/2017 (A/H5N8)	99,8%	17.02.27
PB1	A/Anas platyrhynchos/Belgium/1899/2017 (A/H5N8)	99,7%	17.02.27
PA	A/duck/France/161108h/2016 (A/H5N8)	99,9%	16.11.28
NP	A/duck/France/161108h/2016 (A/H5N8)	99,7%	16.11.28
MP	A/chicken/Kalmykia/2643/2016 (A/H5N8)	99,5%	16.11.21
NS	A/domestic goose/Germany-BY/R677/2017 (A/H5N8)	99,5%	17.01.25

Table 7. Closest strains to H5N8 isolated in Spain, identity (%) and isolation date.

Based on the topology of the HA gene phylogenetic tree, the Spanish H5N8 isolate clustered within the genetic clade 2.3.4.4 group B of Gs/GD lineage, closely related with H5N8 B isolated in wild and domestic birds in Europe, Asia and Africa in 2016, 2017 and 2018 **(Figure 5A).** High homogeneity among H5N8 B 2016/2017 European isolates was found in NA **(Figure 5B)**, M and NS **(Sup. Figure 1A-B).** However, based on PB2, PB1, PA and NP tree topology **(Sup. Figure 1C-F)**, European isolates separated into five different clusters (named CL1, CL2, CL3, CL4, CL5). Spanish H5N8 B fell

in all gene segments into CL2, which includes H5N8 B HPAIVs isolated from several European countries (France, Italy, Poland, Germany, Hungary, Croatia and Macedonia).



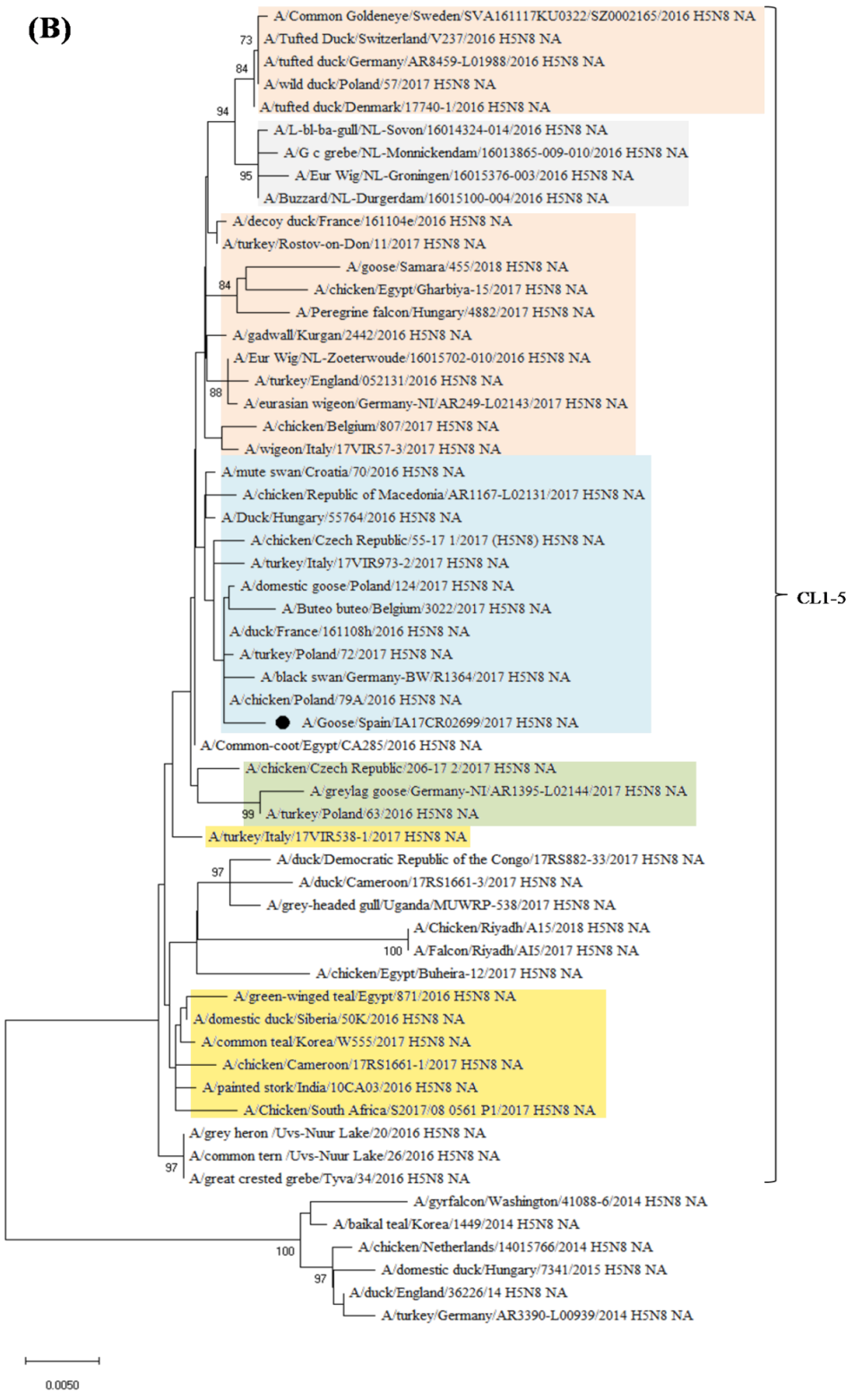


Figure 5. Neighbor-joining phylogenetic trees of HA (A) and NA (B) gene segments. The Spanish H5N8 is highlighted with a black dot. Bootstrap values ≥70% (700/1000 replicates) are shown. The different genetic groups are presented: cluster 1 (orange), cluster 2 (blue), cluster 3 (green), cluster 4 (yellow), cluster 5 (grey). Scale bar indicates nucleotide substitution per site.

Amino acid analyses

The mutations present in surface glycoproteins, internal and non-structural proteins of Spanish H5N8 HPAIV that are associated with a phenotype are listed in **Table 7.** The amino acid characterization revealed that the H5N8 Spanish isolate presents a MBCs identical to other H5N8 HPAIVs: PLREKRRKR↓GLF (↓ denotes cleavage site). Spanish isolate presented eight potential glycosylation sites (H5 numbering): 10, 11, 23, 165, 193, 286, 483, 542, which can affect HA receptor-binding specificity. The HA presented mutations associated with increased virus binding to human-like 2,6 SA and associated with virulence and airborne transmission in mammals **(Table 8).** However, Spanish H5N8 presented Q222 and G224 at the receptor-binding site (RBS), indicating that the virus mainly retained the preferential binding to SA in an α2,3 configuration (avian-like receptors). Two mutations associated with increased virulence in chickens were also detected. The NA presented six potential glycosylation sites at positions 54, 67, 84, 144, 294 and 398. We detected two mutations in NA protein associated with reduced susceptibility to oseltamivir and zanamivir.

We found numerous mutations in the RNP complex (PB2, PB1, PA, NP) associated with increased virulence to mammals and/or mammalian markers. However, the Spanish H5N8 did not present the mammalian markers 627K and 701N in PB2 protein, which are commonly found in lethal human infections. We also detected several mutations associated with increased virulence of HPAIVs in mammals and/or inhibition of host gene expression (e.g interferon pathway) in M1, M2 and NS1 proteins. However, mutations associated with resistance to amantadine were not present in M2 protein. Mutations related to increased pathogenicity, transmissibility and inhibition of host responses in bird species (chickens and ducks) were also detected in PB2, PB1, PA, NP, M1 and NS1 proteins.

Protein	Mutation	Phenotype (Subtype)
PB2	T63I	Increased virulence in mice (H5N1) [571].
	L89V	Increased polymerase activity and virulence in mice (H5N1) [572].
	K123E	Increased virulence in chickens (H7N7) [573]
	K251R	Increased virulence in mice (pH1N1) [574].
	I283M	Increased virulence in mice (H5N8) [575].
	G309D	Enhanced polymerase activity and virulence in mice (H5N1) [572].
	T339K	Enhanced polymerase activity and virulence in mice (H5N1) [572].
	Q368R	Enhanced polymerase activity and virulence in ferrets (H5N1) [576].
	K389R	Increased polymerase activity and replication in mammalian cell line (H7N9) [577].
	H447Q	Increased polymerase activity and virulence in ferrets (H5N1) [576].
	R477G	Increased polymerase activity and virulence in mice (H5N1) [572].
	I495V	Increased polymerase activity and virulence in mice (H5N1) [572].
	I504V	Increased polymerase activity and virulence in mice (H1N1) [578].
	V598T	Increased polymerase activity and replication in mammalian cells, increased virulence in mice (H7N9) [577].
	A676T	Enhanced polymerase activity, increased virulence in mice (H5N1) [572].
PB1	D3V	Increased polymerase activity and viral replication in avian and mammalian cell lines (H5N1) [579].
	L13P	Increased polymerase activity, mammalian host marker (H5N1) [153, 580].
	C38Y	Increased polymerase activity and virulence in chickens (H5N1) [445]
	K328N	Increased polymerase activity and virulence in ferrets (H5N1) [576].
	S375N/T	Increased polymerase activity, increased virulence in ferrets, human host marker (H5N1) [21, 576].
	H436Y	Increased polymerase activity and virulence in mallards, ferrets and mice [581].
	A469T	Increased transmissibility in guinea pigs [582].
	L473V	Increased polymerase activity and replication efficiency in mammalian cells (pH1N1, H5N1) [583].
	D622G	Increased polymerase activity and virulence in mice (H5N1) [584].
	V652A	Increased virulence in mice (pH1N1) [574].
	M677T	Increased virulence in mice (H5N1) [571].
PA	S37A	Increased polymerase activity in mammalian cells (H7N9) [585].
	K237E	Increased polymerase activity and pathogenicity in mallards (H5N1) [313].
	F277S	Adaptation to mammalian hosts (H10N8) [586].
	C278Q	Adaptation to mammalian hosts (H10N8) [586].
	N383D	Increased replication and virulence in mice, increased virulence in ducks (H5N1) [587, 588].
	N409S	Increased polymerasre activity in mammalian cells (H7N9) [585].
	A515T	Increased polymerase activity and virulence in mallards (H5N1) [581].
	I550L	Enhanced polymerase activity and virulence in mice [578].
	L653P	Adaptation to mammalian hosts (H10N8) [586].
	F672L	Increased transmissibility in chickens (H9N2) [589]
HA[1]	S107R	Increased virulence in chickens and mice, increased pH of fusion (H5N1) [438].
	T108I	Increased virulence in chickens and mice, increased replication (H5N1) [438, 590].
	S123P	Increased virus binding to 2,6 SA (H5N1) [591].

	S133A	Increased pseudovirus binding to 2,6 SA (H5N1) [592].
	S154N	Increased virus binding to 2,6 SA (H5N1) [455].
	T156A	Increased pseudovirus binding to 2,6 SA, increased transmissibility in guinea pigs and ferrets (H5N1) [455, 593, 594]
	V182N	Increased virus binding to 2,6 SA, decreased binding to 2,3 SA (H13N6) [595].
	K218Q	Increased virus binding to 2,6 and 2,3 SA (H5N1) [596]
NP	M105V	Increased virulence in chickens (H5N1) [597, 598].
	A184K	Increased replication in avian cells and virulence in chickens, up-regulation of interferon response (H5N1) [446].
	I353V	Increased virulence in mice (pH1N1) [574].
	K398Q	Mammal adaptation marker (H9N2) [599].
NA[2]	I117T	Reduced susceptibility to oseltamivir and zanamivir (H5N1) [600].
	I314V	Reduced susceptibility to oseltamivir (H5N1) [601].
M1	N30D	Increased virulence in mice (H5N1) [602].
	I43M	Increased virulence in mice, chickens and ducks (H5N1) [603].
	T215A	Increased virulence in mice (H5N1) [602].
NS1	P3S	Enhanced replication in mammalian cells and pathogenicity in mice (H7N1 backbone with H5N1 NS) [604].
	R41K	Enhanced replication in mammalian cells and pathogenicity in mice (H7N1 backbone with H5N1 NS) [604].
	P42S	Increased virulence in mice, decreased antiviral response in mice (H5N1) [605].
	K55E	Enhanced replication in mammalian cells, decreased interferon response (H5N1) [606].
	L103F	Increased virulence in mice (H5N1), inhibition of host gene expression (H9N2) [607, 608].
	I106M	Increased viral replication in mammalian cells and virulence in mice (H1N1 with internal genes from H7N9, H5N1), inhibition of host gene expression (H9N2) [607–609].
	P114S	Inhibition of host gene expression (H9N2) [608].
	G125D	Inhibition of host gene expression (H9N2) [608].
	C138F	Increased replication in mammalian cells, decreased interferon response (H5N1) [606].
	V149A	Increased virulence in mice and chickens, antagonism of IFN induction (H5N1) [610].
	V178I	Enhanced virulence and promoted the virus replication *in vivo* (H7N9) [611].

Table 8. Amino acid substitutions present in Spanish H5N8 isolate reported to change the virulence, transmissibility and/or host tropism of HPAIVs in avian and/or mammal species. [1] H5 numbering (A/Vietnam/1203/04 (HPAI)). [2]N2 numbering (A/Aichi/2/1968 (H3N2)).

3.4. DISCUSSION

Our study demonstrates that the cause of the HPAI outbreak occurring in Catalonia, Spain, in early 2017s is a H5N8 belonging to clade 2.3.4.4 group B of Gs/GD HPAIVs. Previous reports indicated the existence of two separately genotypes within H5N8 B: 1 (Siberia) and 2 (European), generated through reassortment of H5N8 B and LPAIVs present in Central Asian flyway [612]. Later on, Voronina *et al.* [613] indicated that at least four genetic groups exist within H5N8 B based on the differential genetic clustering. The European isolates clustered within Base Group, Mediterranean Group and a singleton, demonstrating that more than a single genetic group existed in Europe. More profound investigations of H5N8 B diversity in Germany, Italy, Netherlands and Poland indicated that Europe was affected by at least five different H5N8 B HPAIV reassortants [614–617]. Italian H5N8 B belonging to CL1 further evolved into two main groups, named Italy-A and Italy-B [618]. By means of full-genome characterization of the isolate, we also identified that European isolates fell into five different clusters (named CL1, CL2, CL3, CL4 and CL5). The different clustering would indicate that multiple incursions and/or emergence in Europe of distinct genetic variants of H5N8 B (formed by multiple reassortment events) had occurred. During H5N8 B worldwide spread, multiple reassortants were also detected in African and Asiatic countries, including Egypt [619], Cameroon [620] and India [621], indicating further evolution.

The goose-isolated H5N8 B HPAIV belonging to clade 2.3.4.4 isolated in Spain clusters within CL2, along with H5N8 B isolated in several European countries. The phylogenetic three reveals that the Spanish H5N8 characterized in this study is a reassortant similar to those previously reported in Poland, Italy and Germany. The original reassortment events that lead place to H5N8 B CL2 are not completely unraveled. However, Fusaro *et al.* [614] indicated that A/turkey/Italy/17VIR973-2/201, which clusters within CL2, is a result of the reassortment of three different AIVs: PB2 from A/Environtment/Chongqing/45208/15 (H5N6)-like, NP from European wild birds and the remaining segments from A/Bar-headed goose/Qinghai/2016 (H5N8)-like AI viruses. Using GISAID search, we noted that the NP gene segment of Spanish isolate presented high nucleotide identity (99%) with LPAIVs present in Georgia in 2016 (located in the frontier between Eastern Europe and Western Asia), suggesting a reassortment event or a common ancestor. However, only one isolate was fully-characterized; thus, we cannot rule out the presence of more genotypes in Spain during the outbreaks in 2017.

The epidemiological surveys performed in the infected farms revealed that the most likely cause of infection in the first two farms and in the backyard flocks was contact with migratory wild birds since: 1) the isolation of H5N8 HPAIV in a white stork in the same province indicate that the virus was already circulating in wild birds, 2) the farms and the backyard flocks showed feasible contact with wild birds, and 3) epidemiological and phylogeny information in different countries suggest that wild birds played an important role in the spread of H5N8 B HPAIVs during 2016/2017 epidemics [197]. In the remaining eight commercial farms, the cause of infection was associated to secondary spread from the first two farms (rearing farms), particularly movement of infected ducks.

In Europe, 70% of the domestic outbreaks occurred in duck holdings and only 12% in chicken holdings [71]. In Spain, the species affected were also domestic ducks and mixed backyard flocks and those presented a notorious increase in the mortality with evident clinical signs of HPAI. The higher proportion in waterfowl indicates that particularities of the production in commercial ducks may be associated with higher probability to H5N8 infection. The higher positivity in duck production could also be attributed to major changes in the affinity, tropism and/or virulence of H5N8B in waterfowl species. An experimental study indicates that European H5N8 B HPAIVs are more virulent to domestic ducks than European H5N8 HPAIV belonging to Group A [367]. Despite the viral factors determining the pathogenicity of H5N8 B in waterfowl is unknown, we noticed that H5N8 B viruses present the substitution 237E in the PA protein, which is associated with increased virus replication, polymerase activity and PA nuclear accumulation in ducks [313]. This mutation is absent in H5N8 A isolated during 2014/2015 European epidemics (data not shown). Host factors may also determine the higher incidence of waterfowl in comparison with galliformes species. Since AIVs adapted in different species can present different receptor specificities [622], H5N8 B viruses may present higher affinity towards influenza receptors in a particular configuration present in waterfowl but not galliformes species. However, this hypothesis needs further study.

To date, no human cases of H5N8 have been reported, and experimental studies performed in mice and ferrets indicate that H5N8 B present low zoonotic potential [367]. Moreover, H5N8 B cases in poultry have dramatically decreased in 2018 and 2019, reducing the potential spillover from poultry to human population. Despite the H5N8 HPAIV isolated in Spain presented multiple mutations associated with increased virulence and/or host tropism in mammal species in mostly all gene segments, the isolate retains an avian-like specificity in key points of the viral genome. Since Gs/GD H5 lineage of HPAIVs continues to evolve, mainly by reassortment events, H5N8 could increase its virulence and host-tropism in mammalian species [623].

In Spain, most of the poultry production is intensive and has high biosecurity standards. This, together with the reinforcement of the passive surveillance of the National Avian Influenza Surveillance Program due to the spread of Gs/GD H5N8 HPAIV in Europe in 2016 and the rapid establishment of control measures resulted in the prevention of H5N8-dissemination among the Spanish territory. In late 2018 and early 2019, H5N8 has reemerged in Bulgaria and Iran, respectively, demonstrating the continuous threat of HPAIVs [69]. Continuous surveillance in wild bird and poultry as well as proper biosecurity measures in backyard and commercial premises should be ensured in order to early detect H5N8 and avoid its propagation.

CHAPTER 4

STUDY II: Pathobiology of the highly pathogenic avian influenza viruses H5N8 and H7N1 in different chicken breeds and role of Mx 2032 G/A polymorphism in infection outcome

4.1. INTRODUCTION

Chickens are highly susceptible to HPAIVs; however, the severity of infection varies depending on the viral strain. The inoculation of most HPAIVs in chickens causes evident clinical signs (e.g. apathy, nervous signs) and gross lesions (e.g. cutaneous edema, cyanosis of the comb and wattles, haemorrhages in skin), and chickens usually die in a period ranging from 3 to 6 dpi. However, some HPAIVs produce a peracute infection that kills birds in a shorter period of time (within 3 dpi) [328–332, 334, 399, 402]. Differences can be observed even in close related isolates of the same subtype, indicating that point or few mutations in viral proteins may have a pivotal effect in their virulence [464]. In 2016/2017, the H5N8 HPAIV belonging to clade 2.3.4.4 B of Gs/GD H5 lineage was responsible of the fourth intercontinental wave of this lineage. Despite Europe has been affected by the four waves of Gs/GD H5 lineage, the caused by H5N8 B HPAIV was the largest in magnitude (reported poultry outbreaks and deaths), geographic spread and rapidity of incidents [70, 71]. Since Gs/GD H5N8 B HPAIVs may present altered biological properties in chickens, the evaluation of its pathobiological characteristics is needed.

The pathogenicity of HPAIVs is also influenced by numerous host factors, including species, age at infection and immune responses. Several reports demonstrate that a wide range of susceptibility to HPAIV infection is present between chicken breeds/lines. Some breeds display a comparatively high resistance, whereas other breeds are particularly susceptible [399, 461–466]. Local chicken breeds are generally raised in small-scale farms and in backyards that allow the direct contact with wild and synanthropic birds and their droppings. Consequently, these birds are likely more exposed to AIVs than commercial chicken breeds, which are raised under high biosecurity standards. However, there is a general believe that local chicken breeds are natural resistant to disease, which is associated with the natural selection over the years by autochthonous pathogens, food availability and harmful climate [467]. This assumption is usually a result of empiric experience at the field, and the results of experimental studies addressing the susceptibility of local chicken breeds to HPAIVs do not always support this theory [462, 465]. To date, there is no information regarding the susceptibility of local chicken breeds from Spain to HPAIVs.

Despite the genetic background that confers higher resistance to HPAIVs in chickens remains unknown, it was reported that the G/A polymorphism at position 2032 in chicken Mx gene (substitution of serine to asparagine at position 631 in the protein) conferred an antiviral effect against AIVs *in vitro* [483]. However, several *in vitro* and *in vivo* experiments have failed to demonstrate a clear

correlation between this polymorphism and inhibition of AIVs or survival after infection, respectively [399, 461, 484, 485, 487]. Therefore, the role of Mx in AIVs infections in chickens is still under debate.

Since the pathobiology of HPAIVs in chickens is multifactorial and numerous viral and host factors can largely influence the infection outcome, the aims of this study were to 1) evaluate the pathobiology of a recent H5N8 HPAIV isolated in Spain (Gs/GD lineage, clade 2.3.4.4, Group B) in comparison with a classical H7N1 HPAIV in different local, commercial and experimental chicken lines from Spain with diverse genetic backgrounds; and 2) determine the role of virus factors (differences in the sequence of amino acids in viral proteins between both HPAIVs) and host factors (allele at position 2032 of chicken Mx gene) in the infection outcome.

4.2. MATERIALS AND METHODS

Viruses

The viruses used in this study were: A/Chicken/Italy/5093/1999 (H7N1), isolated in 1999-2000 during an Italian epidemic that mainly affected Veneto and Lombardia regions (kindly provided by Dr. Ana Moreno from the *Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna*), and A/Goose/Spain/IA17CR02699/2017 (H5N8 clade 2.3.4.4. group B), isolated in Catalonia (Northern Spain) during the 2016/2017 European epizootics. Both viruses are highly pathogenic based on the amino acid sequences at the HA0 cleavage site: PEIPKGSRVRR↓GLF (H7N1) and PLREKRRKR↓GLF (H5N8). Virus stocks were produced in 10 days-old SPF embryonated eggs. The allantoic fluid was obtained at 24-48 hpi, filtered and aliquoted at -75ºC until use. Serial ten-fold dilutions of the filtered viruses in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean egg lethal doses (ELD_{50}) were determined by Reed and Muench method [624].

H7N1 was subjected to full-genome characterization using next-generation sequencing methods. Briefly, viral RNA was extracted from virus-containing allantoic fluid using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. The whole-genome sequencing was carried out using an Ilumina Miseq platform. A RNAseq library (Illumina, San Diego, CA, US) was constructed and checked using LabChip. A 250 Nano run of Miseq was performed (Illumina, San Diego, CA, US). Sample reads yielding a QC score >20 were accepted for further filtering. Reads were mapped against reference genomes of H7N1 AIVs, and a consensus sequence for every segment was assembled using

a tailor-made script. The amino acid identity and the differences in the amino acid sequence in the 8 conserved internal and non-structural viral proteins (PB2, PB1, PA, NP, M1, M2, NS1, NS2) between H7N1 and H5N8 HPAIVs, which was already fully-sequenced in Study I, were determined. The consensus full genome sequences corresponding to the eight segments of H7N1 and H5N8 are available in Genbank under accession numbers: DQ991325.2 to DQ991332.2 (H7N1) and MK494920 to MK494927 (H5N8).

Animals and facilities

15 days-old chickens (*Gallus gallus domesticus*) of six different local breeds from Spain (*Empordanesa, Penedesenca, Catalana del Prat, Flor d'Ametller, Castellana negra, Euskal oiloa),* a commercial breed (Ross 308 Broiler) and a commercial-experimental line (SPF White Leghorns) were used. All breeds were obtained from local breeders. The local breeds included in this study are common in non-commercial, small-scale flocks, usually in backyards alone or mixed with other species in different regions of Spain. For their characteristics, these breeds are common in chicken contests, and their meat and sub-products are used for self-consumption or sold in local markets because of their added value in the market chain. Specific programs have been established to ensure their conservation [625].

At arrival, the animals were individually identified and placed in negative-pressured HEPA-filtered isolators present in Biosecurity Level 3 (BSL-3) facilities of *Centre de Recerca en Sanitat Animal* (*Programa de Sanitat Animal*, IRTA). During the 5 days-acclimation period, serum samples were obtained from all birds to ensure that they were seronegative to IAV by an ELISA competition (c-ELISA) test (ID-VET, Montpellier, France). Furthermore, OS and CS were collected from 5 random chickens of each group and confirmed to be negative to AIV RNA by one-step qRT-PCR. During the experimental procedures, food and water were provided *ad libitum*. The experimental design was approved by the ethical commission of IRTA and the Government of Catalonia (*Departament de Territori i Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural*) under reference code CEEA 18/2017-9457.

Experimental design and sampling

After acclimation, 15 chickens of each breed (except for *Castellana negra* and Broilers that consisted in groups of 13 birds, and *Euskal oiola* inoculated with H7N1 that was a group of 10 animals) were intranasally challenged with H7N1 or H5N8 HPAIV 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). Animals belonging to negative control

group (2-5 animals/breed) were intranasally inoculated with 0.05 mL of sterile phosphate-buffered saline (PBS).

All birds were monitored daily for clinical signs until 10 dpi. A standardized OIE clinical scoring system was used [140]. Animals with absence of clinical signs were classified as 0. Birds presenting one of the following clinical signs were considered sick (1) and those showing more than one were considered severely sick (2): respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head and nervous signs. Animals found dead were scored as 3. For ethical reasons, moribund chickens were anesthetized using the combination of ketamine/xylazine (20 mg/kg body weight, Imalgene 100 and 0,3 mg/kg body weight, Rompun 20 mg/ml) via the intramuscular route, euthanized with intravenous pentobarbital (140 mg/kg body weight, Euthasol 400 mg/ml) and scored as dead. The percentage of mortality and MDT were calculated for each virus in all the breeds.

All birds presenting severe clinical signs or found dead were subjected to macroscopic examination. In addition, three chickens of each breed inoculated with H7N1 and H5N8 HPAIVs were killed at 3 dpi using the combination of drugs reported above to collect tissue samples for pathological studies. The selection of birds was biased towards those found dead or presenting evident clinical signs of disease. Two birds of each breed belonging to mock-infected groups were also necropsied at 3 dpi. In order to evaluate viral shedding, OS and CS were collected from the first 9 chickens of each breed (selected previously to the inoculation) challenged with H7N1 and H5N8 HPAIVs, and from negative control animals, at 1, 3, 6 and 10 dpi. The same birds were sampled through the experiment.

Pathologic examination and immunohistochemical testing

Tissue samples collected from the chickens necropsied at 3 dpi were 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, small intestine, large intestine and bursa of Fabricius.

Microtome sections of 3 μm of thickness (Leica RM2255, Nussloch, Germany) from formalin-fixed, paraffin-embedded tissues (FFPE) collected at 3 dpi were processed, stained with haematoxylin and eosin (H/E) and then examined under light microscopy. An IHC technique was performed in the same tissues. Briefly, samples were pretreated with 0.1% protease at 37ºC during 8 minutes. A mouse-derived monoclonal commercial antibody against NP of IAVs (ATCC, HB-65, H16L-10-4R5) was used as a primary antibody. The slides were incubated overnight at 4ºC. The samples were then incubated with

an anti-mouse secondary antibody conjugated to an HRP-Labelled Polymer (Dako, immunoglobulins As, Denmark). The antigen–antibody reaction was visualized using the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with Mayer's haematoxylin and examined under light microscopy. The positivity in the tissues was semi-quantitatively assessed taking into consideration the percentage of NP-positive and negative cells in the tissue. The samples were classified as follows: no positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells (+++) in a tissue section. Positive and negative controls were used. The positive control was a central nervous system from a chicken experimentally infected with H7N1 HPAIV [400], and the negative control consisted in the same tissue incubated with PBS instead of the primary antibody and the tissues collected from negative control chickens.

Viral RNA detection and quantification in swabs

Swabs were placed in 0.5 ml of sterile PBS enriched with Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Nystatin (Sigma-Aldrich, Missouri, USA) at a final concentration of 6%. Swabs were conserved at -75ºC until further use. Viral RNA was extracted using Nucleospin RNA virus kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions. A highly conserved region of 99 bp present in IAV M1 gene was amplified and detected by one-step Taqman RT-PCR technique in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA), using the same primers and probe as well as conditions of amplification previously described [521, 565]. To extrapolate the genome equivalent copies (GEC) present in the swabs, a standard curve obtained by amplification of the same region of M1 gene was used. Briefly, the amplified region was ligated in pGEM-T vector (Promega, Madison, Wisconsin, USA). The ligation product was purified using MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA, USA) and transfected into electrocompetent E.coli cells (Thermo Fisher Scientific, Waltham, Massachusetts, USA) by electroporation. The recombinant plasmid was purified from transformed colonies using NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany) and quantified in Biodrop (Biodrop μLite, Cambridge, England). GEC were calculated using DNA Copy Number Calculation (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Serial ten-fold dilutions were used to obtain the standard curve. The limit of detection of the technique was 1,89 log GEC in both OS and CS.

RFLP-PCR Mx

Prior to infection, total blood in a 1:1 ratio with anticoagulant (Alsever's solution, Sigma-Aldrich, Missouri, USA) was obtained from all chickens belonging to H7N1 and H5N8 HPAIV-inoculated groups. Genomic DNA was isolated from 10 µl anticoagulated blood using a standard DNA purification kit (DNeasy Mini Kit, Qiagen, Valencia, CA, USA), following manufacturer's instructions. To avoid RNA contamination, samples were treated with RNase (RNase A, Qiagen, CA, USA). As described by Sironi *et al.* [626], the following primers were used to amplify a 299 pb region in exon 14 of chicken Mx gene: forward 5'-GCACTGTCACCTCTTAATAGA-3' and reverse 5'-GTATTGGTAGGCTTTGTTGA-3'. PCR reaction mixture included 60 ng genomic DNA, 10 µmol of each primer, 10X buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP and native Taq DNA polymerase (5 U/µL) (Taq DNA Polymerase, native, ThermoFisher Scientific, Massachusetts, USA) in a final volume of 25 µl. Mx region was amplified in GeneAmp PCR System 9700 equipment (Applied Byosistems, CA, USA) as follows: 95°C for 10 min, 35 cycles of 94°C for 1 min, annealing at 53°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min.

5 µl of PCR products were run in a 2% agarose gel in 1X TAE buffer with ethidium bromide (EtBr) to confirm the presence of a specific band at 299 pb. PCR products were incubated at 37ºC during 16 hours with a restriction enzyme (Hpy8I-MjaIV, 10 U/µL, Thermo Fisher Scientific, Massachusetts, USA), following manufacturer's instructions. The restriction enzyme (5'-GTN|NAC-3') cleaves the sequence 2 pb downstream of the Mx polymorphism in presence of guanine (G), whereas the product is not cut in case of an adenine (A) at this position. Digestion products were visualized in a 2% agarose gel in 1X TAE buffer with EtBr. Animals were classified in homozygous-resistant genotype (AA), heterozygous-intermediate genotype (AG) and homozygous-susceptible genotype (GG).

The proportion of birds dead at the end of the study by genotype groups were compared using the Pearson's chi-square test. Then, post-hoc pairwise comparisons with Bonferroni corrections were carried out [627]. Also, for the animals that succumbed to infection, the MDT by genotypes were compared. First, the normality of the data was assessed using the Shapiro-Wilk test. Then groups were compared using either the anova test (in case of normally distributed data), or the Kruskal-Wallis test (in case of non-normally distributed data). Finally, post-hoc comparisons were carried out using the Tukey test (for normally distributed data), or Dunn's test with Bonferroni correction (in case of non-normally distributed data). All calculations were carried out using R statistical software (http://cran.r-project.org/).

4.3. RESULTS

Clinical signs and mortality

Severe clinical signs were observed in H7N1 and H5N8 HPAIVs-inoculated chickens in all breeds, but the frequency varied depending on the viral isolate and the chicken breed**.** At 2 dpi, several chickens of different breeds inoculated with H7N1 HPAIV presented severe apathy, were prostrated or found dead without previous evident clinical signs. Few chickens inoculated with H5N8 HPAIV also presented severe apathy at 2 dpi and were consequently euthanized. From 2 dpi and lasting until 9 (H7N1) or 10 dpi (H5N8), severe clinical signs were detected in several chickens at different times post-inoculation. The main clinical signs observed in both HPAIV infections were moderate apathy that progressed to prostration, and less frequently subcutaneous oedema, cyanosis of the comb and wattles and nervous signs (ataxia, circling, tremor and head shaking). The percentage of animals presenting prostration and nervous signs was higher in chickens challenged with H7N1 HPAIV than in those inoculated with H5N8 HPAIV. Similarly, the onset of nervous signs was earlier in H7N1 HPAIV-inoculated groups than in the inoculated with H5N8 HPAIV (3 dpi *versus* 5 dpi, respectively). Moreover, H7N1 HPAIV produced a higher mortality rate in chickens than H5N8 HPAIV (70 and 47%, respectively), as well as a shorter MDT (3,3 and 4,9 dpi, respectively) **(Figure 6).**

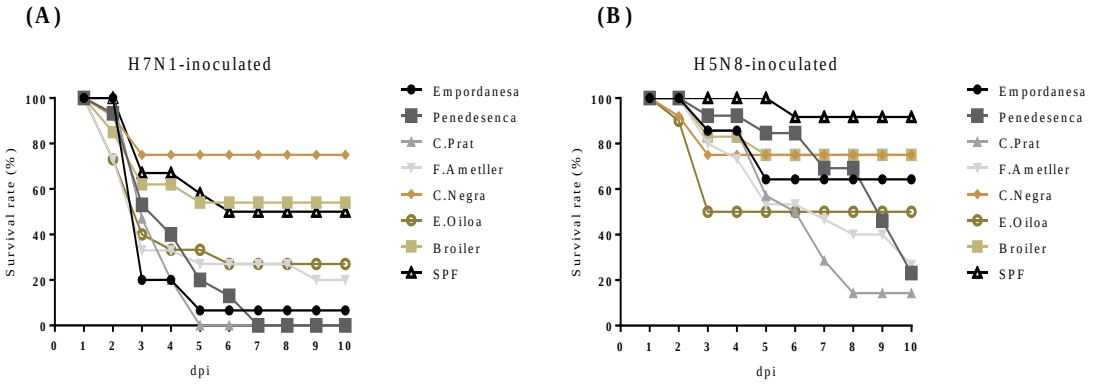


Figure 6. Survival curves of the different chicken breeds experimentally inoculated with H7N1 (A) or H5N8 (B) HPAIVs at a dose of 10^5 ELD_{50}.

Regarding breeds, *Castellana negra*, Broiler and SPF chicken breeds presented less frequency of clinical signs and considerably lower mortality rates (≤50%) than *Penedesenca, Catalana del Prat, Flor d'Ametller and Euskal Oiloa* (≥50%) breeds in both H7N1 and H5N8 HPAIVs-inoculations. Only *Empordanesa* breed presented differing susceptibility depending of the virus tested (93 and 33% mortality after challenge with H7N1 and H5N8, respectively). *Catalana del Prat and Penedesenca* presented the highest incidence of nervous signs and cutaneous edema among all the breeds tested, respectively **(Table 9).**

Virus	Parameter	*Empordanesa.*	*Penedesenca*	*C.Prat*	*F.Ametller*	*C.Negra.*	*E.Oiloa*	Broiler	SPF	**Mean**
H7N1	Percentage of dead birds	93	100	100	80	25	73	46	40	**70**
	Mean death time, dpi	3,3	4,2	3,3	3,3	2,7	3	3	3,8	**3,3**
	Clinical signs (%)									
	Severe apathy	93	73	67	40	15	67	31	40	**55**
	Cutaneous edema/cyanosis	7	20	7	0	0	0	0	7	**5**
	Nervous signs	0	7	20	0	8	20	8	0	**8**
H5N8	Percentage of dead birds	33	76	85	73	25	50	25	8	**47**
	Mean death time (dpi)	4,2	7,9	5,8	5,7	2,7	2,8	3,7	6	**4,9**
	Clinical signs (%)									
	Severe apathy	7	40	40	53	23	40	8	67	**35**
	Cutaneous edema/cyanosis	0	7	7	7	0	0	0	8	**4**
	Nervous signs	0	7	0	0	0	0	0	0	**4**

Table 9. Clinical signs, mortality and MDT of the different chicken breeds challenged with H7N1 or H5N8 HPAIVs. Dpi: Day post-infection.

Gross lesions

Gross examination of the chickens inoculated with H7N1 or H5N8 HPAIVs revealed similar lesions with both viruses in all breeds. At 2 dpi, few chickens inoculated with H7N1 HPAIV exhibited multifocal haemorrhages in proventriculus and gizzard, whereas non- evident lesions were present in the chickens inoculated with H5N8 HPAIV. From 3 dpi to the end of the study, the most common findings in the chickens inoculated with H7N1 and H5N8 HPAIVs were multifocal petechiae and necrotic areas in pancreas, and/or multifocal petechiae in proventriculus, gizzard and in the proventriculus-gizzard junction. Congestion in central nervous system was also a common finding. Less frequently, several chickens exhibited hemorrhages of variable intensity in skin (e.g. legs), subcutaneous edema, lung consolidation and diffuse congestion in internal organs. At 10 dpi, one chicken inoculated with H5N8 HPAIV presented multifocal petechias in bursa of Fabricius. No evident lesions were observed in negative control birds.

Histopathological findings

Microscopic examination of the tissues collected from dead or severely-affected chickens at 3 dpi revealed evident lesions of variable intensity in all breeds in mostly all the collected organs. However, we detected differences in the severity and viral replication in the different tissues between H7N1 and H5N8 HPAIV-inoculated chickens. In both viral infections, the predominant microscopic lesions in the tissues were areas of necrosis and haemorrhages with mixed inflammatory infiltrate (macrophages, lymphoplasmacytic cells and heterophils). The extension and severity of microscopic lesions correlated well with the intensity of IAV antigen (IHC techniques) in the tissue. The main organs affected were similar in all the chicken breeds inoculated with the same virus **(Table 10 and 11).**

H7N1	E	P	C	F	N	O	B	S	NP+ cell types	Microscopic lesions
Skin	+ (1/3)	+ (1/3)	+ (3/3)	+ (2/3)	+ (1/3)	+ (2/3)	+ (2/3)	++ (2/3)	Follicular epithelial cells, keratinocytes, EC, IC	Mixed inflammatory cell infiltrate in dermis, oedema, congestion, microthrombosis, vasculitis.
Pectoral muscle	+ (1/3)	+ (3/3)	+ (3/3)	+ (1/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (2/3)	Myocytes	Focal degenerated and necrotic fibers with mild inflammatory infiltrate.
Nasal cavity	+ (1/3)	+ (1/3)	+ (3/3)	+ (2/3)	+ (2/3)	+ + (3/3)	+ (3/3)	+ (1/3)	Respiratory and olfactory cells, Bowman glands, nasal glands, nasolacrimal duct, EC, IC	Areas of necrosis of epithelial cells (loss of continuity, loss of cilia) with mixed inflammatory cell infiltration, congestion.
Lung	++ (1/3)	+ (2/3)	+ (2/3)	+ (2/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (2/3)	EC, IC	Increase of cellularity (mixed inflammatory cells) in air capillaries interstitium, microthrombosis, interstitial oedema.
Central nervous system	+++ (1/3)	+++ (3/3)	++ (3/3)	+ (3/3)	++ (3/3)	+++ (3/3)	++ (3/3)	++ (3/3)	Neurons, glial cells, Purkinje cells (cerebellum), EC	Areas of necrosis (chromatolysis and spongiosis) with microgliosis, congestion.
Pancreas	+ (1/3)	+++ (3/3)	++ (3/3)	+ (1/3)	++ (1/3)	++ (3/3)	+++ (2/3)	+ (2/3)	Exocrine acinar cells, EC, IC	Areas of necrosis with inflammatory cell infiltration.
Heart	+++ (1/3)	+++ (3/3)	+++ (3/3)	++ (3/3)	++ (3/3)	+++ (3/3)	++ (3/3)	++ (3/3)	Myocytes, EC, IC	Areas of necrosis of myocytes (fiber degeneration and hyalinization) with mixed inflammatory cell infiltrate.
Spleen	++ (2/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (1/3)	EC, IC	Mild areas of necrosis with mixed inflammatory infiltrate.
Thymus	++ (1/3)	+ (2/3)	+ (1/3)	+ (2/3)	+ (2/3)	+ (2/3)	++ (3/3)	+ (1/3)	EC, IC	Mild necrotic areas (primarily in medulla) with few macrophages.
Liver	+ (1/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)	+ (2/3)	+ (3/3)	Kuppfer cells, EC, IC	Mild distension of hepatic sinusoids with presence of inflammatory cells (mainly macrophages), congestion.
Kidney	+ (1/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (3/3)	+ (2/3)	+ (2/3)	Epithelial tubular cells, EC, IC	Acute tubular necrosis with mild mixed inflammatory infiltrate, congestion.
Proventriculus	+ (1/3)	+ (3/3)	+ (3/3)	+ (1/3)	+ (2/3)	+ (2/3)	+ (2/3)	+ (2/3)	EC, IC	Areas of necrosis of lymphoid tissue.
Gizzard	+ (1/3)	+ (3/3)	+ (3/3)	+ (2/3)	+ (2/3)	++ (3/3)	+ (3/3)	+ (3/3)	Epithelial cells of the ventricular glands, smooth muscle cells, EC, IC	Areas of necrosis in glandular cells with mixed inflammatory cell infiltration, muscular cells necrosis and degeneration.
B.Fabricius	+ (1/3)	+ (3/3)	+ (3/3)	+ (1/3)	+ (1/3)	+ (3/3)	+ (2/3)	+ (2/3)	EC, IC	Areas of necrosis with inflammatory cell infiltrate (macrophages and heterophils) in germinal centers and interstitial compartment.
Small intestine	+ (1/3)	+ (1/3)	+ (2/3)	+ (1/3)	-	+ (2/3)	+ (1/3)	+ (2/3)	EC, IC	No apparent lesions.
Large intestine	+ (1/3)	+ (1/3)	+ (2/3)	+ (1/3)	-	+ (1/3)	+ (1/3)	-	EC, IC	No apparent lesions.

Table 10. Average distribution of NP-positive cells and associated lesions in tissues collected at 3 dpi from different chicken breeds inoculated with H7N1 HPAIV. n=3/group. -: no positive cells, +: <10% positive cells, ++: 10-40% positive cells, +++: >40% positive cells. E: *Empordanesa,* P: *Penedesenca*, C: *Catalana del Prat*, F: *Flor d'Ametller*, N: *Castellana Negra*, O: *Euskal Oiloa*, B: Broiler, S: SPF. EC: endotelial cells, IC: inflammatory cells.

H5N8	E	P	C	F	N	O	B	S	NP+ cell types	Microscopic lesions
Skin	+ (2/3)	+ (1/3)	++ (2/3)	+ (3/3)	+ (1/3)	+ (3/3)	+ (1/3)	+ (1/3)	Follicular epithelial cells, keratinocytes, EC, IC	Mixed inflammatory cell infiltrate in dermis, oedema, congestion, microthrombosis, vasculitis.
Pectoral muscle	+ (2/3)	+ (1/3)	+ (2/3)	+ (3/3)	+ (2/3)	+ (3/3)	+ (1/3)	+ (1/3)	Myocytes	Focal degenerated and necrotic fibers with mild inflammatory infiltrate.
Nasal cavity	++ (3/3)	+ (1/3)	++ (2/3)	++ (3/3)	++ (2/3)	++ (3/3)	+ (1/3)	++ (1/3)	Respiratory and olfactory cells, Bowman glands. Nasal glands, nasolacrimal duct, EC, IC	Areas of necrosis of epithelial cells (loss of continuity, loss of cilia) with mixed inflammatory cell infiltration, congestion.
Lung	+++ (2/3)	++ (1/3)	+++ (2/3)	+++ (3/3)	++ (2/3)	++ (3/3)	++ (1/3)	++ (1/3)	Air capillaries cells, EC, IC	Increase of cellularity (mixed inflammatory cells) in air capillaries interstitium, focal areas of necrosis in pneumocytes, microthrombosis, intersticial oedema.
Central nervous system	++ (3/3)	+ (1/3)	+++ (2/3)	++ (3/3)	++ (2/3)	++ (3/3)	+ (1/3)	+ (1/3)	Neurons, glial cells, Purkinje cells (cerebellum), EC	Areas of necrosis (chromatolisis and spongiosis) with microgliosis, congestion.
Pancreas	++ (3/3)	++ (1/3)	++ (2/3)	++ (3/3)	+ (1/3)	++ (3/3)	+ (1/3)	+ (1/3)	Exocrine acinar cells, EC, IC	Areas of necrosis with inflammatory cell infiltration.
Heart	+++ (2/3)	+++ (1/3)	+++ (2/3)	++ (3/3)	++ (2/3)	++ (3/3)	++ (1/3)	+ (1/3)	Myocytes, EC, IC	Areas of necrosis of myocytes (fiber degeneration and hyalinization) with mixed inflammatory cell infiltrate.
Spleen	++ (2/3)	++ (1/3)	++ (2/3)	++ (3/3)	++ (2/3)	++ (3/3)	++ (2/3)	+ (2/3)	EC, IC	Areas of necrosis (including germinal centers) with mixed inflammatory infiltrate.
Thymus	++ (2/3)	++ (1/3)	++ (2/3)	++ (3/3)	++ (2/3)	++ (2/3)	++ (1/3)	+ (1/3)	EC, IC	Areas of necrosis with inflammatory cell infiltrate in medulla and more restricted in cortex, congestion.
Liver	+ (2/3)	++ (1/3)	++ (2/3)	+ (3/3)	+ (2/3)	++ (3/3)	++ (1/3)	+ (1/3)	Hepatocytes, Kuppfer cells, EC, IC	Focal areas of necrosis with mixed inflammatory cell infiltrate, mild distension of hepatic sinusoids, congestion.
Kidney	+ (2/3)	+ (1/3)	+ (2/3)	+ (3/3)	+ (2/3)	+ (3/3)	+ (1/3)	+ (1/3)	Epithelial tubular cells, EC, IC	Acute tubular necrosis with mild mixed inflammatory infiltrate, congestion.
Proventriculus	+ (2/3)	++ (1/3)	++ (2/3)	+ (2/3)	+ (2/3)	+ (3/3)	+ (1/3)	+ (1/3)	Epithelial cells of the proventricular glands, EC, IC	Focal areas of necrosis in glandular cells and mainly in lymphoid tissue.
Gizzard	++ (2/3)	++ (1/3)	++ (2/3)	++ (3/3)	+ (2/3)	++ (3/3)	++ (1/3)	+ (1/3)	Epithelial cells of the ventricular glands, smooth muscle cells, EC, IC	Areas of necrosis in glandular cells with mixed inflammatory cell infiltration, muscular cells necrosis and degeneration.
B.Fabricius	+ (2/3)	+ (1/3)	++ (2/3)	+ (3/3)	+ (2/3)	++ (3/3)	+ (1/3)	+ (1/3)	EC, IC	Areas of necrosis with inflammatory cell infiltrate (macrophages and heterophils) in germinal centers and interstitial compartment.
Small intestine	+ (2/3)	+ (1/3)	+ (2/3)	+ (3/3)	+ (2/3)	++ (3/3)	+ (1/3)	+ (1/3)	EC, IC	Necrosis of lymphoid tissue with mixed inflammatory infiltrate.
Large intestine	+ (2/3)	+ (1/3)	++ (2/3)	+ (3/3)	+ (1/3)	+ (3/3)	-	+ (1/3)	EC, IC	Necrosis of lymphoid tissue with mixed inflammatory infiltrate.

Table 11. Average distribution of NP-positive cells and associated lesions in tissues collected at 3 dpi from different chicken breeds inoculated with H5N8 HPAIV. n=3/group. -: no positive cells, +: <10% positive cells, ++: 10-40% positive cells, +++: >40% positive cells. E: *Empordanesa,* P: *Penedesenca*, C: *Catalana del Prat*, F: *Flor d'Ametller*, N: *Castellana Negra*, O: *Euskal Oiloa*, B: Broiler, S: SPF. EC: endotelial cells, IC: inflammatory cells.

In H7N1 HPAIV-inoculated chickens, the most relevant microscopic lesions and viral replication were observed in heart, followed by central nervous system and pancreas. Viral replication in the heart was associated with fiber degeneration/necrosis and hyalinization of myocytes mixed with mild inflammatory cell infiltration (mainly macrophages) **(Figure 7 A/B)**. In the central nervous system, non-suppurative encephalitis consisting in multifocal areas of necrosis in cerebral hemispheres, intense spongiosis, neuronal chromatolysis and gliosis were commonly observed **(Figure 7 C/D)**. In cerebellum, chromatolysis of Purkinje neurons was a common finding. The lesions observed in pancreas were multifocal areas of lytic necrosis of exocrine gland cells **(Figure 7 E/F)**. The remaining tissues generally presented mild necrotic and/or inflammatory lesions and few positive cells **(Figure 7 H/I; J/K)**.

The chickens inoculated with H5N8 HPAIV presented the most severe lesions and intense viral replication in the lung and heart, but evident lesions and high viral replication were also detected in spleen, thymus, central nervous system, nasal cavity, gizzard, pancreas and liver. The lesions in the heart, central nervous system and pancreas were similar to those described in H7N1 HPAIV-inoculated chickens **(Figure 7 K/L; M/N; and Ñ/O)**. In the lung, interstitial pneumonia consisting on moderate to severe increase of the cellularity (macrophages and lymphoid cells) in air capillaries and focal areas of necrosis associated with intense viral replication was commonly observed **(Figure 7 P/Q)**. In lymphoid tissues, including spleen, thymus and bursa of Fabricius, multifocal areas of necrosis and apoptosis of variable intensity in mononuclear cells were present. Particularly, diffuse necrotic areas and widespread viral replication were present in the spleen of one chicken **(Figure 7 R/S)**. Several animals also presented multifocal areas of necrosis in respiratory and olfactory epithelial cells in the nasal cavity. Multifocal areas of necrosis in glandular cells with mixed inflammatory cell infiltration, muscular cell degeneration and necrosis of lymphoid tissue were detected in gizzard and proventriculus. In the liver, we detected focal areas of necrosis with mild distention of hepatic sinusoids. The remaining tissues (skin, pectoral muscle, kidney and intestines) presented mild necrotic and/or inflammatory lesions and few positive cells.

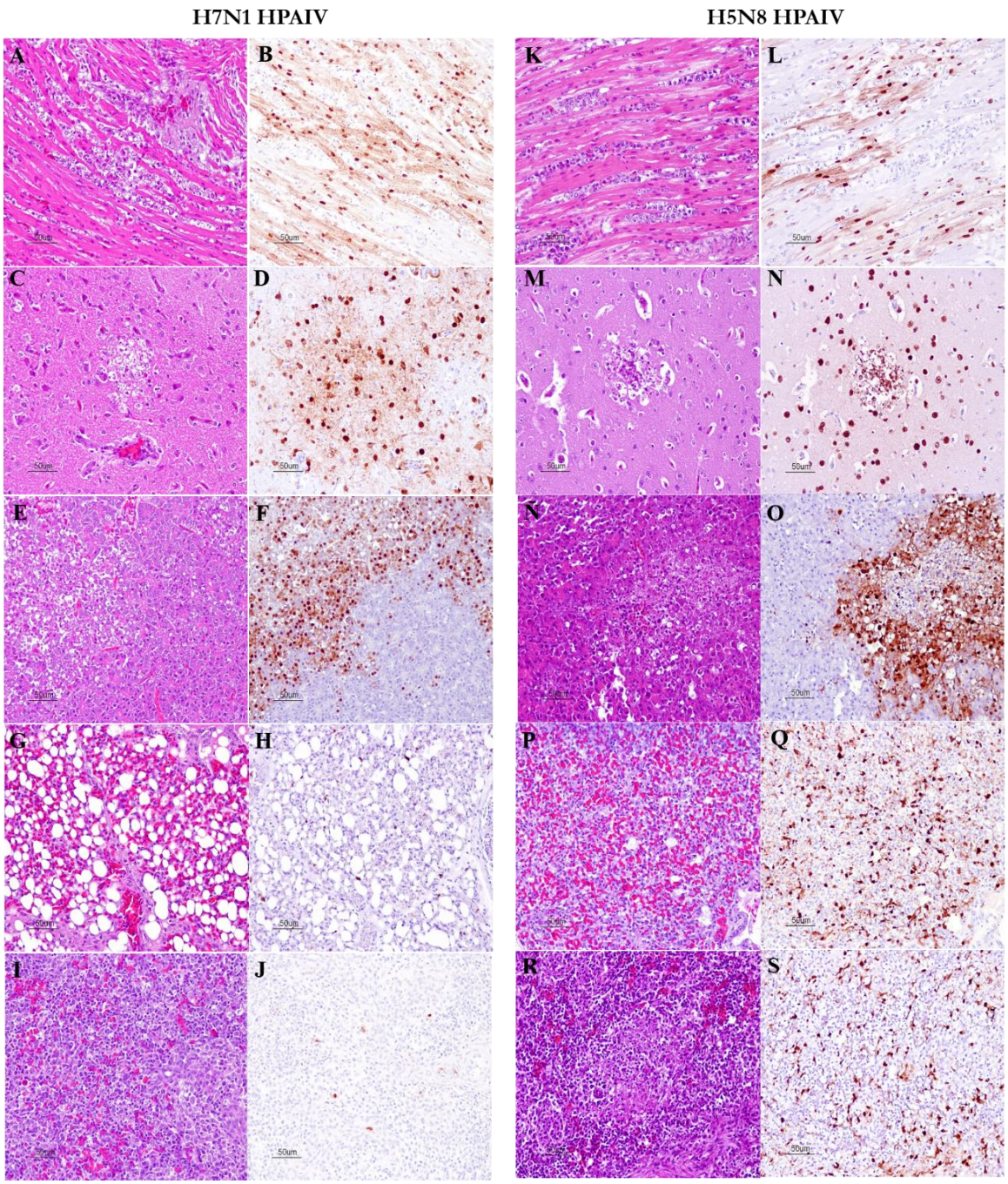


Figure 7. Microscopic lesions (HE staining) and viral replication (IHC staining) at 3 dpi in several organs obtained from chicken breeds experimentally inoculated with H7N1 and H5N8 HPAIVs. Myocardium A/B, K/L: multifocal necrosis of myocardiocytes with inflammatory infiltrate (A,K) and NP-positive myocardiocytes and inflammatory cells (B,L). CNS C/D, M/N: multifocal areas of necrosis in cerebral hemispheres (C,M), widespread NP-positive neurons and glial cells (D/N). Pancreas E/F, Ñ/O: diffuse area of necrosis in pancreatic acinar cells (E/Ñ) associated with widespread NP-positive cells in necrotic areas and surrounding acinar pancreatic cells (F/O). Lung G/H, P/Q: mild increase of increase of cellularity (mixed inflammatory cells) in air capillaries interstitium (G) and few NP-positive inflammatory cells (H). Severe increase of increase of cellularity in air capillaries interstitium, focal areas of necrosis in pneumocytes, microthrombosis and diffuse oedema (P), widespread NP-positive cells in inflammatory cells, endothelial cells and air capillary cells (Q). Spleen I/J, R/S: non-apparent lesions (I) and few NP-positive lymphoid-cells (J). Areas of necrosis with mixed inflammatory cell infiltration (R), widespread NP-cells in inflammatory and endothelial cells (S).

Viral shedding

Differences in the viral shedding between H7N1 and H5N8 HPAIV-inoculated chickens were detected **(Figure 8).** High viral excretion by both oropharyngeal and cloacal routes was detected in chickens inoculated with H7N1 HPAIV. At 1 dpi, viral RNA was detected in several OS but not in CS. The peak of shedding by means of proportion of positive swabs and levels of viral RNA occurred at 3 dpi. By 6 dpi, few birds presented detectable levels of virus RNA in OS and CS, but the levels detected were similar with those collected at 3 dpi. No viral RNA was present in the OS and CS collected at 10 dpi.

Regarding H5N8 HPAIV-inoculated groups, a low number OS presented detectable levels of viral RNA at 1 dpi. The proportion of positive OS and levels of viral RNA peaked at 3 dpi. In contrast, a low number of birds inoculated with H5N8 HPAIV presented cloacal shedding at the different dpi tested. However, the levels of viral RNA in the positive CS at 3 dpi were similar to those present in OS. By 6 and 10 dpi, viral shedding was still detected in several birds by both the oral and cloacal routes and in some samples, the levels were high.

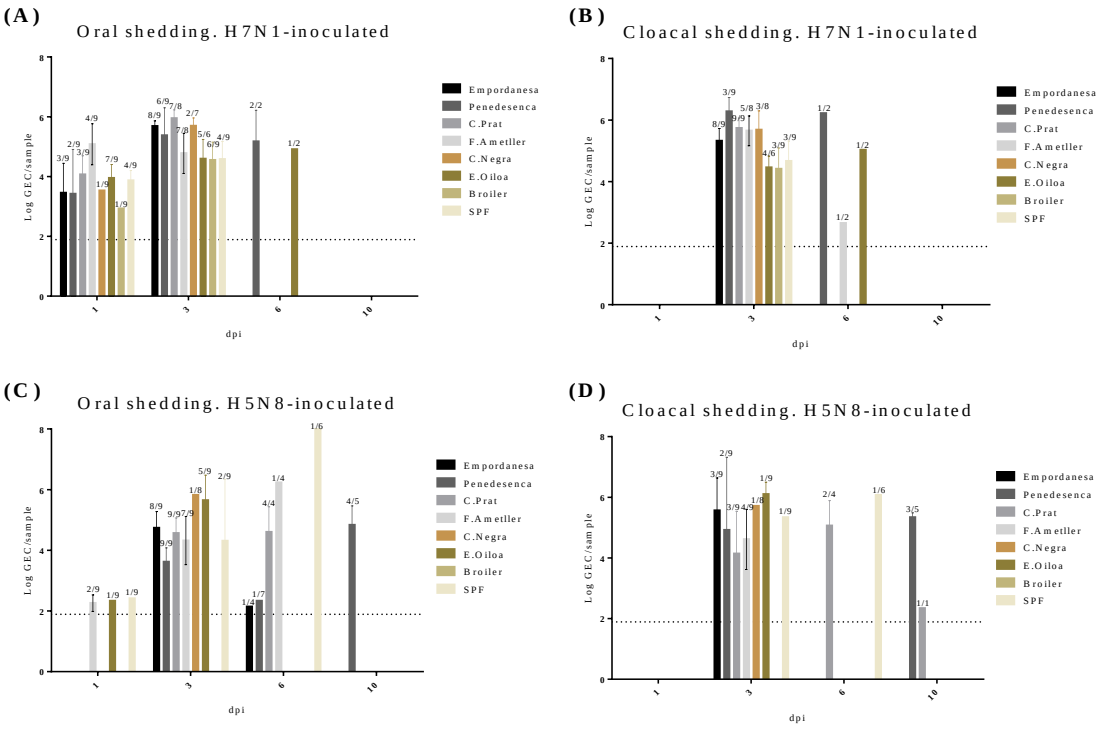


Figure 8. Viral titers expressed as log GEC in OS and CS obtained from chickens inoculated with H7N1 (A, B) or H5N8 (C, D) HPAIVs at different time points. Viral titers are represented as the mean values ± SEM. The numbers above the columns represent the number of chickens shedding virus out of the total sampled. GEC: Genome equivalent copies; Dpi: day post-infection.

Despite the levels of viral RNA in OS and CS obtained through the experiment were quantitatively similar in all the chicken breeds included in the study, a higher proportion of chickens of the *Empordanesa, Penedesenca, Catalana del Prat, Flor d'Ametller and Euskal Oiloa* breeds excreted virus by oral and cloacal routes in both viral infections than those belonging to *Castellana negra*, Broiler and SPF breeds.

Amino acid characterization

High amino acid identity between H7N1 and H5N8 HPAIVs was found in PB2, PB1, PA, NP and MP proteins, but not in NS protein **(Table 12).** Both HPAIVs present amino acids in particular positions associated to virulence and/or transmission of HPAIVs in chickens and ducks (PB2: 123E; PB1: 3V, 38Y, 436Y; PA: 237E, 383D, 515T, 672L; NP: 105V, 184K; M1: 43M; NS: 106M, 125D, 149A) [313, 445, 446, 573, 579, 581, 587–589, 597, 598, 603, 607–610]. Only 5 differing amino acids between the proteins sequence of H7N1 and H5N8 HPAIVs associated with a biological function were detected, and all were found in H5N8 HPAIV. This virus presented 375N and 42S-55E-103F in PB1 and NS1 proteins, respectively, which are associated with increased virulence and/or inhibition of host immune response in mammals [21, 576, 605–607], and 103F and 114S in NS1 that produce the inhibition of host gene expression in chicken cells [608].

Protein	Aa Id (%)	Differing amino acids (H5N8 vs H7N1)
PB2	98,9	K80R, M90I, N127H, I255V, S286G, V451I, R508M, R555K
PB1	98,5	A110T, G154D, R168K, S216G, G261S, T374N, **N375S**, K586R, V606I, S694N, K745E
PA	98,6	G59E, I61T, H96N, N115K, N184S, E252K, L261M, S453C, I459M, Y503F
NP	99,2	I201V, T350A, S377N, S403A
M1	98	V33A, L144F, I165M, N207S, R230K
M2	98,9	V50I
NS1	67,3	M6I, L7T, F14Y, Y17H, V18I, R21K, F22L, A23S, D24M, Q25R, E26D, G28C, L33D, **S42A**, R44K, N48S, G53D, I54C, **E55R,** T56V, R59M, A60E, Q63K, R67D, E70K, E71S, S73T, A76N, M79I, T80A, V81I, S86A, S87P, L90I, T94S, L95I, M98I, D101E, **F103Y**, K108R, A112T, **S114G,** C116M, I117V, N127R, V129I, V136I, I137L, R140Q, A143T, I145V, L146S, E153D, G158A, L163I, F166M, T170S, D171T, V180I, T191S, V192I, V194A, T197N, L198I, R204G, N205I, S206R, N207D, D209N, R211G, S213P
NS2	81,8	M6I, L7T, M14Q, G22E, E26V, G36E, S37R, L40I, Y48S, G63A, K64T, E67D, Q68E, G70S, E81A, V83C, H85N, R86I, K88T, I89K, M100L, Q111S

Table 12. Amino acid identity and differing amino acids in the viral proteins sequence between A/Goose/Spain/IA17CR02699/2017 (H5N8) and A/Chicken/Italy/5093/1999 (H7N1) HPAIVs. The amino acids previously associated with a phenotype are highlighted in black..

Polymorphism at position 2032 of Mx gene and association with infection outcome

We found wide differences regarding genotype and allele distribution at position 2032 of chicken Mx gene in the breeds included in this study **(Table 10)**. The three genotypes AA, AG, GG were present in *Empordanesa, Penedesenca, Catalana del Prat and Castellana negra* breeds with variable frequency. The heterozygous genotype (AG) was the predominant in *Empordanesa and Catalana del Prat* breeds. *Penedesenca* presented a higher frequency of the homozygous-resistant genotype (AA), whereas *Castellana negra* had more frequently the homozygous-susceptible genotype (GG). Almost all *Flor d'Ametller* and *Euskal Oiloa* chickens presented the homozygous-susceptible genotype (0.094 and 0.923, respectively), and any the resistant genotype. SPF chickens presented predominantly the homozygous-resistant genotype (AA), and in a minor amount the heterozygous one, but not the homozygous-susceptible one. Broiler chickens were fixed for the homozygous-susceptible genotype (GG). Overall, the average genotype frequency of the susceptible genotype GG (0.50) was higher than AG (0.29) and AA (0.21) genotypes. The A allele was present in all the chicken breeds except in Broilers, but the frequency varied from 0.030 and 0.038 in *Flor d'Ametller* and *Euskal oiloa* breeds, respectively, to 0.903 in SPF chickens. G allele was present in all the chicken breeds. In this case, the range of frequencies varied in the interval of 1.00 present in Broiler chickens to 0.097 in SPF chickens. G allele (0.65) was more predominant in the chicken population tested than A allele (0.36).

In order to study the association of Mx genotypes at position 2032 with percentage of survival at the end of the study and mean days of death in the birds that succumbed to infection, statistical analyses were performed. The differences in the proportion of dead birds were not statistically significant among genotypes. However, the Kruskal-Wallis test indicated statistically significant differences in the MDTs among genotype groups (p.value=$6.3x10^{-5}$). Post-hoc pairwise using the Dunn's test with Bonferroni correction indicates differences were statistically significant between groups AA and GG (5 *versus* 3.6 dpi, p=0.0015), and between groups AG and GG (4.7 *versus* 3.6 dpi, p=0.0006).

Breed	Genotype frequency			Allele frequency	
	AA	AG	GG	A	G
Empordanesa	0.129	0.452	0.419	0.355	0.645
Penedesenca	0.485	0.364	0.152	0.667	0.333
Catalana del Prat	0.156	0.750	0.094	0.531	0.469
Flor d'Ametller	0.000	0.061	0.939	0.030	0.970
Castellana negra	0.100	0.400	0.500	0.300	0.700
Euskal oiloa	0.000	0.077	0.923	0.038	0.962
Broiler	0.000	0.000	1.000	0.000	1.000
SPF	0.806	0.194	0.000	0.903	0.097

Table 13. Genotype AA, AG and GG and allele frequencies of A and G alleles in exon 14, position 2032 of chicken Mx gene in different chicken breeds. AA, resistant genotype; AG, heterozygous genotype; GG, susceptible genotype.

4.4. DISCUSSION

Available data demonstrates that the susceptibility to HPAIVs varies largely depending of the viral isolate and the genetic background of the host. In order to evaluate the existence of viral- and host-dependent differences in HPAIV infections in chickens, we selected a classical HPAIV (H7N1 isolated from a chicken in Italy) and a recent HPAIV of the Gs/GD H5 lineage (H5N8 Gs/GD clade 2.3.4.4 group B isolated from a domestic goose in Spain) and assessed their pathobiology in a broad spectrum of chicken breeds from Spain (local and commercial breeds).

Both HPAIVs used in this study were highly virulent for chickens, as expected based on the presence of a MBCs site in the HA protein and demonstrated experimentally by the severe clinical signs and fatal outcomes observed through the experiment. However, H7N1 and H5N8 HPAIVs differed in the progression of the disease they caused in chickens. With the highest frequency of prostration and neurological signs, highest mortality rates and shortest MDT, H7N1 HPAIV is considerably more virulent for chickens than H5N8 HPAIV. The viral shedding pattern also varied between H7N1 and H5N8 HPAIVs. The differences in oral excretion between H7N1 and H5N8-inoculated chickens were minor, with both groups presenting a similar number of birds shedding virus (70% and 58% by 3 dpi, respectively) and mean levels of viral RNA in OS (5.1 and 4.6 by 3 dpi, respectively). However, a low number of chickens inoculated with H5N8 HPAIV presented cloacal excretion. Despite some birds

were still shedding at the end of the study, our findings suggest the potential for decreased horizontal transmission efficiency of Gs/GD H5N8 clade 2.3.4.4 B HPAIV among chickens. In concordance with our results, previous studies demonstrate that Italian H7N1 HPAIV exhibit high virulence and transmissibility in several galliformes species [400, 534, 628], whereas Gs/GD clade 2.3.4.4 H5Nx reassortants (including H5N8 HPAIVs) generally cause lower mortalities, longer MDTs and present lower transmissibility in chickens in comparison with the ancestral Gs/GD H5N1 HPAIVs [334–338]. These results confirm the variable pathogenicity and potential transmissibility of HPAIVs of different lineages and host-origin in the chicken species.

The comparatively lower frequency of clinical signs and mortality, longer MDT and reduced excretion in H5N8 HPAIV-inoculated chickens suggest a suboptimal affinity and/or adaptation of H5N8 HPAIV to chickens. This could have partially contributed to the limited number of H5N8 HPAIV outbreaks in chicken holdings during the 2016-2017 epidemics in Europe (11 % of the total reported outbreaks), in comparison with its detections in waterfowl holdings, including in Spain [71]. However, since different genotypes of H5N8 HPAIV circulated in Europe at that time [617], important differences in the biological properties of H5N8 HPAIV between European strains may exist. The production characteristics of this species could also be an important factor. Migratory wild birds are thought to have played a pivotal role in the worldwide dissemination of Gs/GD H5N8 HPAIVs. Since chicken production in Europe in mostly intensive and farms present high biosecurity standards, the low detection in chicken holdings during the 2016/2017 epidemics may also be due to a low exposure to the virus.

Despite the different course of infection caused by H7N1 and H5N8 HPAIVs, the clinical signs and macroscopic lesions in the severely affected chickens were similar and consistent with HPAIV infection. The viral antigen detected in mostly all collected organs in H7N1 and H5N8 HPAIVs-inoculated chickens that succumbed to infection demonstrate the widespread dissemination of both HPAIVs. However, the intensity of replication and associated microscopic lesions in the different tissues were virus-dependent, indicating differences in their tissue tropism. Chickens inoculated with H7N1 presented severe lesions and high viral replication in heart, central nervous system and pancreas. In addition to those observed in H7N1-inoculated birds, inflammatory and necrotizing lesions associated to intense viral replication were detected in the lungs and primary lymphoid organs collected from chickens inoculated with H5N8 HPAIV. Widespread staining was generally detected in lymphocytes in these tissues, indicating a high avidity of H5N8 HPAIV for lymphoid-cell populations. The cause of death in the chickens that succumbed to infection after H7N1 and H5N8 HPAIV inoculation appear to be the result of the multi-organ replication of these viruses. However, H5N8 HPAIV presents a

reduced neurotropism, a hallmark of HPAIV pathogenesis, based on the comparatively lower amounts of viral antigen and lesions detected in the brain of H5N8 HPAIV-inoculated chickens in comparison with those inoculated with H7N1 HPAIV. The reduced neurotropism could be a reason of the lower mortalities caused by H5N8 HPAIV. Other mechanisms of pathogenicity not evaluated in the present study may also impact the differences in virulence between H7N1 and H5N8 HPAIVs in chickens, including an aberrant innate immune response in H7N1 HPAIV-inoculated chickens after infection [308].

Particular amino acids in specific positions of PB2, PB1, PA, NP, MP and NS proteins sequence have been associated with increased pathogenicity and transmissibility of HPAIVs in chickens and/or ducks [445, 446, 629]. Even closely related HPAIV isolates differ in their virulence in chickens, indicating that point or few mutations in the viral genome may produce significant biological effects [464]. Whereas there is no evidence of sustained circulation of Spanish H5N8 HPAIV in galliformes species, the Italian H7N1 HPAIV emerged from a LPAIV precursor that had been circulating in gallinaceous poultry for several months [630]. Therefore, H7N1 HPAIV may present markers of adaptation and/or virulence in internal and non-structural proteins that are lacking in H5N8 HPAIV. With the exception of NS protein, the amino acid identity in internal and non-structural proteins was high (>98%). Several amino acid substitutions associated with increased virulence of HPAIVs in chickens or chicken-derived cells were present in PB2, PB1, PA, NP, M1 and NS1 proteins in both H7N1 and H5N8 HPAIVs [445, 446, 573, 579, 589, 597, 598, 603, 608]. Only two differing amino acids in the sequence of the proteins between H7N1 and H5N8 HPAIVs have been reported to have a biological effect in the chicken species: 103F and 114S that are present in NS1 protein of H5N8 HPAIV. These mutations are associated with inhibition of host gene expression [608]. However, they were reported in a LPAIV strain and their effect *in vivo* was not evaluated. Therefore, the differences in infection outcome between the two HPAIVs may be due to a single or a combination of amino acid substitutions whose effects have not been yet characterized. Since they belong to different subtypes, the HA and NA surface glycoproteins are also expected to have played a critical role in the differential outcomes. Actually, we detected that H7N1 HPAIV presented an amino acid substitution in HA (388T, H5 numbering) and NA proteins (401T, N2 numbering) which are associated with increased pathogenicity in chickens and increased binding to a2,3 SA, respectively [573, 631], and are not present in H5N8 HPAIV (data not shown). However, the biological implications of the amino acid substitutions reported here require further evaluation.

The outcome after infection with HPAIVs is also largely influenced by host factors. Several reports demonstrate a wide range of susceptibilities to AIVs among breeds and lines of chickens. Specifically, significant variations in mortalities after experimental inoculation with Gs/GD H5N1, H5N6, H5N8

HPAIVs [338, 412, 416, 462–465] and the Italian HPAIV H7N1 [399, 461] have been reported. We then evaluated the existence of breed-related differences in the susceptibility to HPAIVs in a broad range of local chicken breeds, and in two commercial breeds. In the present study, four breeds (*Empordanesa, Penedesenca, Catalana del Prat, Flor d'Ametller*, *Euskal Oiloa*) were highly susceptible to HPAIV infection, whereas three breeds (*Castellana negra*, Broiler and SPF chickens) were considerably more resistant. The breeds that were more resistant exhibited less frequency of severe clinical signs, lower mortality rates and lower number of animals shedding virus to both HPAIVs infections than susceptible breeds, demonstrating that the genetic background of particular chicken breeds confer a higher natural resistance to diverse HPAIVs subtypes. Moreover, two of the highly susceptible breeds presented a higher incidence of cutaneous edema and nervous signs, suggesting that the clinical presentation may vary to some extent dependent of the breed.

Local chicken breeds are believed to be more resistant to disease as a result of the natural selection by autochthonous pathogens and minor artificial selection towards productive-associated genes [467]. However, almost all the local chicken breeds included in our study were highly susceptible to both HPAIVs. This is in concordance with previous reports [462, 465], demonstrating that local breeds do not necessarily present an improved resistance to infectious diseases. The high susceptibility of the local chicken breeds included in our study is a concern; these breeds are usually raised in backyards in the absence or little biosecurity measures. Since Spain is located within natural migratory routes between Eurasia and Africa, these particular breeds are expected to be highly vulnerable to infection with HPAIVs carried by migratory birds. Because of that, local chicken breeds could act as sentinels for HPAIV environmental contamination.

The genetics of resistance to HPAIVs remains unknown. However, particular alleles present in immune response-related genes have shown a positive correlation with antiviral activity [481, 488]. Mx proteins are induced by type 1 interferons and interfere with viral functions by inhibiting viral polymerases in the nucleus [480]. The substitution of serine with asparagine at position 631 of Mx protein, which is produced by a particular non-synonymous G/A polymorphism in exon 14 of chicken Mx gene, was associated with higher antiviral activity *in vitro* [481]. *In vitro* and *in vivo* studies do not always show a clear correlation between this allele and inhibition of AIV replication and/or survival after HPAIV infection, respectively [399, 461, 484, 485, 487]. Therefore, the impact of this particular amino acid substitution is still unclear. In the present study, we evaluated the genotype frequencies of that particular SNP in the different chicken breeds and evaluated their association with infection outcome. Similar as in other studies reporting high diversity in genotype and allele frequency in that position between breeds [632], we detected huge differences in the frequency of the three genotypes among the spectrum of local,

commercial and experimental breeds included in our study. As reported previously, White Leghorns and Broilers appear to be almost fixed for the resistant and susceptible genotype, respectively [482, 632, 633]. In contrast, the results in local breeds were more variable, which could be associated with the higher genetic diversity generally present in unselected breeds. Overall, the susceptible G allele was more prevalent in Spanish chickens, while the resistant A allele prevails in chickens from India [634], Indonesia [635] and Egypt [636]. The statistical analyses showed that the different genotypes in the target Mx region were not associated with significant differences in mortality ratios. However, the birds carrying the AA and AG presented a statistically significant longer MDT than those carrying the GG genotype. In concordance with our results, the study carried out by Ewald *et al.* [486] observed that chickens homozygous for GG allele presented a delayed MDT. These suggest that the presence of an asparagine at position 631 in Mx protein may result in a higher antiviral effect response of Mx protein against HPAIVs, but, as shown in our studies, the biological implications of this change *in vivo* are probably limited.

This study represents an exhaustive characterization of the pathobiology of two HPAIVs in a broad range of chicken breeds. Our results demonstrate that the outcome after infection with HPAIVs is influenced by numerous tightly interconnected factors, including the viral isolate, the genetic background of the breed and particular alleles in genes encoding antiviral proteins, underlining the complexity of HPAIV infections. A proper surveillance and education of caretakers in commercial but also in local chicken holdings are required to early detect the circulation of HPAIVs in the territory.

CHAPTER 5

STUDY III: Experimental infection of domestic geese (*Anser anser var. domesticus*) with highly pathogenic avian influenza viruses H7N1 and H5N8 reveals large differences in their virulence and potential transmissibility

5.1. INTRODUCTION

The isolation of HPAIVs in waterbirds has been more sporadic than in galliformes species. With the exception for A/tern/South Africa/1961 (H5N3) [18, 19], evident data indicating that HPAIVs could be maintained or cause extensive mortality in waterbird populations is lacking. However, since the emergence of the Gs/GD H5 lineage of HPAIVs, the role of wild and domestic waterfowl in the epidemiology of HPAIVs has radically changed. Domestic waterfowl, especially ducks and geese, has been fundamental in the emergence, perpetuation, genetic evolution and transmission of Gs/GD H5 HPAIVs [53–55, 243, 637]. During their evolution, Gs/GD H5 HPAIVs have also acquired altered pathobiological characteristics for waterfowl species. In comparison with the resistance or minimal susceptibility of domestic waterfowl to most HPAIVs in terms of clinical disease [193, 279, 285, 286, 330, 331, 340–343], infection with Gs/GD H5 HPAIVs may result lethal in these species. The clinical outcome ranges from subclinical to severe (100% mortalities) depending on viral factors, including the clade, genetic group and particular mutations in viral gene segments, and host factors, such as the species and age at infection [364, 638–640].

Despite chicken farming is the leading producer in poultry sector with approximately the 90% of world poultry meat and egg production, rearing minor avian species such as domestic waterfowl also represents a significant part of the national agriculture in different countries of the world [29]. Descendants of the wild Greylag goose (*Anser anser,* Western breeds) and the Swan goose (*Anser cygnoidse,* Eastern breeds), geese were one of the first birds to be domesticated [641]. Used as a multi-purpose poultry species, most goose breeds are raised for their meat, but also for feathers, down and fatty livers, in several production systems that range from backyards to specialized commercial farms. Overall, production of domestic geese is economically important in China and Central Europe [642]. Moreover, local breeds of domestic geese are used for controlling weeds in several crops, kept as guard birds or pets. As in ducks, the isolation of HPAIVs in domestic geese populations have been sporadic and generally associated to low mortalities [28], and the few studies performed in domestic geese indicate that classical lineages of HPAIVs are avirulent for this species under experimental conditions [333, 339]. In contrast, data clearly demonstrates that domestic geese have played a main role in the epidemiology of Gs/GD H5 HPAIVs [643], and the presence of geese on farms is considered a risk factor of Gs/GD H5 HPAIVs outbreaks in poultry flocks [644]. In addition, natural or experimental infections with several Gs/GD HPAIVs have produced severe disease in domestic geese, generally higher than in domestic ducks [352, 354–361].

During the 2016-2017 European epidemics caused by H5N8 clade 2.3.4.4 group B of Gs/GD lineage of HPAIVs, an unprecedented number of outbreaks were recorded in duck and geese holdings (up to 70% of reported outbreaks) [71]. This was not observed during previous Gs/GD H5 HPAIV epidemics, including the phylogenetically related H5N8 HPAIV belonging to Group A that caused several outbreaks in Europe in 2014/2015. The higher proportion of outbreaks in domestic waterfowl suggests that particularities of the production in these species are associated with higher probability to H5N8 Group B HPAIV infection. However, it could also reflect the acquisition of an increased affinity, tropism and/or virulence towards waterfowl species. Grund *et al.* [367] reported that the increased virulence of H5N8 Group B HPAIV to domestic ducks was associated with the neuro- and hepato-tropism characteristics of the virus. Despite domestic geese were the second domestic species most affected, the pathobiological features of H5N8 Group B HPAIV in this species have not been characterized to date.

Taking into consideration the large number of outbreaks in geese holdings caused by Gs/GD H5N8 clade 2.3.4.4 Group B HPAIV, the low isolation rates of HPAIVs others than those belonging to Gs/GD lineage in domestic geese, and the lack of direct comparison of different HPAIVs in this species, the aims of this study were to: 1) perform a profound investigation of the differential pathobiology of a H5N8 Gs/GD Group B and a classical H7N1 HPAIVs in domestic geese, and 2) evaluate the susceptibility and potential role of local and commercial breeds of geese in the epidemiology of these HPAIVs.

5.2. MATERIALS AND METHODS

Viruses

The viruses used in this study were: A/Chicken/Italy/5093/1999 (H7N1), isolated in 1999-2000 during an Italian epidemic that mainly affected Veneto and Lombardia regions (kindly provided by Dr. Ana Moreno from the *Instituto Zoopfifilattico Sperimentale della Lombardia e dell'Emilia Romagna*) and A/Goose/Spain/IA17CR02699/2017 (H5N8 clade 2.3.4.4. group B), isolated in Catalonia (Northeastern Spain) during the 2016/2017 European epizootics. Both viruses are highly pathogenic based on the aminoacid sequences at the HA0 cleavage site: PEIPKGSRVRR↓GLF (H7N1) and PLREKRRKR↓GLF (H5N8). Virus stocks were produced in 10 days-old SPF embryonated eggs. The

allantoic fluid was obtained at 24-48 hpi, filtered and aliquoted at -75ºC until use. Serial ten-fold dilutions of the filtered viruses in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean egg lethal doses (ELD_{50}) were determined by Reed and Muench method [624]. The consensus full genome sequences corresponding to the eight segments of H7N1 and H5N8 are available in Genbank under accession numbers: DQ991325.2 to DQ991332.2 (H7N1) and MK494920 to MK494927 (H5N8).

Animals and facilities

A total of 29 geese (*Anser anser var.domestica*) of approximately 3-5 months of age were used in this study. Two breeds were included: 18 birds of the *Empordanesa* breed, a local geese breed present in backyards in Spain alone or mixed with other domestic avian species, and 11 G35-line geese, which is a commercial breed raised in specialized farms. At arrival, the birds were individually identified and placed in different negative-pressured HEPA-filtered boxes present in BSL-3 facilities in *Centre de Recerca en Sanitat Animal* (*Programa de Sanitat Animal*, IRTA). Water pools under the minimum size required by the Spanish Royal Decree 53/2013, which lays down the basic obligations and general principles concerning the animal protection in experimentation, were included in the boxes **(Figure 9)**. The birds were kept 5 days for acclimation. Prior to infection, serum samples were obtained from all birds to ensure that they were seronegative to IAVs by a cELISA (ID-VET, Montpellier, France). Furthermore, OS and CS were collected from all birds and confirmed to be negative to AIV by one-step qRT-PCR. During the experimental procedures, food and water were provided *ad libitum*. The experimental design was approved by the ethical commission of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) and the Government of Catalonia (*Departament de Territori i Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural*) under reference code CEEA 57/2017- 10185.

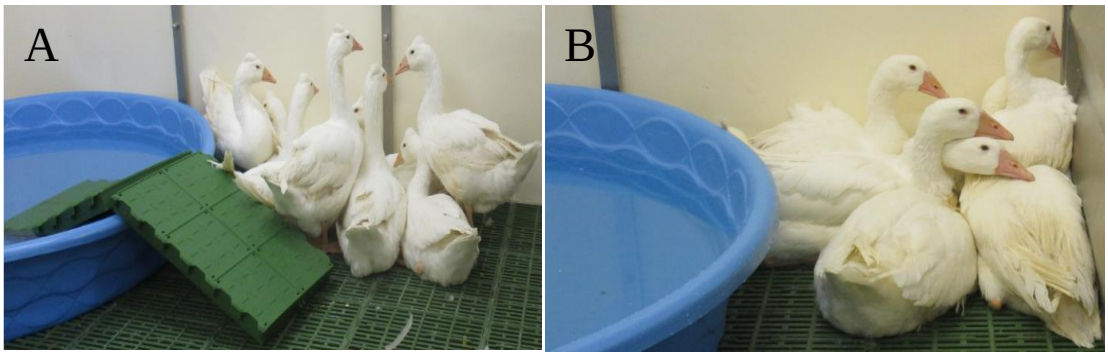


Figure 9. Water pools installed in the HEPA-filtered boxes containing local (A) and commercial (B) domestic geese.

Experimental design and sampling

29 geese were randomly separated into four challenged groups. For each HPAIV (H7N1 and H5N8), 5 commercial and 8 local geese were challenged via the intranasal route. The viruses were 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). 1 commercial and 2 local geese used as negative control birds, and due to space limitations, they were euthanized prior to infection in order to collect samples (as described below).

All birds were monitored daily for clinical signs until 15 dpi. A standardized OIE clinical scoring system was used [140]. Moribund geese were anesthetized using ketamine/xylazine (20 mg/kg body weight, Imalgene 100 and 0,3 mg/kg body weight, Rompun 20 mg/ml) via the intramuscular route, euthanized with intravenous pentobarbital (140 mg/kg body weight, Euthasol 400 mg/ml) and scored as dead. The clinical signs, mortality and MDT were recorded for each virus and breed.

Programmed necropsies were performed at 4 dpi and at the end of the study (15 dpi) in order to evaluate gross lesions and collect tissues for pathological studies and viral detection and quantification. 3 birds (2 local and 1 commercial) inoculated with H7N1 and H5N8 HPAIVs were randomly selected and sacrificed at 4 dpi. At the end of the study, 2 geese of each breed were necropsied. All birds found dead as well as those euthanized for ethical reasons during the experiment were included. In order to evaluate viral shedding, OS and CS were obtained from all birds at 1, 3, 6 and 10 dpi. 1,5 ml of pool water were collected from all pools at the same time points. Furthermore, approximately 0.75 ml of total blood in a 1:1 ratio with anticoagulant (Alsever's solution, Sigma-Aldrich, Missouri, USA) was extracted from the medial brachial vein of all geese at 3, 6 and 10 dpi. At the end of the study, serum samples were obtained from all survivor birds. All samples were appropriately conserved at -75ºC until further use.

Pathological examination and immunohistochemical testing

Tissues collected in necropsies were immersed in 10% formalin for fixation during 48 hours and embedded in paraffin wax. These samples included skin, thymus, ocular conjunctiva, pectoral muscle, nasal cavity, trachea, lung, central nervous system, heart, spleen, liver, kidney, proventriculus, gizzard, pancreas, duodenum, cecum, colon and bursa of Fabricius. Microtome sections of 3 µm of thickness (Leica RM2255, Nussloch, Germany) from FFPE tissues were processed, stained with H/E and then examined under light microscopy. An IHC technique was performed in the same tissues as described in Study II on serial sections of the tissues. The positivity in the tissues was semiquantitatively assessed taking into consideration the percentage of NP-positive and negative cells in the tissue. The samples

were classified as follows: no positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells (+++) in a tissue section. Positive and negative controls were used. The positive control was a central nervous system from a chicken experimentally infected with H7N1 HPAIV [400], and the negative control consisted in the same tissue incubated with PBS instead of the primary antibody the tissues collected from negative control geese.

AIV RNA detection and quantitation

Swabs were placed in 0.5 ml of sterile PBS enriched with Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Nystatin (Sigma-Aldrich, Missouri, USA) at a final concentration of 6%. Blood was centrifuged at 3100 revolutions per minute for 10 minutes and plasma was collected. Thin sections of spleen, central nervous system and lung were obtained during necropsies and placed in 1 ml of RNAlater Stabilization Solution (Invitrogen, Carlsbad, CA, USA). After overnight conservation at 4º, RNAlater was removed from samples and 30 mg of each tissue were weighted, homogenized in 400 µl of Nuclease-free water using a pestle, centrifuged for 3 minutes and the supernatant collected. Viral RNA was extracted from OS and CS, pool water, plasma and from homogenized RNAlater-stabilised tissues using Nucleospin RNA virus kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions. A highly conserved region of IAVs M1 gene 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 [521, 565]. To extrapolate the GEC present in the samples, the same standard curve obtained by amplification of M1 gene fragment used in Study II was included in the qRT-PCR reactions. The limit of detection of the technique was 1.89 log GEC in OS and CS, 2.37 log GEC in plasma and water and 1.79 log GEC in tissue samples.

Seroconversion

To evaluate seroconversion, serums were tested by a cELISA test that detects Abs against the NP of IAVs (ID Screen® Influenza A Antibody Competition Multi-species, ID-VET, Montpellier, France). The technique was performed following manufacturer's instructions.

5.3. RESULTS

Clinical signs and mortality

Clinical signs were only observed in H5N8 HPAIV-inoculated geese, being apathy and neurological signs the most frequently observed. Until 5 dpi, no evident clinical signs or mortality were recorded. At 5 dpi, one local goose was found dead without previous evident clinical signs. At 6 dpi, two geese (one local and one commercial) presented severe nervous signs, including ataxia and head shaking, and were consequently euthanized. Furthermore, two geese (one of each breed) were found dead without showing prior clinical signs. At 7 dpi, two local geese presented severe apathy and incoordination. The remaining geese showed mild apathy and tremor. At 9 dpi, one commercial goose was found dead. At 10 dpi, the remaining geese (one local and one commercial) were euthanized for ethical reasons, reaching a mortality of 100% in both breeds **(Figure 10).**

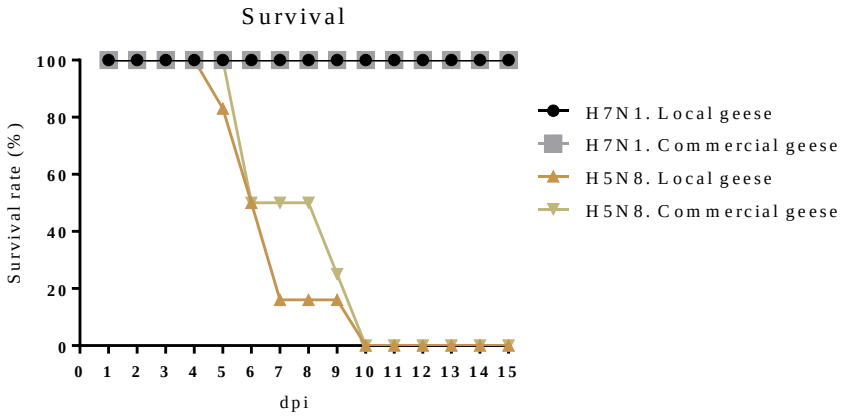


Figure 10. Survival curves of domestic geese experimentally inoculated with H7N1 o H5N8 HPAIVs at a dose of 10^5 ELD_{50}.

The MDT was 6,2 and 7 dpi in commercial and local geese, respectively. No differential susceptibility between local and commercial breeds to H5N8 HPAIV infection was present, considering mortality was 100% in both breeds and no major differences in MDT. Neither clinical signs nor mortality were recorded in H7N1 HPAIV-inoculated geese along the experimental period **(Figure 10).**

Gross lesions

Evident macroscopic lesions were only detected in the group of domestic geese inoculated with H5N8 HPAIV, being the pancreas the most affected organ. Macroscopic examination of H5N8 HPAIV-

inoculated geese serially euthanized at 4 dpi revealed moderate congestion in nasal turbinates and intestinal blood vessels. Moreover, two geese presented splenomegaly. However, evident lesions were not detected until 5 dpi, when the geese found dead presented multifocal areas of hemorrhages and necrosis in pancreas **(Figure 11A),** and tracheal congestion. Similar lesions in pancreas were observed in the necropsies performed in the severely-affected geese from 6 to 10 dpi. At 6 dpi, two geese also presented multifocal areas of necrosis in the liver associated with a moderate hepatomegaly. At 6 dpi, one geese presented multifocal petechiae in bursa of Fabricius, and another exhibited petechiae in gizzard. From 6 to 10 dpi, moderate to severe congestion in several organs, such as cecal tonsil, subcutaneous tissue and central nervous system, as well as necrotic areas in heart, were also frequently observed. At 10 dpi, one goose presented moderate friability of the liver, severe congestion of the intestinal mucosa and marked multifocal hemorrhages in central nervous system **(Figure 11B).**

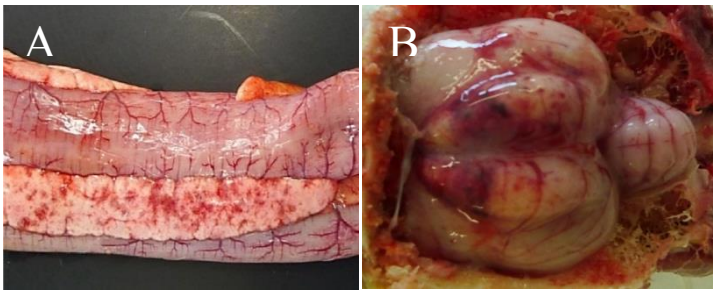


Figure 11. Diffuse hemorrhagic areas in pancreas (A) and central nervous system (B) found in geese experimentally inoculated with H5N8 HPAIV.

Regarding H7N1 HPAIV-inoculated geese, birds necropsied at 4 and 15 dpi presented non-specific gross lesions, including mild to moderate congestion in nasal turbinates and intestinal blood vessels. One goose also presented splenomegaly at 4 dpi.

Histopathological findings

Microscopic observation of tissues revealed evident lesions in mostly all H5N8 HPAIV-inoculated geese tissues, independently of the tested breed. The main microscopic findings were multifocal to diffuse areas of necrosis and hemorrhages associated with inflammatory cell infiltration of variable intensity. NP-positive cells in H5N8 HPAIV-inoculated geese were observed in mostly all collected tissues and correlated well with pathological findings. NP-positive staining was mostly detected in parenchymal cells, epithelial cells and inflammatory cells **(Table 14).**

Tissue	4 dpi		5 dpi		6 dpi		7 dpi		9 dpi		10 dpi		NP+ cells	Microscopic lesions
	L	C	L	C	L	C	L	C	L	C	L	C		
Central nervous system	-	-	+++	nd	+++	+++	+++	nd	nd	+	+	+	Neurons, glial cells, ependymal cells, Purkinje cells	Multifocal areas of necrosis, diffuse congestion, perivascular cuffing (10 dpi)
Pancreas	+	-	++	nd	++	+++	++	nd	nd	+	-	-	Acinar cells, macrophages	Multifocal areas of necrosis with inflammatory infiltrate
Liver	+	-	++	nd	++	++	++	nd	nd	+	-	-	Hepatocytes, Kupffer cells, macrophages	Multifocal areas of necrosis and hemorrhages with inflammatory infiltrate
Spleen	+	-	+	nd	++	++	++	nd	nd	+	-	-	Lymphoid cells, macrophages,	Multifocal areas of necrosis and hemorrhages with inflammatory infiltrate
Thymus	+	-	++	nd	++	+	++	nd	nd	+	-	-	Lymphoid cells, macrophages,	Multifocal areas of necrosis with inflammatory infiltrate
Heart	-	-	+	nd	+	++	+	nd	nd	+	-	-	Myocardyocytes	Multifocal areas of necrosis with inflammatory infiltrate
Skin	-	-	-	nd	+	+	-	nd	nd	-	-	-	Feather follicles	No apparent lesions
Nasal turbinates	+	-	+	nd	+	+	++	nd	nd	-	-	-	Respiratory epithelial cells, inflammatory cells	Diffuse congestion and edema with inflammatory infiltrate
Lung	+	-	+	nd	+	+	+	nd	nd	-	-	-	Macrophages	Diffuse congestion
B.Fabricius	+	-	+	nd	+	-	+	nd	nd	-	-	-	Lymphocytes, macrophages	Bursal depletion, focal hemorrhagic areas
Kidney	-	-	-	nd	+	-	+	nd	nd	-	-	-	Tubular epithelial cells, inflammatory cells	Congestion, focal hemorrhagic areas, focal necrosis of tubular cells
Proventriculus	-	-	-	nd	+	+	-	nd	nd	-	-	-	Epithelial cells of the gastric glands	No apparent lesions
Small intestine	-	-	-	nd	+	+	+	nd	nd	-	-	-	Lymphoid cells	No apparent lesions

Table 14. Average distribution of NP-positive cells and associated microscopic lesions in tissues collected from local and commercial geese experimentally inoculated with HPAIV H5N8.

No positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells, nd: not determined. L: local geese; C: commercial geese.

The most severely affected organ was the central nervous system, followed by pancreas, liver, spleen, thymus and heart. In central nervous system, non-suppurative encephalitis characterized by multifocal areas of necrosis, spongiosis of the neuropil, chromatolysis, karyolysis, gliosis and diffuse congestion associated to widespread AIV antigen were present in cerebral cortex at 5 dpi, which correlated with the onset of mortality. In the cerebellum, necrosis of Purkinje cells associated to AIV antigen was also observed. Severe lesions and high viral antigen were observed in central nervous system in all birds until 7 dpi, and declined by 9 dpi **(Figure 12A/B).** At 10 dpi, the geese presented perivascular cuffing in brain, and was the only tissue that presented AIV-positive cells. Acinar cells lytic necrosis in pancreas was observed in nearly all geese, in association with low (4 dpi) to intense (6 dpi) AIV antigen in necrotic areas and surrounding acinar pancreatic cells **(Figure 12C/D).** Starting at 4 dpi and peaking at 6-7 dpi, severe multifocal areas of lytic necrosis and hemorrhages associated to widespread viral antigen were detected in liver **(Figure 12E/F).** In thymus and spleen, multifocal areas of necrosis and inflammatory infiltrate as well as moderate amounts of viral antigen were present from 4 to 9 dpi, reaching the maximum levels by 6-7 dpi **(Figure 12G/H and I/J, respectively)**. In heart, multifocal areas of degenerated and/or necrotic myocytes and mononuclear cell infiltration associated to low-moderate amounts of AI antigen were present from 5 to 9 dpi **(Figure 12K/L).** Mild focal areas of necrosis and hemorrhages associated to low viral antigen were detected in other organs from 4 to 7 dpi, including kidney and bursa of Fabricius. In nasal cavity and lung, diffuse congestion associated to low amounts of viral antigen were also present. Single positive cells without evident microscopic lesions were detected in proventriculus and lamina propria of small intestine at 6 dpi and in 6-7 dpi, respectively. In general, higher amounts of AIV-positive cells were present in H5N8 HPAIV-inoculated local geese than in the commercial breed. No evident microscopic lesions or AIV-antigen positive cells were observed in H7N1 HPAIV-inoculated or negative control geese.

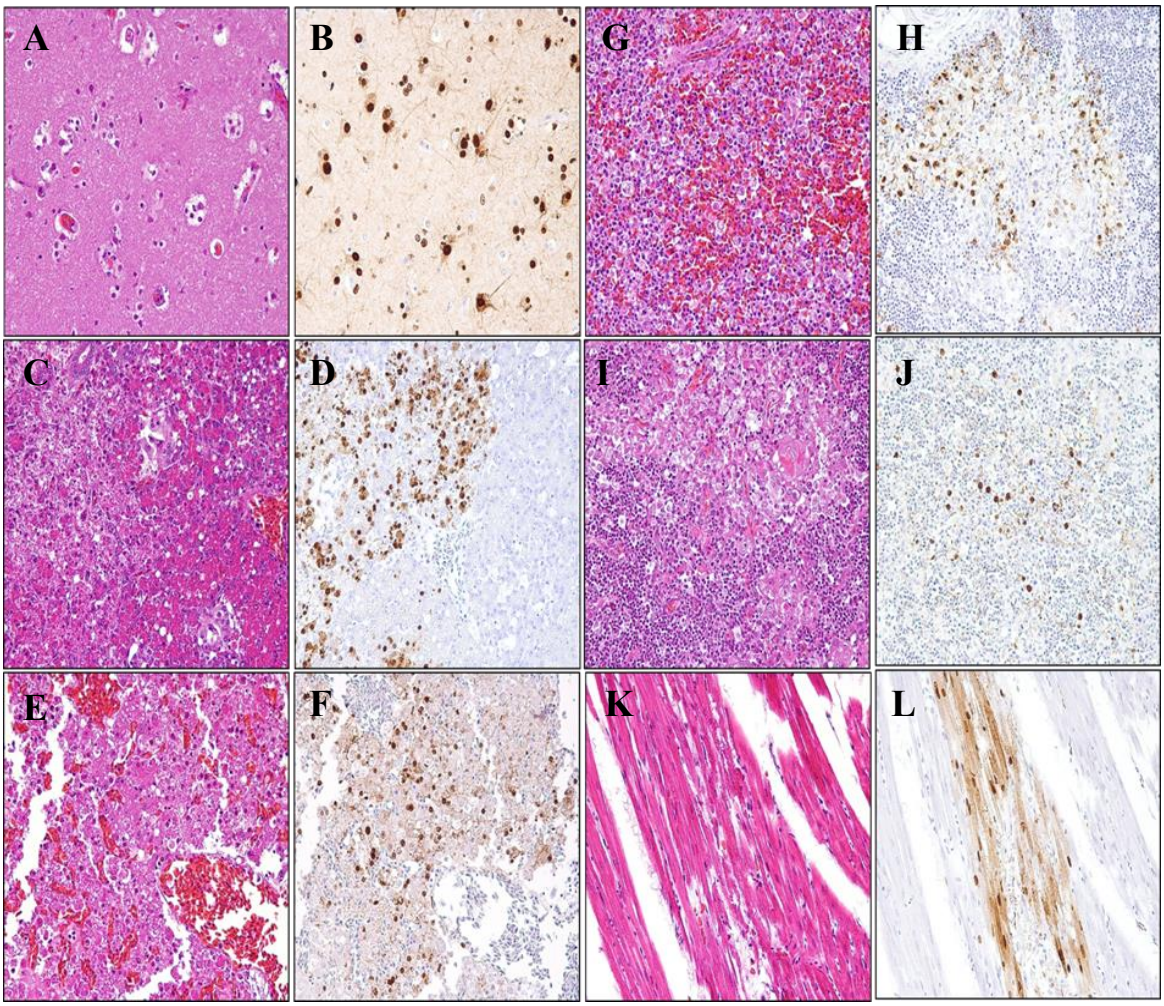


Figure 12. Serial sections of different organs of geese experimentally inoculated with H5N8 HPAIV stained with conventional HE staining and IHC techniques against NP nucleoprotein, respectively (20x). CNS A/B: diffuse areas of spongiosis and gliosis of cerebral parenchyma (A) and NP-positive neurons and glial cells (B). Pancreas C/D: diffuse areas of lytic necrosis of acinar pancreatic cells (C) and NP-positive cells in necrotic areas and surrounding acinar pancreatic cells (D). Liver E/F: multifocal areas of necrosis in liver parenchyma (E) and diffuse NP-positive hepatocytes, Kupfer cells and inflammatory cells (F). Spleen G/H: multifocal areas of mild necrosis and diffuse congestion (G) and presence of inflammatory cells positive to NP (H). Thymus I/J: areas of necrosis in medulla (I) and NP-positive lymphoid cells (J). Myocardium K/L: multifocal necrosis of myocardiocytes with mild inflammatory infiltrate (K) and NP-positive myocardiocytes and inflammatory cells (L).

Viral shedding

High viral RNA excretion was present in H5N8 HPAIV-inoculated geese in both OS and CS from 3 dpi to 6 dpi **(Figure 13A-B).** Virus was firstly detected at 1 dpi only in OS from two commercial geese. At 3 dpi, moderate levels of viral RNA were present in nearly all local and commercial geese in both OS and CS samples. At 6 dpi, all birds presented viral RNA from OS and CS. At that day, viral RNA reached the maximum levels in OS in both breeds, and it was maintained at levels similar as in 3 dpi in CS also in both breeds. At 10 dpi, only one local goose presented detectable levels of viral RNA.

Regarding H7N1 HPAIV-inoculated geese, a very low number of birds presented viral shedding and it was principally restricted to samples from OS **(Figure 13C-D).** At 3 dpi, one local goose presented high levels of viral RNA from OS and in lower amounts from CS. At 6 dpi, the same local goose and one commercial goose tested positive to viral RNA only from OS. Lastly, at 10 dpi, viral RNA was detected again only from OS in one local goose and in two commercial geese. The local and one commercial geese positive at 10 dpi were negative the previous days of sampling. We did not detect differences concerning viral shedding between local and commercial geese neither in H5N8 nor H7N1 HPAIVs -inoculated geese.

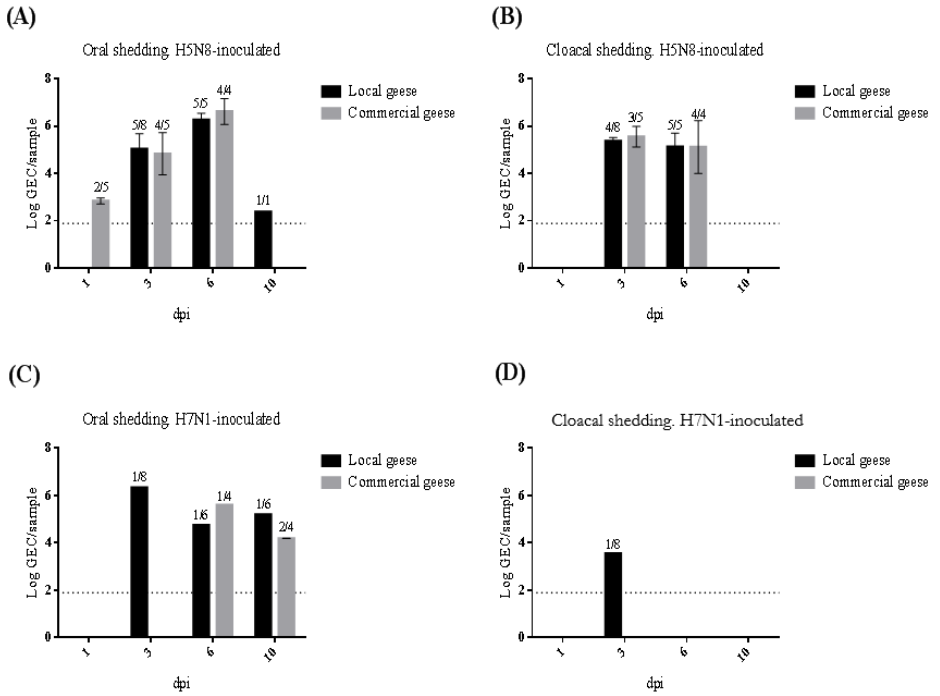


Figure 13. Viral titers expressed as log GEC in OS and CS obtained from domestic geese (local and commercial) inoculated with H5N8 (A, B) or H7N1 (C, D) HPAIVs at different time points post-inoculation. The ratios above the columns represent the number of birds shedding virus out of the total sampled. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

Viral RNA in pool water was detected in both enclosures of H5N8 and H7N1 HPAIVs experimental groups. Viral RNA in the case of H5N8 HPAIV-inoculated groups was firstly detected at 6 dpi in both local (4,37 log CGE) and commercial geese (4,33 log CGE) groups, and the levels were constant until 10 dpi (4,28 log CGE, 4 log CGE, respectively). In contrast, viral RNA in the case of H7N1 HPAIV-challenged group was only present at 10 dpi in local geese, and was near to undetectable levels (2,49 log CGE).

Viral RNA in plasma

High levels of viral RNA were detected in plasma from almost all H5N8 HPAIV-inoculated local and commercial geese at 3 dpi, and all were positive at 6 dpi **(Figure 14A)**. In general, the levels in plasma correlated well with levels presented in swabs. At 10 dpi, no viral RNA was detected in plasma. Viral RNA in plasma of H7N1 HPAIV-inoculated geese was only detected in one local goose from 3 to 6 dpi and in one commercial goose at 3 dpi. The levels of viral RNA were generally lower and the decay was considerably faster than in H5N8 HPAIV-inoculated geese **(Figure 14B)**. We did not detect differences concerning viral RNA in plasma between local and commercial geese neither in H5N8 nor in H7N1 HPAIV-inoculated geese.

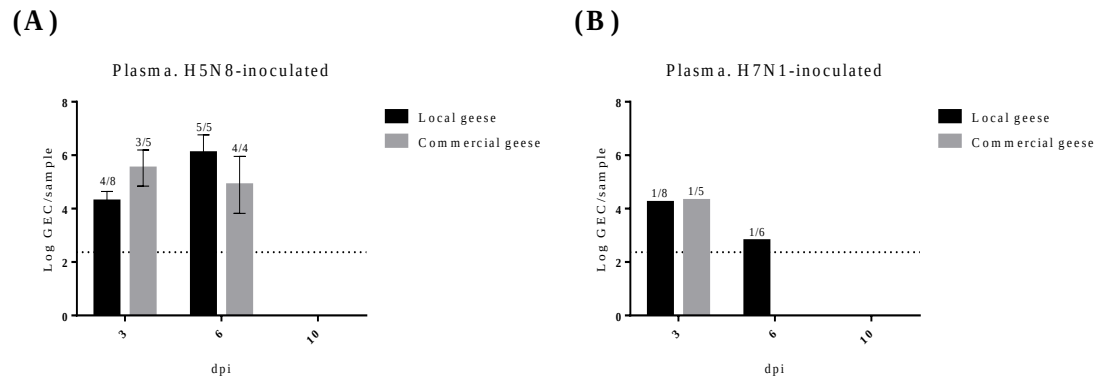


Figure 14. Viral titers expressed as log GEC in plasma obtained from domestic geese (local and commercial) inoculated with H5N8 (A) or H7N1 (B) HPAIVs at different time points post-inoculation. The ratios above the columns represent the number of birds showing viremia out of the total sampled. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

Viral RNA in tissues

Since we did not found evident differences in mortality and in the main organs affected between local and commercial geese, the quantitation of viral RNA in brain, spleen and lung in both breeds were grouped. In H5N8 HPAIV-inoculated groups **(Figure 15A)**, high amounts of viral RNA were detected in the spleen and lung at 4 dpi in all geese euthanized for pathological purpose. Low levels were present at that time in brain. Viral RNA in brain notably increased in all naturally-dead and severely-affected geese from 5 to 10 dpi, reaching titers higher than 9 log CGE at 6, 7 and 9 dpi. At 10 dpi, high levels of virus were still detected in brain samples. Viral RNA in spleen and lung were detected in all birds and remained high at the different time points, reaching the maximum levels at 7 dpi. At 10 dpi, low levels were present in lung, and were undetectable in spleen.

Regarding H7N1 HPAIV-inoculated geese **(Figure 15B).**, moderate amounts of viral RNA were detected in spleen and in lung at 4 dpi, but not in brain. At 15 dpi, both geese tested positive to AIV RNA in brain samples. One bird also presented AIV RNA in lung, but near to the limit of detection of the technique. No viral RNA was detected in control birds.

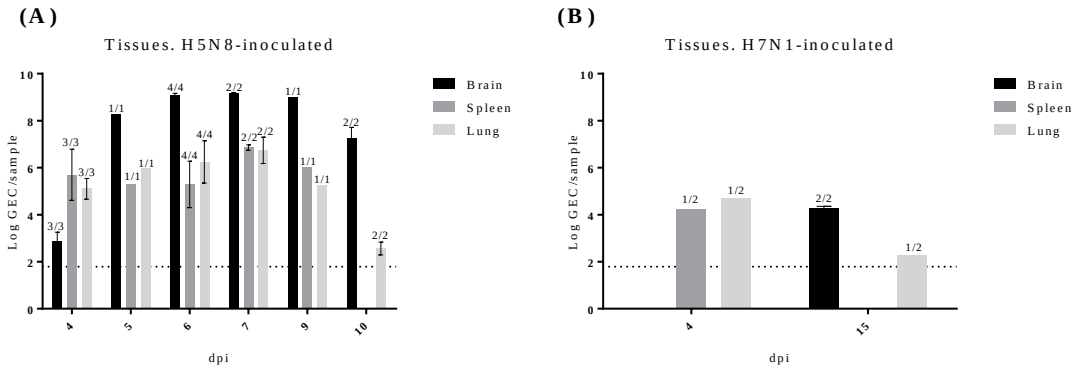


Figure 15. Viral titers expressed as log GEC in tissues (brain, spleen and lung) obtained from domestic geese (local and commercial) inoculated with H5N8 (A) or H7N1 (B) HPAIVs at different time points post-inoculation. The ratios above the columns represent the number of birds were viral RNA was detected out of the total sampled. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

Seroconversion

All included birds were seronegative prior to infection. At 15 dpi, 33,3% (2/6) and 75% (3/4) of H7N1-inoculated local and commercial survivor geese, respectively, seroconverted.

5.4. DISCUSSION

Before the emergence of Gs/GD H5 lineage, the isolation of HPAIVs in domestic geese was sporadic and not associated to high mortalities [28]. In addition, several studies demonstrate that domestic geese have played an important role in the emergence, evolution, perpetuation and interspecies transmission of HPAIVs belonging to Gs/GD H5 lineage [643], but the information about their potential role in the epidemiology of other HPAIVs is lacking. In 2016-2017, the HPAIV H5N8 belonging to clade 2.3.4.4 Group B of Gs/GD lineage caused an unprecedented number of outbreaks in domestic duck and geese holdings in Europe, which was not observed during the previous intercontinental waves of this lineage. Here, a local and a commercial breed of domestic geese were intranasally inoculated with a classical HPAIV strain (Italian H7N1) and a Gs/GD-HPAIV strain (Spanish H5N8 clade 2.3.4.4 B). To our knowledge, this is the first study performing a direct comparison of the pathobiological features of a classical and a Gs/GD-lineage strain in domestic geese.

Our data clearly demonstrated that domestic geese were highly susceptible to H5N8 HPAIV infection. Starting at 5 and lasting until 10 dpi, the mortality rate in the domestic geese inoculated with H5N8 HPAIV reached the 100%. Previous studies have reported a wide variation of susceptibility of domestic geese to Gs/GD H5 HPAIVs. The lethality ranges from 0 to 100% depending on the subtype, clade and genetic group of the Gs/GD H5 HPAIV, dose of inocula, the species (*Anser anser, Anser cygnoides*), breed and age at infection [352–361, 367, 393]. In the present study, two factors could influence the pathogenicity of H5N8 HPAIV in geese, most probably by reducing it. First, we challenged domestic geese with a dose of inoculum comparatively lower than the generally used for experimental infections in waterfowl species (10^5 ELD_{50} *versus* 10^6 ELD_{50} or higher). Second, we used geese of approximately 3-5 months of age, while most studies have performed the experimental infections in younger birds [352, 354–361]. The high mortality rates observed in our study despite the factors mentioned above, together with the mortalities reported by Grund *et al.* [367] and Slomka *et al.* [369] in domestic ducks demonstrate that the H5N8 HPAIVs belonging to clade 2.3.4.4 Group B of Gs/GD lineage circulating in Europe in 2016-2017 acquired virulence to domestic waterfowl populations.

The virulence of Gs/GD H5 HPAIVs in a wide range of avian and mammal species has been associated with the strong neurotropism characteristics of this lineage. Domestic geese infected with Gs/GD H5 HPAIVs usually exhibit neurological signs and microscopic lesions and viral antigen/RNA are detected in the central nervous system with or without mortality associated [352, 354–361]. In our study, the main clinical signs observed in the severely-affected geese were neurological, including tremor,

ataxia and head shaking. The histological lesions of the tissues coincided with the clinical manifestations: birds infected with H5N8 HPAIV showed multifocal to diffuse areas of necrosis in the central nervous system associated to widespread presence of AIV antigen demonstrated by IHC. These findings correlated well with the onset of mortality at 5 dpi and were common until the end of the study. As expected, viral RNA quantification in brain was in concordance with IHC results, being the organ that presented the highest viral loads. Thus, our results demonstrate that Gs/GD H5N8 2.3.4.4 Group B HPAIV is highly neurovirulent in domestic geese. Despite neurological dysfunction was considered the main cause of the high mortalities, lesions in other organs could have an important effect in the infection outcome. Our data indicated that H5N8 HPAIV presented a multi-organ tropism in domestic geese. Multifocal areas of hemorrhages in the pancreas were commonly observed during the macroscopic examination of the birds. Microscopically, the birds exhibited large necrotic and inflammatory lesions and high levels of AIV antigen presence in pancreas, liver, and to a lesser extent in spleen and thymus. The intensity of the lesions produced by H5N8 HPAIV in these organs could lead to multi-organ failure, and compromise the cellular immunity in case birds survive to infection. Similarly, Grund *et al.* [367] reported that the high virulence of the H5N8 clade 2.3.4.4 Group B HPAIV in domestic ducks was also probably associated with the intense hepato-tropism of this virus.

Previous reports have demonstrated that HPAIVs other than those belonging to Gs/GD H5 lineage can readily infect domestic waterfowl and replicate in different internal organs, but in the majority of cases they produce a subclinical to mild disease [193, 279, 285, 286, 330, 331, 333, 339–343]. However, experimental inoculation of particular H7 HPAIVs have caused severe clinical signs and mortality in ducks, in variable proportion depending on the species, virus strain and route of inoculation [329, 345, 346]. These studies demonstrate the potential virulence of particular classical lineages of HPAIVs for domestic waterfowl. Therefore, the low mortalities reported in domestic waterfowl during most HPAIV outbreaks may have been in some cases the result of low exposure needed to initiate infection rather than to low virulence of the viruses for these species. During the epidemics caused by the HPAIV H7N1 in Italy in 1999/2000, several flocks consisting in mixed poultry species reported high mortalities. However, domestic waterfowl were generally unaffected. In contrast, a particular outbreak was characterized by mortality and nervous signs in domestic ducks and geese [190]. By means of IHC techniques, the authors demonstrated AIV antigen in pancreas and in the central nervous system in these birds [190]. In the present study, domestic geese were susceptible to infection with the Italian H7N1 HPAIV as demonstrated by the seroconversion in several individuals. However, none of the birds showed evident clinical signs, gross or microscopic lesions, and all of them survived. Our results, together with those obtained by Nayaran *et al.* [333] and Röhm *et al.* [339] support the theory that most classical lineages of HPAIVs appear to be avirulent for domestic geese under experimental conditions.

However, we detected AIV RNA in plasma and in the three collected organs (central nervous system, pancreas and spleen) at different time-points in several geese inoculated with H7N1 HPAIV. Interestingly, one goose still presented detectable levels of viral RNA in brain and lung at the end of the study. Even though, the detection of viral RNA in tissues and plasma was inconsistent and at lower levels in comparison with those obtained from H5N8 HPAIV-inoculated geese, and all birds lacked AIV-positive cells by IHC techniques. These results provide evidence for a poor capacity of H7N1 HPAIV to produce a robust systemic infection in domestic geese. Therefore, differences related to the viral isolate, to the exposed dose, or to underlying factors in the birds (e.g. immunosupression, concomitant pathogens) could have facilitated the systemic dissemination of H7N1 HPAIV in that particular flock during the Italian 1999/2000 epidemics, and consequently, contributed to the mortality.

In the present study, we also evaluated the potential role of domestic geese in the epidemiology of the selected HPAIVs. Previous reports indicate the potentially important role of domestic geese in the epidemiology of Gs/GD H5 HPAIVs, as determined by high viral shedding and transmission to contact birds, and in some cases in a subclinical way [352, 354–361]. In our study, H5N8 HPAIV-inoculated geese shed large amounts of virus by oral (ranging from 2,16 to 7,75 log GEC) and cloacal (ranging from 2,43 to 7,52 log GEC) routes, suggesting that both fecal-oral and oral-oral routes could play a major role in the transmission of H5N8 HPAIVs in domestic waterfowl populations. Oral excretion did not significantly surpassed cloacal excretion in our study. Since the shift from predominantly cloacal shedding to oral is usually associated to adaptation to galliformes species [194], our findings suggest that the local H5N8 HPAIV strain may retain the wild waterfowl-shedding pattern. In addition, previous studies observed that Mandarin ducks and mallards inoculated with H5N8 HPAIV (clade 2.3.4.4 Group A and B) presented higher cloacal viral shedding than those infected with H5N1 (Clade 2.2 and 2.3.2.1) [645] and H5N6 (clade 2.3.4.4 Group C) HPAIVs [387]. These findings suggest a higher efficiency of particular strains of Gs/GD H5N8 subtype in the horizontal transmission among infected waterfowl species.

We detected a moderate viral load in the pool water at the later stages of infection. The detection in water despite we did not concentrated the samples suggests that sharing contaminated water can play an important epidemiological role as a source of H5N8 HPAIV infection. For instance, proximity to outdoor water, ponds and lakes frequently visited by domestic and wild waterfowl are considered a potential risk factor in the transmission of Gs/GD H5 HPAIVs to land-based poultry flocks [646]. The detection of a high viral load in plasma indicates that blood (i.e. as a result of fighting between infected geese) may represent an additional source of environmental contamination.

Despite the absence of clinical signs and mortality until 5 dpi, high viral shedding was already detected at 3 dpi. This, together with the high viral loads detected in all samples including water, suggest the possible involvement of domestic geese in the dissemination of H5N8 B HPAIV between waterfowl holdings and spill back to wild birds, either directly or through a common source of water, during the 2016-2017 H5N8 B European epidemics.

The study conducted by Pantin-Jackwood *et al.* [194] demonstrated that mallards experimentally inoculated with a battery of classical H5 and H7 HPAIVs strains transmitted the virus to contact mallards. In addition, lesser scaups (*Aythya affinis*) infected with two North-American H7 lineages of HPAIVs shed virus up to 14 days after infection [343]. Domestic geese inoculated with H7N7 isolated in Germany also excreted titers similar to those in chickens up to 7 days [339]. H7N2 and H7N3 HPAIVs derived from the Asian H7N9 lineage also appears to be in process of adaptation to waterfowl, with domestic and mallard ducks shedding virus for several days after experimental inoculation [286, 647]. The high and/or prolonged viral excretion reported in these studies suggest that waterfowl could play a role in the dispersal of HPAIVs other than those belonging to Gs/GD lineage in case the infection is established. However, the literature studying the potential role of domestic waterfowl in the epidemiology of classical HPAIVs is still scarce. In our study, a low number of H7N1 HPAIV-inoculated geese shed virus during the experiment and was mostly restricted by the oral route, suggesting a high degree of adaptation of the virus to gallinaceous species. Similarly, few birds presented detectable levels of viral RNA in plasma and were close to the limits of detection of the technique in pool water. However, the levels detected in the positive OS were comparable with those collected from H5N8 HPAIV-inoculated geese (up to 6,32 log GEC). In addition, the detection of viral RNA in swabs in two geese at 10 dpi that were negative the prior days of sampling could be indicative of intra-species transmission of the virus, either by aerosols or through the pool water. Since geese did not present any evident clinical sign through the study, we suggest that domestic geese may play, to some extent, a role in the perpetuation and transmission of classical HPAIVs to more susceptible avian species without being noticed.

Several mutations in viral gene segments that lead to amino acid substitutions in AIV proteins have been associated as markers of adaptation, and/or to increased virulence and transmissibility of HPAIVs in birds. PB2, PB1, PB1-F2, PA, HA, NP, and NS gene segments are all responsible for Gs/GD H5N1 HPAIV pathogenicity in ducks [313, 581, 588, 648, 649]. In geese, no information is available. The H7N1 and H5N8 HPAIVs strains used in the present study presented numerous amino acid differences in NS protein (67.3% identity) and to a lesser extent in PB2, PB1, PA, NP and M proteins (≥95% identity). Both HPAIVs presented amino acid substitutions associated with increased virulence in ducks (PB1:

436Y; PA: 237EW, 383D; M1: 43M) [313, 581, 587, 588, 603]. Since the majority of these amino acid mutations have been reported in HPAIVs of Gs/GD lineage and the effect of some amino acid mutations are dependent on the virus lineage [650], some of these mutations could explain the higher pathogenicity of H5N8 HPAIV. The differences observed between viruses in our study could also be associated to molecular markers of adaptation and virulence to waterfowl species that are still unidentified.

Some studies demonstrate wide differences in the susceptibility to HPAIVs between chicken breeds/lines [338, 399, 412, 416, 461–465], whereas in ducks the differences appear to be minor [469, 470]. In the present study, we did not detect evident differences in susceptibility between the local and commercial breed. However, the high mortalities of both, local and commercial geese, after infection with H5N8 HPAIV makes them suitable sentinels for the presence of the virus in the domestic-wild interface (local breed) and its introduction into commercial holdings (commercial breed). Domestic geese should be also targeted in active surveillance programs to early detect the circulation of HPAIVs of the Gs/GD lineage since they presented high viral loads of virus in different samples (OS, CS and blood) before the presence of evident clinical signs. The susceptibility of local geese to H5N8 and, to a lesser extent, to H7N1 HPAIVs is of particular interest. This breed is mostly reared in backyards, usually mixed with other domestic poultry species under minor biosecurity measures, which facilitates the exchange of HPAIVs between wild and domestic avian species and the potential generation of novel HPAIV reassortants with unknown biological characteristic to avian and mammal species.

The results of the present study demonstrate that domestic geese are susceptible to H5N8 and H7N1 HPAIVs. However, we demonstrate the lower infectivity, virulence and excretion of the classical H7N1 HPAIV strain in domestic geese in comparison with the Gs/GD lineage H5N8 strain when compared side by side. Since viral shedding were detected in both H7N1 and H5N8-inoculated geese, and HPAIVs continue to evolve and acquire new biological characteristics, an enhanced monitoring in a broad range of avian species, including backyard and commercial geese must be guaranteed in order to avoid the perpetuation of HPAIVs in the domestic-wild interface.

CHAPTER 6

STUDY IV: Experimental inoculation of local and urban pigeons (*Columba livia var. domestica*) with a classical and a Gs/GD highly pathogenic avian influenza virus

6.1. INTRODUCTION

Pigeons are synanthropic birds that congregate in habitats where large quantities of food, water and shelters for roosting and nesting are accessible. Pigeons preferentially forage on agricultural areas and a high number of these birds can be present in the vicinities of poultry holdings [651]. Their habits facilitate the direct contact with domestic poultry, particularly with those present in backyards and in free-range husbandry, and the contamination of feed and water with feces during their storage. In these areas, pigeons are also exposed to numerous species of wild birds, including their predators, and they can be the gate to wild birds pathogens go through reaching poultry species. Pigeons are one of the most common birds found in urban areas, where forage is based on spilled food, then living in proximity with humans and their beings and sharing habitats with aquatic birds (e.g. with mallards in parks) [651]. Pigeons are also present in LBMs, backyards and professional lofts, especially the breeds raised for meat or for contests, including the multi-million dollar pigeon racing industry [652].

The close association with wild birds, poultry and humans suggests that pigeons are likely to be recurrently exposed to AIVs. Therefore, infected pigeons could act spreaders of AIVs between farms and be involved in the zoonotic transmission into human population as a "bridging species". However, available data indicates that pigeons play a minimum role in the epidemiology of AI. Several studies have detected specific antibodies and several subtypes of AIVs in free-living and captive pigeons but the overall prevalences are low [653–656]. In most experimental trials, pigeons were resistant or minimally susceptible to HPAIV infection, with several studies reporting an inconsistent presence of clinical signs, gross and microscopic lesions, lack of mortality, reisolation of the virus and viral antigen in tissues, and/or low seroconversion rates [316, 347, 348, 375, 390, 657, 658]. Moreover, pigeons are considered poor propagators of HPAIVs by the transient and low viral shedding observed after experimental inoculation and the lack of transmission to co-housed pigeons and/or chickens [316, 375, 379, 389, 390, 657]. Thus, they are usually referred as dead-end hosts.

In general, the limited replication of AIVs in pigeons is associated with the expression pattern of SA in this species: the upper respiratory tract of pigeons contain predominantly SA in an a2,6 configuration (human-like), whereas a2,3 SA (avian-like) are almost restricted to the lung alveolar cells and rectum [659]. However, a degree of variation exists in the susceptibility of pigeons to HPAIVs, especially evident since the emergence of the Gs/GD H5 lineage of HPAIVs. Particular HPAIVs belonging to Gs/GD H5 lineage, including H5N1 and H5N8, have been isolated from pigeons that died naturally or presented evident signs of infection [392, 660–662]. Moreover, particular Gs/GD H5N1 HPAIVs isolates

acquired virulence to pigeons: several birds presented clear nervous signs before death or died in the absence of clinical signs, in association to viral replication in internal organs [375–379]. Presence of pigeons near poultry holdings has been considered a risk factor of introduction of Gs/GD H5 HPAIVs in backyard chickens [663], indicating the possible involvement of pigeons in Gs/GD H5 HPAIVs outbreaks in poultry. In addition, Gs/GD H5N1 HPAIV of clade 1 was efficiently transmitted to co-housed chickens [378]. Since particular HPAIVs may have acquired unusual avidity towards pigeons, it is important to characterize the pathobiology of different HPAIVs in this species.

The outcome after infection with HPAIVs is influenced by the virus isolate, but also largely by numerous host factors. Several experiments in chickens demonstrated that the susceptibility to infection is highly dependent of the genetic background of the breed/line [338, 399, 412, 416, 461–465]. However, the existence of breed-specific differences in peri-domestic avian species, including pigeons, has not been evaluated to date. Moreover, the different breeds of pigeons are present in diverse environments, and some of them are raised as domestic birds; thus, they could play a different role in the epidemiology of AI in case of infection.

Considering the differences in outcome and shedding after infection with different HPAIVs in pigeons and the potential breed-specific variations in susceptibility between breeds, herein we evaluated the differential pathobiology of two HPAIVs in two breeds of pigeons. A classical H7N1 isolated in Italy during the 1999-2000 epidemics and a H5N8 belonging to Gs/Gd H5 lineage isolated in Spain during the 2016/2017 European epidemics were inoculated in local and urban pigeons. The infectivity, the pathogenesis and the viral shedding were assessed.

6.2. MATERIALS AND METHODS

Viruses

The viruses used in this study were: A/Chicken/Italy/5093/1999 (H7N1), isolated in 1999-2000 during an Italian epidemic that mainly affected Veneto and Lombardia regions (kindly provided by Dr. Ana Moreno from the *Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna*) and A/Goose/Spain/IA17CR02699/2017 (H5N8 clade 2.3.4.4. group B), isolated in Catalonia (Northern Spain) during the 2016/2017 European epizootics. Both viruses are highly pathogenic based on the aminoacid sequences at the HA0 cleavage site: PEIPKGSRVRR↓GLF (H7N1) and

PLREKRRKR↓GLF (H5N8). Virus stocks were produced in 10 days-old SPF embryonated eggs. The allantoic fluid was obtained at 24-48 hpi, filtered and aliquoted at -75ºC until use. Serial ten-fold dilutions of the filtered viruses in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean egg lethal doses (ELD_{50}) were determined by Reed and Muench method [624]. The consensus full genome sequences corresponding to the eight segments of H7N1 and H5N8 are available in Genbank under accession numbers: DQ991325.2 to DQ991332.2 (H7N1) and MK494920 to MK494927 (H5N8).

Animals and facilities

In total, 70 pigeons (*Columba livia domestica*) of approximately 6 months of age were used in the present study. Two breeds were included: *Colom del vol català* (35 birds), which is a local breed of Catalonia (northern Spain) generally present in backyard flocks that has been selected for flight in flock and plumage colours, and urban pigeons (35 birds), extensively present in urban and peri-domestic areas.

At arrival, the animals were individually identified and placed in separated negative-pressured HEPA-filtered boxes present in BSL-3 facilities in *Centre de Recerca en Sanitat Animal* (*Programa de Sanitat Animal*, IRTA). In order to ensure animal welfare the installations were enriched with perches, as described in the Spanish Royal Decree 53/2013 that lays down the basic obligations and general principles concerning the animal protection in experimentation. Prior to infection, serum samples were obtained from all animals to ensure that they were seronegative to IAV and Newcastle disease virus (NDV) by cELISA (ID-VET, Montpellier, France). In addition, OS and CS were collected from 5 random pigeons of each group (10 animals/breed) and confirmed to be negative to IAV by one-step qRT-PCR.

During the experimental procedures, food and water were provided *ad libitum*. The experimental design was approved by the ethical commission of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) and the Government of Catalonia (*Departament de Territori i Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural*) under reference code CEEA 92/2018-10253.

Experimental design and sampling

64 pigeons (32 local and 32 urban) were randomly separated into 4 infected groups of 16 birds each. After 5 days of acclimation, for each virus (H7N1 and H5N8) 16 local and 16 urban pigeons were inoculated with the corresponding virus 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). 6 pigeons that were previously demonstrated

seronegative to IAV and NDV by cELISA test were euthanized prior to infection in order to collect tissue samples as negative control birds.

All birds were monitored daily for clinical signs until 14 dpi. An OIE scoring for AIV infection was used [140]. Moribund pigeons were anesthetized using ketamine/xylazine (20 mg/kg body weight, Imalgene 100 and 0,3 mg/kg body weight, Rompun 20 mg/ml) via the intramuscular route, euthanized with intravenous pentobarbital (140 mg/kg body weight, Euthasol 400 mg/ml) and scored as dead. The mortalities and MDT were recorded in each group. In order to evaluate viral shedding, OS and CS were obtained from the first 9 birds of each group (selected previously to the inoculation) at 1, 3, 6, 10 and 14 dpi. Pulps from immature feathers (FP) were collected from the ventral area in the same birds at 3 and 6 dpi. 2 birds were sacrificed, using the combination of sedation and euthanasia described above, at 3, 6, 10 and 14 dpi (at 14 dpi only one pigeon was euthanized) to evaluate gross lesions, and tissues were collected and formalin-fixed from the necropsies performed for pathological studies. The selection of birds was biased towards those found dead or presenting evident clinical signs of disease. At the end of the study, serum samples were obtained from all survivor pigeons in order to evaluate seroconversion.

Pathological examination and immunohistochemical testing

A standardized necropsy was performed in order to detect gross lesions and collect tissues for pathological studies. Tissue samples were collected, immersed in 10% formalin for fixation during 72 hours and embedded in paraffin wax. Samples included skin, thymus, ocular conjunctiva, pectoral muscle, nasal cavity, trachea, lung, central nervous system, heart, spleen, liver, kidney, proventriculum, gizzard, pancreas, duodenum, cecum, colon and bursa of Fabricius. Tissue samples collected at 3, 6 and 10 dpi were subjected to microscopic examination. Microtome sections of 3 μm of thickness (Leica RM2255, Nussloch, Germany) from FFPE tissues were processed, stained with H/E and then examined under light microscopy. An IHC technique was performed as described in Study II on serial sections of the tissues. The positivity in the tissues was semi-quantitatively assessed taking into consideration the percentage of NP-positive and negative cells. The samples were classified as follows: no positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells (+++) in a tissue section. Positive and negative controls were used. The positive control was a central nervous system from a chicken experimentally infected with H7N1 HPAIV [400], and the negative controls consisted in the same tissue incubated with PBS instead of the primary antibody and the tissues collected from seronegative pigeons that were euthanized prior to infection.

Viral RNA quantitation in swabs and feather pulps

Swabs were placed in 0.5 ml of sterile PBS enriched with Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Nystatin (Sigma-Aldrich, Missouri, USA) at a final concentration of 6% The pulps were separated from the feathers and homogenized in 0.5 ml of sterile PBS with 6% antibiotics. All samples described above were conserved at -75ºC until further use. Viral RNA was extracted from OS, CS and FP using Magattract 96 cador pathogen kit and BioSprint 96 equipment (Qiagen, Valencia, CA, USA), following manufacturer's instructions. A highly conserved region of AIV M1 gene 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 [521, 565]. Samples presenting a Ct value lower than 40 were considered positive to IAV RNA. To extrapolate the GEC present in the samples, a standard curve obtained by amplification of M1 gene fragment (99 bp) was used. The limit of detection of the technique was 2,07 log GEC in swabs and in FPs.

Seroconversion

To evaluate seroconversion, sera of all survivor animals were tested by a cELISA test (ID Screen® Influenza A Antibody Competition Multi-species, ID-VET, Montpellier, France), following manufacturer's instructions.

6.3. RESULTS

Clinical signs and mortality

Clinical signs were only observed in a local pigeon inoculated with H5N8 HPAIV. At 5 dpi, this pigeon presented reluctance to movement, severe apathy and nervous signs, including tremor and ataxia. At 6 dpi, the bird presented similar nervous signs and was prostrated, and was consequently euthanized for ethical reasons. The remaining local and urban pigeons inoculated with H5N8 HPAIV did not present any evident clinical sign. Local and urban pigeons inoculated with H7N1 HPAIV did not exhibit any evident clinical sign through the study.

Gross lesions

Consistent gross lesions were only observed in the pigeon inoculated with H5N8 HPAIV that was euthanized at 6 dpi. The lesions were restricted to the pancreas, which presented a generalized mild discoloration with small petechial haemorrhages. The remaining birds inoculated with H5N8 and H7N1 HPAIVs serially necropsied at 3, 6, 10 and 14 dpi did not present any evident macroscopic lesion, neither in negative control animals.

Histopathological findings

Pigeons serially necropsied at 3, 6 and 10 dpi were subjected to microscopic examination. Only the pigeon inoculated with H5N8 HPAIV euthanized at 6 dpi presented microscopic lesions and NP+ cells, which were restricted to the central nervous system and myocardium. The most severe microscopic lesions and the higher percentage (>40%) of cells expressing the presence of viral antigen by means of IHC techniques were observed in the brain. The cerebral hemispheres presented extensive areas of non-suppurative encephalitis consisting in severe spongiosis, gliosis and neuronal chromatolysis, and lymphocytic cuffing; the IHC technique revealed widespread AIV antigen in neurons and glial cells **(Figure 16A-B).** The myocardium of the same bird presented focal areas of moderate necrosis and degeneration of myocytes with mixed inflammatory infiltrate (lymphoplasmacytic and heterophils); in these areas IHC techniques revealed a low percentage (<10%) of myocytes and inflammatory cells expressing the presence of viral antigen **(Figure 16C-D).** No microscopic lesions neither NP-positive cells were detected in the pancreas. The remaining tissues of the same bird and the other examined pigeons inoculated with H7N1 or H5N8 HPAIVs did not present evident microscopic lesions nor NP-cells positivity. Negative control animals showed neither microscopic lesions nor viral antigen in tissues.

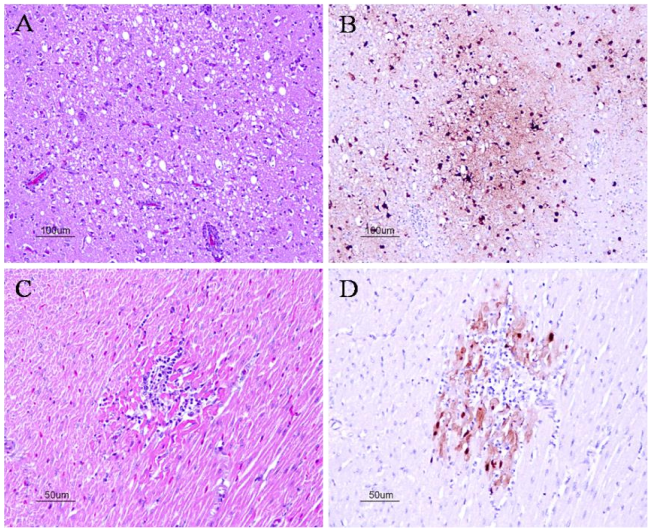


Figure 16. Serial sections of the central nervous system and heart of a local pigeon experimentally inoculated with H5N8 HPAIV stained with conventional HE staining and IHC techniques against NP nucleoprotein, respectively. CNS A/B, diffuse areas of necrosis, gliosis and perivascular cuffing (A) and NP-positive neurons and glial cells (B).Myocardium C/D., multifocal necrosis of myocardiocytes with mild inflammatory infiltrate (C) and NP-positive myocardiocytes (D).

Viral shedding

In H7N1 HPAIV-inoculated birds, three OS collected from urban pigeons were positive at 1 dpi, and two urban pigeons different from those at 1 dpi tested positive at 3 dpi. In CS, one urban pigeon maintained detectable levels from 3 to 6 dpi. The levels of viral RNA in OS and CS remained low through the experiment (ranging from 2,93 to 3,86 log GEC and from 3,16 to 4,23, respectively) **(Figure 17)**.

In H5N8 HPAIV-inoculated birds, the OS collected from one local and one urban pigeon tested positive to AIV RNA at 1 dpi; at 3 dpi, one pigeon of each breed different from those that were positive at 1 dpi presented detectable levels of viral RNA. Similarly, one pigeon of each breed different from those positive at 1 and 3 dpi were positive at 6 dpi. In the case of the CS, viral RNA was detected in two urban pigeons at 1 dpi, and only a local pigeon tested positive at 3 dpi. The levels of AIV RNA in OS and CS were generally low; only one CS collected at 1 dpi (5,16 log GEC) and one OS collected at 6 dpi (5,02 log GEC) presented moderate amounts of viral RNA **(Figure 17).**

No viral RNA was detected in the OS and CS collected at 10 and 14 dpi in any experimental group, or in the swabs collected in the local pigeons inoculated with H7N1 HPAIV. No viral RNA was detected in the feather pulps collected at 3 and 6 dpi in any bird.

In summary, viral RNA from OS and CS was only in 4 local pigeons inoculated with H7N1 HPAIV, and in two local and three urban pigeons inoculated with H5N8 HPAIV. Oral and cloacal shedding in the same bird was only observed in a local pigeon and in an urban pigeon inoculated with H5N8 HPAIV at 3 and 1 dpi, respectively. The detection in the birds was transient, inconsistent, and the viral shedding restricted from 1 to 6 dpi.

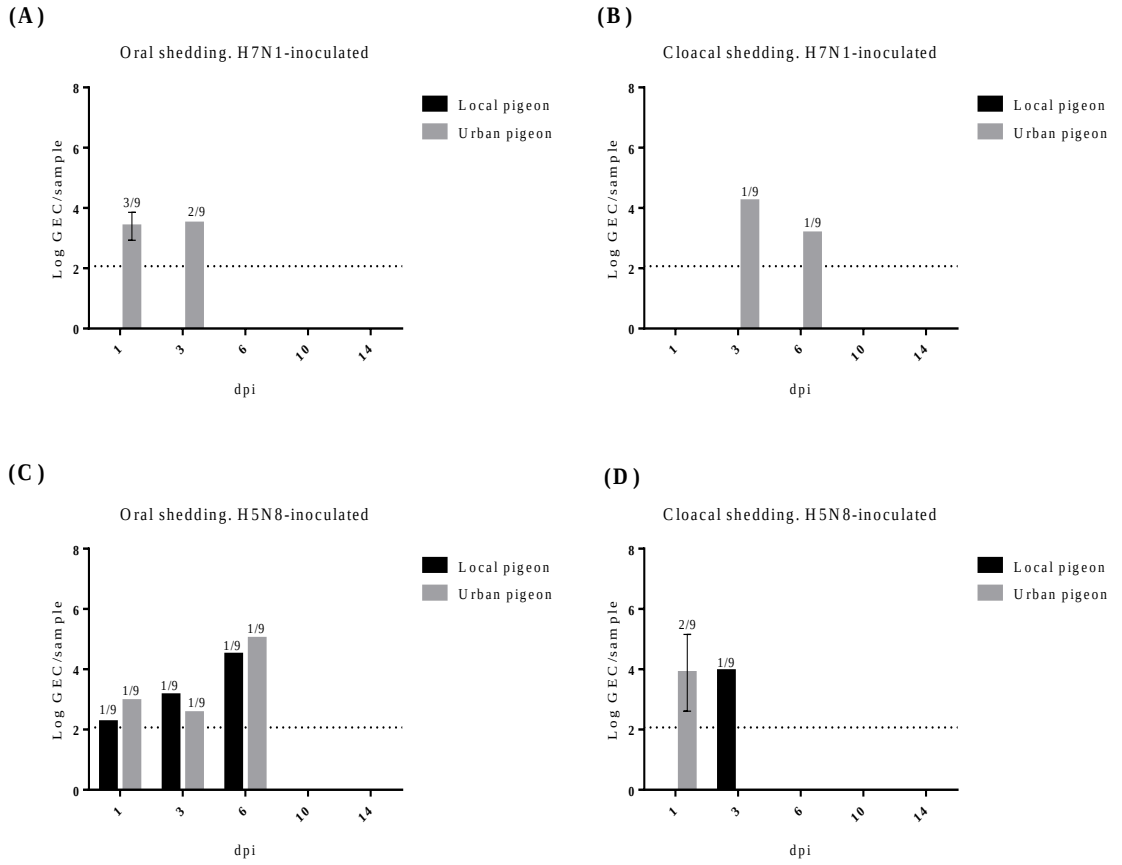


Figure 17. Viral titers expressed as log GEC in OS (A, C) and CS (B, D) obtained from local and urban pigeons inoculated with H7N1 (A,B) or H5N8 (C,D) HPAIVs at different times post-inoculation. The ratios above the columns represent the number of birds shedding virus out of the total sampled. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

Seroconversion

All included pigeons were seronegative to AIV prior to infection. At the end of the study (14 dpi), 60% (6/10) of the urban pigeons inoculated with H7N1 HPAIV seroconverted, and no local pigeons tested positive. Regarding H5N8 HPAIV-inoculated birds, 30% (3/10) of the local and 30% (3/10) of the urban pigeons seroconverted.

6.4. DISCUSSION

Available data indicate that the susceptibility of pigeons to HPAIVs is dependent of the virus isolate, and may also be influenced by the genetic background of the breed as in chickens. In the present study, the differential pathobiology of a classical H7N1 HPAIV and a Gs/GD H5N8 HPAIV in pigeons and the existence of breed-specific variations in susceptibility between local and urban pigeons were evaluated.

Pigeons can be infected with HPAIVs, but birds usually lack evident signs of disease and if so, recover entirely within a short period of time [347, 348, 375, 388, 390, 657, 658]. However, some HPAIVs have produced severe clinical signs and mortality in this species. Despite in the majority of cases the mortality ratios have been minimal, infection with particular Gs/GD H5N1 HPAIVs resulted in mortalities up to 37.5% [375–379], demonstrating important differences in virulence between HPAIV strains for pigeons. In the present study, the experimental inoculation of a classical H7N1 HPAIV and a Gs/GD H5N8 HPAIV in pigeons resulted in the lack of evident clinical signs, mortality, gross lesions, microscopic lesions and viral replication in tissues by IHC techniques in all pigeons, except in one local pigeon inoculated with H5N8 HPAIV. However, the infection was established in several birds, as demonstrated by the viral shedding and seroconversion. Thus, the results of the present study support the existing literature that pigeons become infected by diverse HPAIVs, although the infection is subclinical [664].

Our data suggest that HPAIVs belonging to Gs/GD H5 lineage are potentially more virulent to pigeons than other lineages of HPAIVs. Whereas neither clinical signs nor mortality was recorded in any bird inoculated with H7N1 HPAIV, one local pigeon inoculated with H5N8 HPAIV exhibited severe apathy and evident nervous signs, including tremor and ataxia, and had to be euthanized for ethical reasons. Despite the bird was euthanized, it is likely that the infection would have resulted in death within a short period of time. The clinical presentation observed was similar to that previously reported in pigeons inoculated with particular Gs/GD H5N1 HPAIVs [375–379], but to our knowledge, this is the first experimental study reporting severe clinical signs in pigeons after inoculation with Gs/GD H5N8 HPAIV of clade 2.3.4.4. The present study demonstrates that despite marginal, Gs/Gd H5 HPAIVs harboring distinct NA subtypes other than N1 can produce deadly infections in pigeons.

Herein, we report for the first time mortalities in pigeons using a titer of inoculum lower than 10^6 ELD_{50}. The dose of inoculum is also known to largely influence the infection outcome; however, it has been suggested that the relatively high mortalities caused by particular Gs/GD H5N1 HPAIVs in pigeons

could be associated with excessive titers of inoculum (10^{6-8} ELD_{50}) that could contribute to cell damage from inflammatory processes [664].

The severe clinical signs in the local pigeon inoculated with H5N8 HPAIV correlated well with presence of microscopic lesions and viral antigen in particular tissues. This bird presented large areas of necrosis in the central nervous system associated to widespread presence of viral antigen, indicating that the fatal outcome was likely associated with neurological dysfunction. Presence of viral antigen and microscopic lesions in the central nervous system are common in pigeons, even in asymptomatic infections, and in other species infected with Gs/GD H5 HPAIVs, demonstrating the strong neurotropism of this lineage [367, 375–379, 390, 423–426]. In lethal infections in pigeons, the central nervous system generally presents the highest viral loads and more severe microscopic lesions [376]. This particular pigeon also presented mild multifocal necrotic and inflammatory lesions in the myocardium, associated to moderate amounts of viral antigen in myocardiocytes, indicating that the virus was circulating in the bloodstream. However, no microscopic lesions and viral antigen were observed in any other organ in that pigeon. This, together with the absence of viral antigen in the remaining pigeons and the lack of detection of viral RNA in FPs in all birds indicate that despite H5N8 HPAIV can replicate at high titers in the central nervous system, the virus is not prone to produce a robust systemic infection in pigeons.

The outcome after infection with HPAIVs is dependent on numerous viral but also host factors. Previous reports have demonstrated that the genetic background of the breed largely influences the infection outcome [338, 399, 412, 416, 461–465]. To our knowledge, the existence of breed-specific differences in susceptibility to AIVs in peri-domestic avian species, including pigeons, has not been tested to date. Herein, we did not detect any evidence of differential susceptibility between the local and urban breed, neither in clinical presentation, pathogenesis nor viral shedding. Taken into account the low mortality rates present in this study, we cannot discard that the highly virulent infection caused by H5N8 HPAIV could be related to the possible existence of other infectious agents and non-infectious factors, such as nutritional deficiencies, stress and immune status that aggravated the infection in that particular pigeon, rather than to breed-specific factors. However, why only a particular bird presented higher susceptibility could not be unraveled. All pigeons tested seronegative to NDV, and a qRT-PCR targeting a highly conserved region of NDV was performed in brain and pancreas of this bird in order to detect an early infection, with negative results (data not shown).

Pigeons are naturally present in a wide range of habitats driven by the availability of resources, and could play a role in the transmission of HPAIVs by direct contact with susceptible species and/or indirectly by contamination of the environment with infective secretions. Most experimental studies demonstrate

that the viral shedding after HPAIV infection in pigeons is brief and viral titers are under the minimum threshold required to infect other species, even in sick individuals [375, 379, 389, 390, 657]. Therefore, the transmission of HPAIVs between poultry holdings by pigeons is more likely to occur mechanically (e.g. carrying the virus on their feet and feathers). However, one study demonstrated effective transmission of Gs/GD H5N1 HPAIV to co-housed chickens [378], suggesting that pigeons could actively act as a biological vector of particular HPAIVs. Therefore, the shedding pattern of pigeons infected with different HPAIVs should be further assessed to determine if they represent a risk for HPAIV interspecies transmission. In the present study, viral RNA was detected in oral and cloacal swabs in few pigeons inoculated with the classical H7N1 HPAIV and the Gs/GD H5N8 HPAIV, but the detection was inconsistent, short and generally at low titers. These results suggest a minimum role of pigeons as amplifiers of HPAIVs regardless of the viral lineage. However, moderate levels of viral RNA were detected in some OS and CS in H5N8 HPAIV-inoculated groups (up to 4,23 log GEC), including in the pigeon that succumbed to infection. Since pigeons often gather in large numbers, we speculate that even a small percentage of pigeons shedding moderate levels of virus could represent a risk of H5N8 HPAIV environmental contamination and spill over into avian species that are more susceptible. However, transmission studies are required to test this hypothesis.

Several studies detected high titers of HPAIVs within the feather of HPAIV-infected chickens and ducks, and the transmission following feather consumption has been already demonstrated [665]. In this study, the lack of viral RNA and antigen in all FP despite some animals became subclinically infected indicate that feathers from pigeons do not likely play an important role in the interspecies transmission of HPAIVs. In contrast, the large quantities of viral antigen detected in the brain of one pigeon inoculated with H5N8 HPAIV indicate that a high viral load may be present in this organ. It was previously reported that a cat succumbed to Gs/GD H5N1 HPAIV after consuming a pigeon carcass infected with the virus [267]. This fact and the apparent increased virulence of Gs/GD H5N8 Group B in a variety of wild bird species suggest that pigeons represent a potential risk of infection to H5N8 to predators (e.g. cats, crow, and hawk).

In summary, we found that pigeons are susceptible to HPAIVs belonging to classical and Gs/GD lineages, but the infection is asymptomatic. However, the severe clinical signs, microscopic lesions and viral antigen detected in one pigeon inoculated with H5N8 HPAIV suggest some differences in the pathobiology of classical HPAIVs and Gs/GD HPAIVs in this species. Since Gs/GD lineage continues to evolve, novel HPAIVs with unprecedented pathobiological characteristics in pigeons and other peri-domestic avian species could emerge. Moreover, the viral excretion in some pigeons indicate

than increased surveillance in synanthropic avian species during active outbreaks in poultry are needed in order to avoid the spread between farms and the potential introduction into human population.

PART III

General discussion and conclusions

CHAPTER 7

GENERAL DISCUSSION

Through the history there have been several reports of HPAIVs that expanded through broad geographic regions, affected a high number of flocks and/or caused outbreaks during long periods of time before their successful eradication [35–42]. Gallinaceous species, particularly chickens and turkeys, have been the main species affected during HPAIV epidemics. In contrast, the isolation of HPAIVs in domestic waterfowl, wild birds or peridomestic avian species have been more sporadic, rarely accompanied by high mortalities, and in most cases associated to active HPAIV outbreaks in land-based poultry in the vicinities [28]. To date, any HPAIV epidemics have reached the severity in terms of economic losses and geographical extent that those caused by the Gs/GD H5 lineage of HPAIVs [47]. Moreover, these viruses produced a change of paradigm in the pathobiology and epidemiology of HPAIVs. First, these viruses have been isolated and present unique infectivity and virulence in a broad range of hosts, including numerous species of wild and domestic birds, mammals and humans [49]. Second, these viruses are thought to be carried through large geographic regions (even intercontinentally) by migratory wild birds, and in particular species in a subclinical way [196–198]. Third, it has been the unique lineage to become established in domestic waterfowl [203–206].

Numerous authors have evaluated the pathobiology of HPAIVs in different avian species belonging to different taxonomic groups. The studies have used a variety of strains, different doses of inocula and routes of inoculation, and birds of different ages and immune status. Altogether, existing literature demonstrate that the outcome after infection with HPAIVs in birds is complex and highly dependent of numerous viral and host factors, underlining the necessity to study the pathobiology of HPAIVs in different virus-host combinations. However, fewer studies have determined the pathobiology of a single HPAIV isolate in different avian species within the same experimental conditions. In addition, there are a low number of studies comparing side by side the pathobiology of HPAIV belonging to classical lineages and HPAIVs of Gs/GD H5 lineage in the same species. Consequently, one objective of the present dissertation was to systematically evaluate the pathobiology of two distinct HPAIVs in a range of avian species belonging to different orders. The H7N1 virus which caused important economic losses in 1999-2000 in Italy was used as a classical strain of HPAIV. The H5N8 virus is a descendant of the Gs/GD H5N1 lineage first detected in Southern Asia in 1996. This particular strain was isolated in Spain during the 2016-2017 European epidemics and belongs to the most widespread clade of this lineage at the present time. Chickens (*Gallus gallus domesticus*), geese (*Anser anser var. domestica*) and pigeons (*Columbia livia var. domestica*) were selected as representative members of the Orders Galliformes, Anseriformes and Columbiformes, respectively.

Several reports reveal the existence of important breed-related differences in the infection outcome after experimental inoculation with HPAIVs. Particularly, there is a general believe that local breeds are more

resistant to disease than commercial breeds, but existing studies do not always support this theory. Most of the studies addressing this issue have been carried out in developing countries, where local chicken breeds represent an important source of protein intake [29]. To our knowledge, the differences in susceptibility to HPAIVs between chicken breeds from Spain have not been evaluated. Moreover, studies evaluating the differences related to the breed in other species than can play a role in the epidemiology of HPAIVs are lacking. Therefore, another objective was to determine the variation in susceptibility to HPAIVs in several breeds of chickens, geese and pigeons with different genetic backgrounds. The main findings of the thesis are presented in this general discussion, as well as the limitations and potential improvements of the different studies. However, the specific results of each study are discussed in more detail in each section and therefore will not be subjected to analysis again in this general discussion.

The probability of introduction of AIVs by legal trade of live poultry or poultry products in Spain is considered low [666]. However, Spain harbours a broad diversity of resident wild bird species, is located within natural migration routes between Eurasia and Africa and present a high number of wetlands where a variety of birds congregate. All these characteristics likely provide the Spanish territory the optimal conditions for the circulation of LPAIVs in the wild bird population. Areas with a considerably high relative risk for the introduction of the H5N1 HPAIV by migratory birds have also been identified [667, 668]. However, surveillance programs carried out in poultry in Spain demonstrate that they are usually free of AIVs [669], and HPAIVs have not been detected in the wild bird population [507, 565, 670, 671]. The exceptions include the isolation of H5N1 HPAIV of Gs/GD lineage in a dead great crested grebe (*Podiceps cristatus*) in 2006 [672], the H5N3 LPAIV detected in a duck meat production farm [673] and H7N7 HPAIV outbreak in a layer farm in 2009 [674], and the isolation of H7N1 LPAIV in a breeding hen farm in 2013 [675]. In 2017, the H5N8 HPAIV reached Spain. Following the detection in two geese and a white stork in early 2017, the virus was detected in commercial duck farms and in several backyard flocks. Before the biological characterization of the virus, the virus isolate was fully-sequenced. The genetic characterization of the H5N8 HPAIV isolated in a domestic goose **(Study I)** revealed that the virus belonged to clade 2.3.4.4 Group B (Gochang-like) of Gs/Gd lineage, and presented high identity (>99.5%) with H5N8 HPAIVs isolated in Eurasia during the autumn-winter period of 2016-2017. All segments of Spanish H5N8 clustered within cluster 2, suggesting that the phenotype of Spanish isolate may be representative of all H5N8 HPAIVs classified in that particular group. However, only one isolate was fully-sequenced; thus, we cannot rule-out that genetically and biologically-distinct H5N8 isolates circulated in Spain at that time.

The H5N8 HPAIV was then phenotypically compared with the classical H7N1 HPAIV by means of three different experimental infections **(Studies II, III and IV).** We focused in the differences in clinical presentation, gross and microscopic lesions (HE staining), viral antigen in tissues (IHC technique), viral shedding (qRT-PCR technique) and seroconversion (cELISA) between both HPAIV isolates in chickens (study II), geese (study III) and pigeons (study IV).

With the dose of inocula selected (10^5 ELD_{50}), which represents a medium dose, we could demonstrate successful infection (by means of clinical signs, mortality, viral shedding and/or seroconversion) in the three species with both H7N1 and H5N8 HPAIVs. However, we found huge differences in the infectivity, clinical presentation, pathogenesis and viral shedding dependent of the virus isolate and the host.

Chickens represent the predominant species in poultry production, and provide approximately the 90% of the poultry meat and eggs produced worldwide [43]. Their pivotal importance in poultry production and the generally high mortalities reported in this species and other major galliforms such as turkeys during HPAIV outbreaks have resulted in a large collection of experimental studies evaluating the pathobiology of different HPAIVs in these species. In contrast, the number of experimental studies evaluating the pathobiology of HPAIVs others than those belonging to Gs/GD lineage in minor galliformes species and in other taxonomic groups of birds is more limited. As expected, based on previous results in different galliformes species [400, 534, 628], H7N1 HPAIV infection in chickens resulted in severe clinical signs and high mortalities. A complete different scenario was observed in geese and pigeons. Inoculation of these species with H7N1 HPAIV resulted in the lack of evident clinical signs, mortality, gross lesions, microscopic lesions and viral antigen in tissues by IHC in all birds. The infection could only be demonstrated in some individuals by means of viral shedding at some point post-inoculation and/or seroconversion. The majority of studies demonstrate that experimental inoculation with classical lineages of HPAIVs can result in infection in domestic waterfowl and pigeons, but they rarely show signs of disease or are mild, similar as reported herein [193, 279, 285, 286, 330, 331, 333, 339–343, 347–351]. In case of mortalities, they are usually limited to a low proportion of birds out of the total inoculated [341]. However, recent studies demonstrate that HPAIVs of the H7 subtype can also cause high mortalities in waterbirds [329, 345, 346]. The lack of mortality in domestic geese differ from the results reported recently by Scheibner *et al.* [345], where inoculation of 10 days-old domestic ducks with the same strain of Italian H7N1 HPAIV used in our study caused 20 to 50% mortality in Pekin and Muscovy ducks, respectively. The differences in susceptibility between ducks and geese suggest species-related differences in susceptibility to HPAIVs of the H7 subtype. However, other host factors such as differences in age of the birds probably have played a more important role.

Since Gs/GD H5 HPAIVs have been isolated in a broad range of hosts in the field, the pathobiology of these viruses in non-galliformes birds has been subjected to more profound investigation. Existing literature show enormous differences in mortality after experimental inoculation, which vary dependent on the viral isolate and the host. In our study, the inoculation of H5N8 HPAIV caused mortality in all inoculated domestic geese. Our results, together with the mortalities reported in domestic ducks [367, 369], demonstrate that the H5N8 HPAIV belonging to clade 2.3.4.4 Group B circulating in Europe in 2016-2017 were more virulent to domestic waterfowl than those H5N8 clade 2.3.4.4 B HPAIVs circulating in South Korea [387] and most H5N8 clade 2.3.4.4 Group A HPAIVs [359, 367, 386, 645]. Scheibner *et al*.. [367] showed that all domestic ducks inoculated with H5N8 B succumbed to infection when inoculated by the intra-muscular route, but the mortality rates in those inoculated by the natural route (oro-nasal) decreased to the 20%. Similarly, the mortalities in domestic ducks were approximately 17% in the study of Slomka *et al*.. [369]. Herein, the mortalities after intranasal inoculation reached the 100%, indicating that domestic geese are more susceptible to H5N8 B HPAIV than domestic ducks. The higher severity of clinical signs after inoculation with Gs/GD H5 HPAIVs in geese compared to ducks has also been observed in other experimental studies comparing side by side both species [288, 352, 355, 359, 676].

Several experimental studies demonstrate that H5N8 HPAIVs of clade 2.3.4.4 of variable origin present a reduced pathogenicty to chickens in comparison with their parental H5N1 [334–338]. Despite H5N8 HPAIV isolated in Spain was highly virulent to chickens, the infection resulted in a lower frequency of severe clinical signs, lower mortality and longer MDT than in those inoculated with H7N1 HPAIV. Since H5N8 HPAIV was isolated from a domestic goose and infected easily this species after experimental inoculation, our results may be an indication that the local strain retains a strong preference for waterbirds that may result in a lower affinity to galliformes species.

Gs/GD H5 HPAIVs have been detected in tissues of dead pigeons and doves, including H5N8 B in South Africa [392, 660–662].. Conversely, under experimental conditions, Gs/Gd H5 HPAIVs have caused high mortality ratios only when high doses of inocula were used [375–379]. In our experiment, with the exception of one pigeon inoculated with H5N8 HPAIV that presented severe clinical signs and lesions associated to viral antigen, H5N8 HPAIV produced a subclinical infection in this species. This is in agreement with the majority of available literature describing the refractory nature of pigeons to disease after Gs/GD H5 HPAIV infection [347, 348, 375, 388, 390, 657, 658]. Even though, the presence of a pigeon inoculated with H5N8 HPAIV presenting severe clinical signs despite using a comparatively lower dose of inoculum suggest that this local strain also gained the potential to cause lethal infections in columbiformes species.

The mortalities recorded in waterbirds after experimental infection with HPAIVs of the H7 subtype suggest that HPAIVs others than those belonging to Gs/GD H5 lineage can acquire virulence to these species and potentially, to other taxonomic groups of birds. Therefore, the pathobiology of new emergent HPAIVs others than those belonging to Gs/GD H5 lineage should also be evaluated in different species. However, we demonstrate that within the same experimental conditions (same dose of inocula and birds in the same range of age), H5N8 HPAIV is more infective and/or virulent in a broader range of hosts than H7N1 HPAIV. This could explain the higher detection of HPAIVs belonging to Gs/GD H5 lineage in comparison with other lineages of HPAIVs. The big limitation of the present dissertation is that we only selected one classical strain of HPAIV and one HPAIV of Gs/GD H5 lineage. Taking into consideration the differences in infection outcome between HPAIVs, even in those classified within the same genetic group [464], the situation could be completely different if other strains had been selected. However, this demonstrates that the continuous evolution of HPAIVs may result in the acquisition of unprecedented pathobiological features to different species.

Regardless of the virus and the species, the presence of nervous signs in severely-affected birds was a common feature. This finding has been reported in numerous experimental studies using a variety of HPAIVs and avian species [270, 317, 677]. Despite the neurological involvement was frequent, we detected some variations in the clinical presentation dependent of the species. Several chickens inoculated with either H7N1 or H5N8 HPAIVs died during the peracute stage of infection (2 dpi) without previous evident clinical signs of disease. In contrast, all geese and the single pigeon that succumbed to H5N8 HPAIV infection presented a delayed clinical course, being recorded the first clinical signs at 5 dpi. Moreover, haemorrhages and oedema in skin that were observed in several infected chickens were not seen in any goose or pigeon. In agreement with our results, experimental infection of domestic waterfowl and pigeons generally results in the absence of cyanotic and edematous lesions in skin and in extended MDTs than the reported in galliformes [302, 352, 354–361, 375–379]. These distinct features could be associated with the excessive replication of HPAIVs in endothelial cells of galliformes, which contribute to increased haemorrhagic and edematous lesions and rapid death [296–299]. As in most studies [295], we did not detect extensive viral replication in endothelium in infected geese and pigeons in any collected organ, included in dead individuals. Thus, the distinct ability of HPAIVs to replicate efficiently in endothelial cells of different species appears to be one important factor determining the different clinical presentations reported here, and demonstrates that the main mechanisms of pathogenicity of HPAIVs differ largely between species.

The macroscopic findings observed in birds naturally or experimentally inoculated with HPAIVs are diverse. The results of our study demonstrate that the tissue tropism of a particular HPAIVs strain can differ largely between severely affected individuals of different species. In H5N8 HPAIV-inoculated groups, the pancreas was the only organ that presented evident gross lesions in the three avian species and in case of the pigeon, it was not associated with viral antigen by IHC techniques. Chickens and geese inoculated with both viruses presented AIV antigen in almost all collected organs. Particularly, the central nervous system, pancreas and heart of both species recurrently presented large amounts of viral antigen associated to severe necrotico-inflammatory lesions. In chickens, H5N8 HPAIV also replicated at high levels in lung, whereas in geese the virus presented a marked hepato-tropism. However, comparatively low levels of viral antigen were detected in the liver and lung in chickens and geese, respectively. In the pigeon that succumbed to infection, the presence of viral antigen was restricted to the central nervous system and to a much lesser extent, to the heart. All in all, the results of the IHC technique reveal that the virus was circulating systemically in severely-affected birds; however, the variation in AIV antigen distribution between species infected with the same strain suggest that host factors shape to a large degree the tissue tropism of HPAIVs. The study of Kwon *et al.* [676] assessed the tissue tropism of a Gs/GD H5N1 HPAIV in five waterfowl species and found similar results to those presented here: whereas some presented viral antigen in multiple organs, in other species the replication was restricted to two or three organs, and mallard ducks lacked viral antigen in all tissues. Therefore, large differences in the main tissues affected exist even between closely related species. Differences in the distribution, type and substructures of SA receptors in tissues and in the immune responses after infection could be key factors that influence the differential tissue distribution of HPAIVs.

Despite the variability of affected tissues dependent on the virus and the species, the intense lesions and viral replication in numerous organs in chickens suggest that multi-organ failure is the likely cause for the lethal outcome. In geese, the onset of mortality clearly corresponded with an intense replication in the central nervous system; however, the high viral replication detected in other vital organs suggests that a similar situation as in chickens occurred. In contrast, in the pigeon the death was clearly associated with neurological dysfunction. In agreement with existing literature [365, 367, 372–374, 376, 423–426], our results confirm that the central nervous system seems to be a primary target organ and a strong determinant of the pathogenesis of HPAIVs in a wide variety of avian species.

In the present dissertation, we also evaluated the viral shedding in order to assess the potential role of each species to transmit different HPAIVs by direct contact or to contaminate the environment with infective secretions. The intense replication and severe infection of H7N1 and H5N8 HPAIVs in chickens and H5N8 HPAIV in geese corresponded with high viral shedding by both the oral and cloacal

routes. Numerous experimental studies in different species also found that severity of infection correlates with intensity of viral excretion [354–356, 400, 534, 628]. Our results demonstrate that chickens and domestic geese can play an important role as amplifiers and disseminators of these HPAIVs in case of infection, but it is unlikely that viral shedding may occur during long periods of time without being noticed by farmers.

High viral shedding can also occur in the absence of evident clinical signs, as demonstrated in domestic waterfowl infected with HPAIVs of the Gs/GD H5 lineage, which can shed virus during relatively long periods of time in a subclinical way [207–209]. In our studies, several domestic geese inoculated with H7N1 HPAIV and few pigeons inoculated with H7N1 and H5N8 HPAIV became subclinically infected and presented viral shedding by either the oral or cloacal route, or both. Despite the overall viral excretion in these birds were considered low, the viral loads in some OS collected from geese were considerably high. In addition, the detection of viral shedding in two geese at the last day of sampling (by 10 dpi) that were negative in the previous days of sampling suggest that intra-group transmission was occurring without being unnoticed. Therefore, our results suggest that domestic geese could play a role in the epidemiology of HPAIVs other than those of Gs/GD H5 lineage, even in those strains that are apparently adapted to gallinaceous species. Similar results were reported by Pantin-Jackwood *et al.* [194], when several non-Gs/GD H5 and H7 HPAIVs were transmitted between mallards in the absence of disease. Other studies have also demonstrated the capacity of waterbirds to excrete the Asian H7N9 lineage HPAIV at similar levels to those in chickens, and virus excretion have been detected up to 14 days after infection [286]. Thus, the low detection in waterfowl during outbreaks caused by classical lineages of HPAIVs in some cases could be the result of low exposure rather than to the poor biological compatibility. The potential role of different species in the dispersal, interspecies transmission and environmental contamination to different HPAIVs should be re-evaluated.

More and more, different studies are associating particular nucleotide changes that lead to amino acid substitutions in PB2, PB1, PA, HA, NP, NA, MP and NS viral proteins with adaptation, virulence and/or transmissibility of HPAIVs [629]. However, their effect appears to be lineage- specific and the residues involved could also vary between species [650]. Herein, the higher infectivity and virulence of the HPAIVs corresponded with their host of isolation (H7N1 HPAIV in a chicken, H5N8 HPAIV in a domestic goose). This suggests that the viruses may have acquired some mutations during their natural passage that increased their affinity to replicate easily in these species. In general, the amino acid identity between H7N1 and H5N8 HPAIVs was high in all viral proteins, and both HPAIVs shared the same amino acids in particular positions associated to adaptation, virulence and/transmission of HPAIVs in chickens and ducks (PB2: 123E; PB1: 3V, 38Y, 436Y; PA: 237E, 383D, 515T, 672L; NP: 105V, 184K;

M1: 43M; NS: 106M, 125D, 149A) [313, 445, 446, 573, 579, 581, 587–589, 597, 598, 603, 607–610]. For example, we detected that H5N8 clade 2.3.4.4 B present a glutamic acid at position 237 in PA protein, which is associated with increased virus replication, polymerase activity and PA nuclear accumulation in ducks [313]. In case this mutation also provides H5N8 HPAIV a higher virulence to domestic geese, this substitution could partially contribute to the apparent high virulence of H5N8 B HPAIV in domestic geese in comparison with H5N8 A HPAIVs. However, H7N1 also presents that particular amino acid in that position despite it is avirulent to domestic geese. Despite this particular amino acid may have a biological effect in H5N8 but not in H7N1 HPAIV, it is likely that pathogenic differences cannot be determined by mutations of single amino acids and is more probable to be caused by the synergistic effect of different amino acid substitutions in the same or other viral proteins that are still not described. Only two amino acids associated with a change in the phenotype differed between H7N1 and H5N8 HPAIVs (114S and 103F in NS1 protein), and their effect *in vivo* has not been evaluated [608]. Since we did not performed "gain of function" studies, the viral segments and particular amino acids associated with the differential infectivity and virulence of H7N1 and H5N8 HPAIVs in the different species could not be established. Since they belong to different HA and NA subtypes, their pathobiology could be attributed in large part to HA and NA proteins and their compatibility, both known to contribute largely to the HPAIV outcome [450–452, 629].

Another objective of the dissertation was to evaluate the existence of breed-related differences to HPAIV infection in different species. Several studies demonstrate a huge variation in susceptibility to infection with viruses (e.g. Infectious Bursal disease), bacteria (e.g. *Salmonella gallinarum*) and parasites (e.g. *Eimeria spp*) among chicken breeds/lines [467]. In waterfowl species, few studies have evaluated this assumption, whereas in other species such as in pigeons this information is lacking. Similarly as in two previous published studies using the same H7N1 HPAIV [399, 461], we detected significant differences in the severity of clinical presentation between chicken breeds. Interestingly, the breeds presenting higher resistance to H7N1 HPAIV were also more resistant to H5N8 HPAIV infection. This demonstrates that the resistance appear not to be subtype-specific, and the genetic background of particular breeds may confer an innate protection to different HPAIVs. In contrast, we did not found evident differences related to the breed in domestic geese and pigeons, but it could be due to the low number of breeds included and the extreme variation in virulence between both strains. In concordance with our studies, the differences in susceptibility to HPAIV infection between domestic duck breeds in two previous study were also reported to be low [469, 470].

As in most developed countries, the poultry industry in Spain is based on the production of highly-performance breeds of broilers and layer hens reared on commercial farms with high biosecurity

standards, and a smaller percentage is estimated to be outdoors or rear local breeds [536]. The worldwide presence of highly productive chicken breeds is considered to be a main reason of the decreasing genetic diversity and population of local breeds, which in some cases can be at risk of extinction [678]. There is a general believe that local breeds have acquired a natural resistance to disease as a result of the natural selection over the years by autochthonous pathogens, harmful climate and nutritional deficiencies [467]. In contrast, the high artificial selection in commercial breeds towards production-driven genes may be associated to undesirable side effects, including a potentially increased susceptibility to pathogens [468]. Therefore, valuable genetic resources would be lost if local breeds become extinct [678]. However, if local breeds present a higher immunological competence to HPAIVs in comparison with commercial breeds is still controversial. We found that 5 out of the 6 local chicken breeds included in the studies were more susceptible to HPAIV infection than commercial broilers and White-Leghorn SPF chickens, as demonstrated by more frequency of birds showing clinical signs and succumbing to infection, and higher number of birds shedding virus. These results are in agreement with other studies [462, 465], demonstrating that selection towards productive efficiency do not necessarily has a negative impact in resistance to HPAIV infection

In the case of chickens, regardless of the breed, we detected huge differences in susceptibility to HPAIV infection between individuals in the same group. With variable proportion depending on the overall susceptibility of the breed and the virulence of the virus, some birds died before the presence of evident signs of disease, several presented severe clinical signs prior to death associated to high viral shedding, whereas others in the same group did not show any evident sign of disease and did not shed detectable levels of virus by any route through all the experiment. Therefore, our results demonstrate the existence of individual-related factors and suggest that in independence of the breed, some birds are able to clear the virus in the earliest stages of infection.

Taking into account the huge individual differences in mortality in each breed group of chickens, we evaluated the possible role of the presence of a serine or an asparagine at position 631 of chicken Mx protein, which depends of the G to A nucleotide substitution, respectively. The presence of an asparagine at this position has been associated with a higher antiviral response against AIVs, but its effect *in vivo* remains unresolved [399, 461, 483, 487]. When grouping the birds of all breeds based on their genotype, the association of the different genotypes (AA, AG, GG) with percentage of mortality resulted not significant. However, AA and AG individuals presented a statistically significantly longer MDT than GG individuals. However, the MDT between groups only varied by 1 day. Despite the presence of an asparagines could confer chickens a higher capacity to impair viral replication in the early stages of infection, the biological implications *in vivo* are low. Therefore, selection based on other antiviral

genes is likely to contribute more to improve poultry breeding than Mx gene. Since the resistance to HPAIVs is multi-genic and we only measured a particular SNP in one exon of an antiviral gene, a broader battery of genes should be analysed to interfere the reason of the huge differences in susceptibility observed between chickens.

The HPAIV surveillance strategies depend on the target species (poultry or wild birds), and the approximation may vary among countries, but in general requires the collection of OS, CS, blood and/or tissues [503]. In our studies, the highest frequency of positive cases in OS in the different virus-host combinations suggests that this sample is the one of choice to detect HPAIVs in live birds. In geese, the detection in plasma correlated well with that in swabs; however, the difficulties in the sampling and their lack of improved sensitivity in comparison with OS discard plasma as a routine sample. Taking into account the high viral loads of HPAIV detected in feather pulps in several avian species after experimental inoculation, this sample have also been suggested to be useful for HPAIV diagnosis [679, 680]. In pigeons, the lack of a robust systemic infection indicates that feathers are not suitable for HPAIV screening and diagnosis. In addition, the detection of high amounts of viral antigen in the brain of severely affected birds in the three species indicate that this tissue is the sample of choice in birds found dead.

A proper surveillance approach should include sampling of those birds that are at a higher risk of infection. The permissiveness of local chickens but also of the local geese and pigeons to be infected by the HPAIVs used in the present study is a concern. These breeds can be found in backyards under low biosecurity measures, which allow the direct contact between them and with wild and peri-domestic birds. In these conditions, domestic geese can act as an intermediate host between wild birds and other species, increasing the risk of interspecies transmission and the potential generation of reassortants with altered pathogenic characteristics. In addition, these viruses may have the potential to spill back to wild birds. Since the range of susceptibilities varied from subclinical infection to severe associated to high and fast mortalities, local birds raised in backyards should be targeted during surveillance programs, and could serve as sentinels for the circulation of HPAIVs in the area. Therefore, a proper education of animal handlers to early recognize signs compatible with AIV infection is needed.

The present dissertation presents some limitations that are necessary to discuss. First, we used birds within a specific range of ages within the same study, but not between studies. Chickens were less than one month of age at the time of infection, whereas geese and pigeons where approximately of 3-5 and 6 months of age, respectively. Age has been demonstrated to be a main determinant of HPAIV infection In ducks, geese and turkeys, the mortality varies largely between young and adult birds, being

the juveniles considerably more susceptible [300, 457–459, 681, 682]. Thus, it is possible that the mortality in geese and pigeons could be higher than the reported in the present dissertation if younger birds were used. In fact, the higher mortality reported in ducks after inoculation with the same H7N1 HPAIV strain used in the present dissertation could be caused by differences in age [345]. A similar situation could occur in pigeons. Second, we used qRT-PCR in order to evaluate the viral load in the different samples. Molecular detection techniques represent a good alternative to viral isolation and titration in embryonated eggs and/or cells, which are more time-consuming. However, these techniques can also detect non-infectious particles. Since we did not conduct transmission studies, the biological significance of the viral excretion can be struggling when the viral loads are low, such as in pool water in H7N1 HPAIV-inoculated geese, and in OS and CS collected from pigeons.

In summary, the results of this dissertation demonstrate the complexity of infections with HPAIVs. The result of the infection depends greatly on the virus, and the host, including species, breed and individual factors, indicating that HPAIVs can acquire unusual characteristics during their evolution. Therefore, we highlight the necessity to continuously evaluate the pathobiological features of the emergent HPAIVs in different hosts. In addition, the establishment of infection in a sublinical way in domestic geese indicates that they could play a more important role than the one considered so far. We believe that the results of the present dissertation also provide a start point for future studies oriented to investigate the host-virus factors associated with the infection outcome in the different hosts.

CHAPTER 8

CONCLUSIONS

1. H5N8 HPAIV causing outbreaks in birds in Catalonia (northern Spain) in February-March 2017 belongs to clade 2.3.4.4 Group B of the Asian Gs/GD lineage of HPAIVs. The virus presents high homology with H5N8 B HPAIVs isolated in several European countries during 2016/2017 epidemics and clusters within genetic group 2.

2. The lower frequency of clinical signs, lower mortalities, longer MDTs and lower cloacal excretion in chickens inoculated with H5N8 HPAIV in comparison with those inoculated with H7N1 HPAIV indicate a lower affinity and/or adaptation of the H5N8 HPAIV to chickens.

3. The genetic background at individual and breed levels are important factors influencing the HPAIV infection outcome in chickens. However, almost all the local chicken breeds included presented a higher susceptibility to HPAIV infection than commercial breeds.

4. The genotypes AA and AG at position 2032 of chicken Mx gene are associated to slight but statistically significant longer mean times of survival after HPAIV infection, but not to the survival rate, contributing to the idea that HPAIV infection outcome is a polygenic trait.

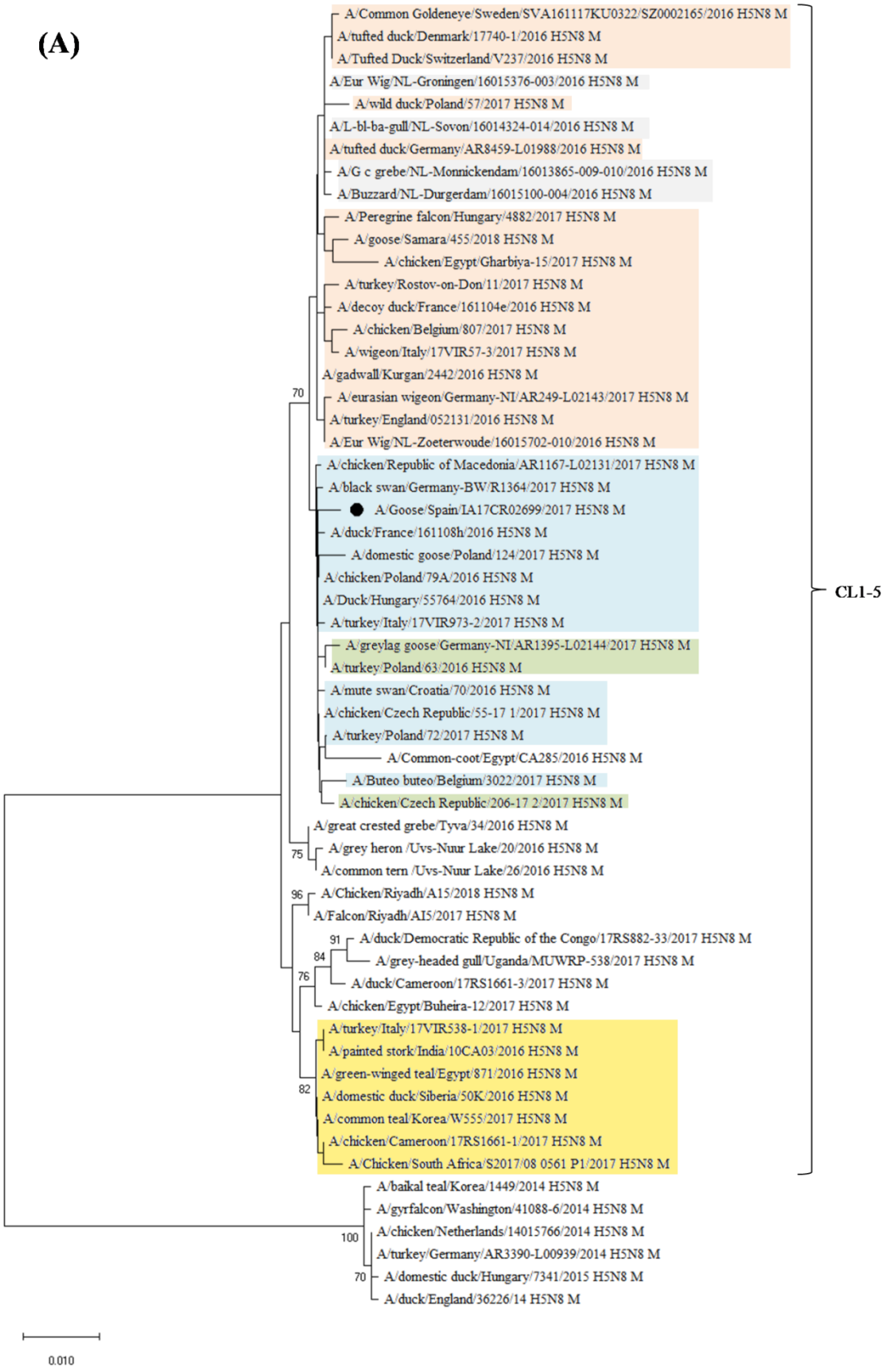
5. The high mortalities and systemic infections in the geese inoculated with HPAIV H5N8 demonstrate that the H5N8 Group B viruses circulating in Europe have acquired high virulence for domestic waterfowl. The intense viral shedding by the oral and cloacal routes and the detection of the virus in pool water indicate that geese could play an important role in the epidemiology of this virus.

6. Domestic geese are susceptible to H7N1 HPAIV infection but the virus is avirulent for this species. The high levels of viral RNA in some oral swabs and the evidences for intra-group transmission suggest that domestic geese can play a role in the perpetuation and interspecies transmission of classical lineages of HPAIVs.

7. Pigeons can be infected with H7N1 and H5N8 HPAIVs without presenting evident signs of disease. However, the presence of a pigeon with severe signs indicates that H5N8 can produce lethal infection in this species. The low shedding indicate a minimum role of pigeons as amplifiers of HPAIVs, but considering the wide habitat utilization of pigeons, they could play a role in the environmental dissemination of HPAIVs.

8. The high amounts of viral antigen detected in the brain in all the included species that succumbed to infection confirm that the neurotropism is a strong determinant of virulence of HPAIVs.

SUPP. MATERIAL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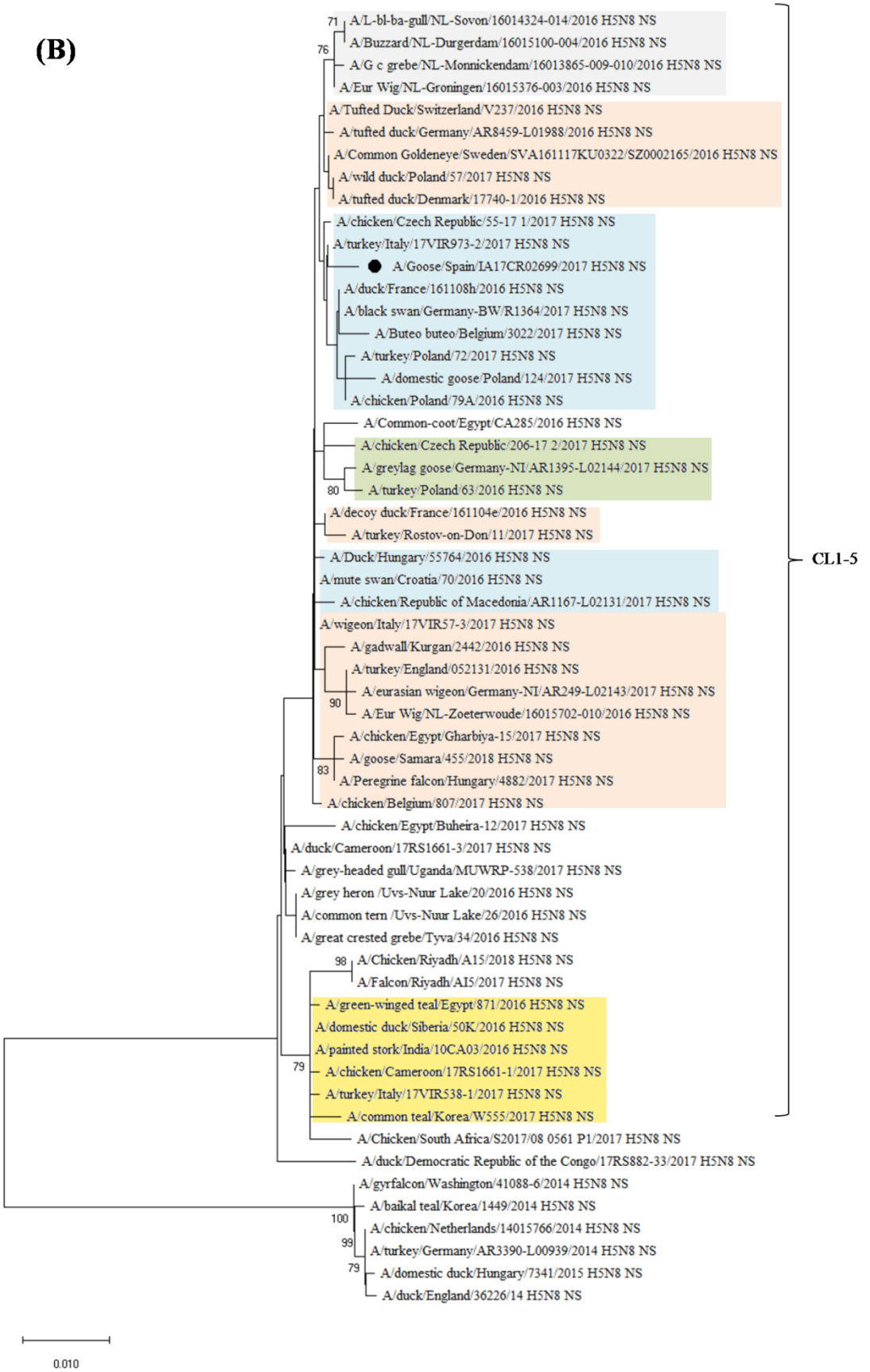
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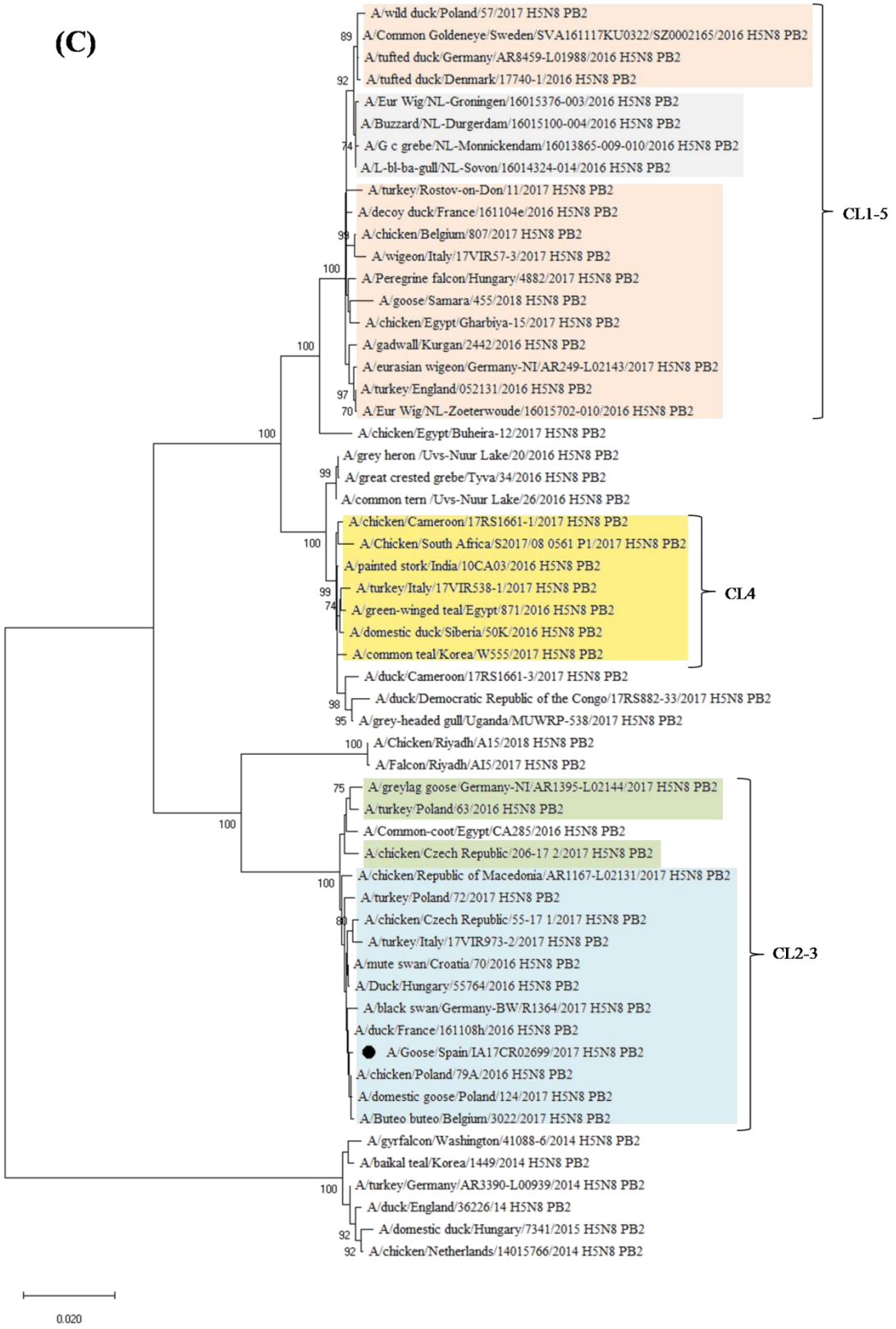
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A/chicken/Netherlands/14015766/2014 H5N8 PB2
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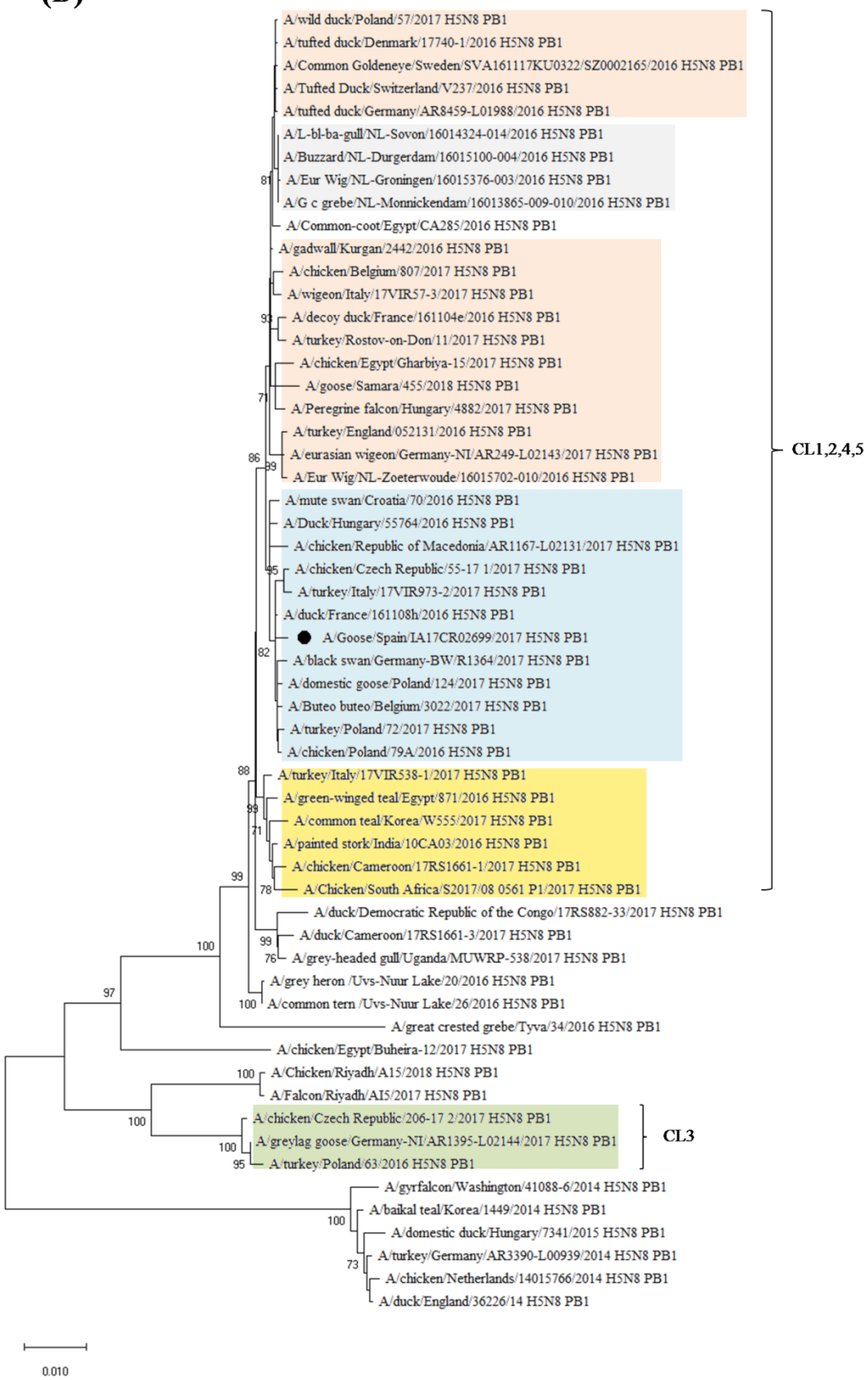
(D)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A/domestic goose/Poland/124/2017 H5N8 PB1
A/Buteo buteo/Belgium/3022/2017 H5N8 PB1
A/turkey/Poland/72/2017 H5N8 PB1
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CL1,2,4,5
A/duck/Democratic Republic of the Congo/17RS882-33/2017 H5N8 PB1
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A/grey heron /Uvs-Nuur Lake/20/2016 H5N8 PB1
A/common tern /Uvs-Nuur Lake/26/2016 H5N8 PB1
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CL3
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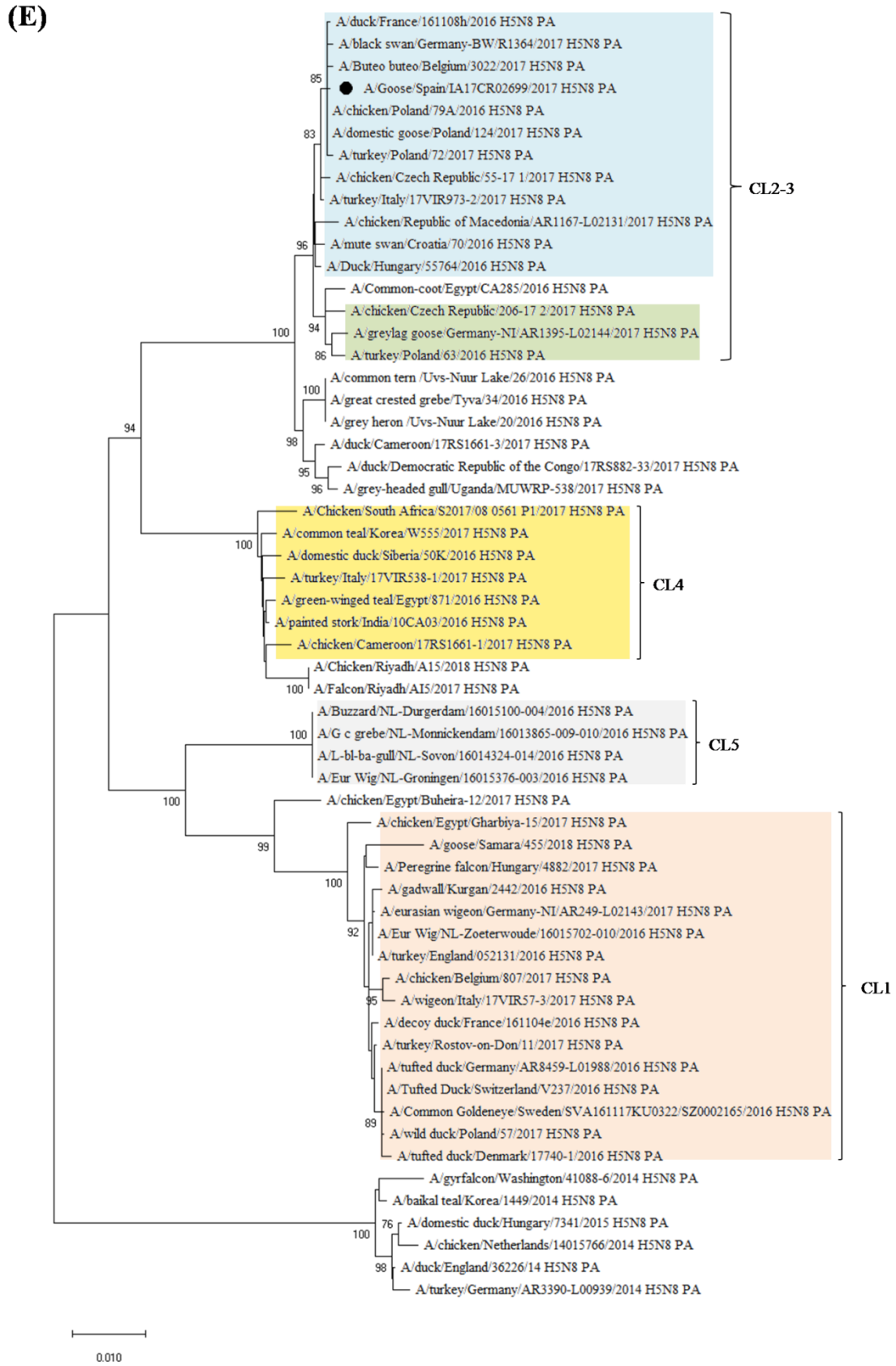
(E)
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A/Duck/Hungary/55764/2016 H5N8 PA
A/Common-coot/Egypt/CA285/2016 H5N8 PA
A/chicken/Czech Republic/206-17 2/2017 H5N8 PA
A/greylag goose/Germany-NI/AR1395-L02144/2017 H5N8 PA
A/turkey/Poland/63/2016 H5N8 PA
CL2-3
A/common tern /Uvs-Nuur Lake/26/2016 H5N8 PA
A/great crested grebe/Tyva/34/2016 H5N8 PA
A/grey heron /Uvs-Nuur Lake/20/2016 H5N8 PA
A/duck/Cameroon/17RS1661-3/2017 H5N8 PA
A/duck/Democratic Republic of the Congo/17RS882-33/2017 H5N8 PA
A/grey-headed gull/Uganda/MUWRP-538/2017 H5N8 PA
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A/turkey/Italy/17VIR538-1/2017 H5N8 PA
A/green-winged teal/Egypt/871/2016 H5N8 PA
A/painted stork/India/10CA03/2016 H5N8 PA
A/chicken/Cameroon/17RS1661-1/2017 H5N8 PA
CL4
A/Chicken/Riyadh/A15/2018 H5N8 PA
A/Falcon/Riyadh/AI5/2017 H5N8 PA
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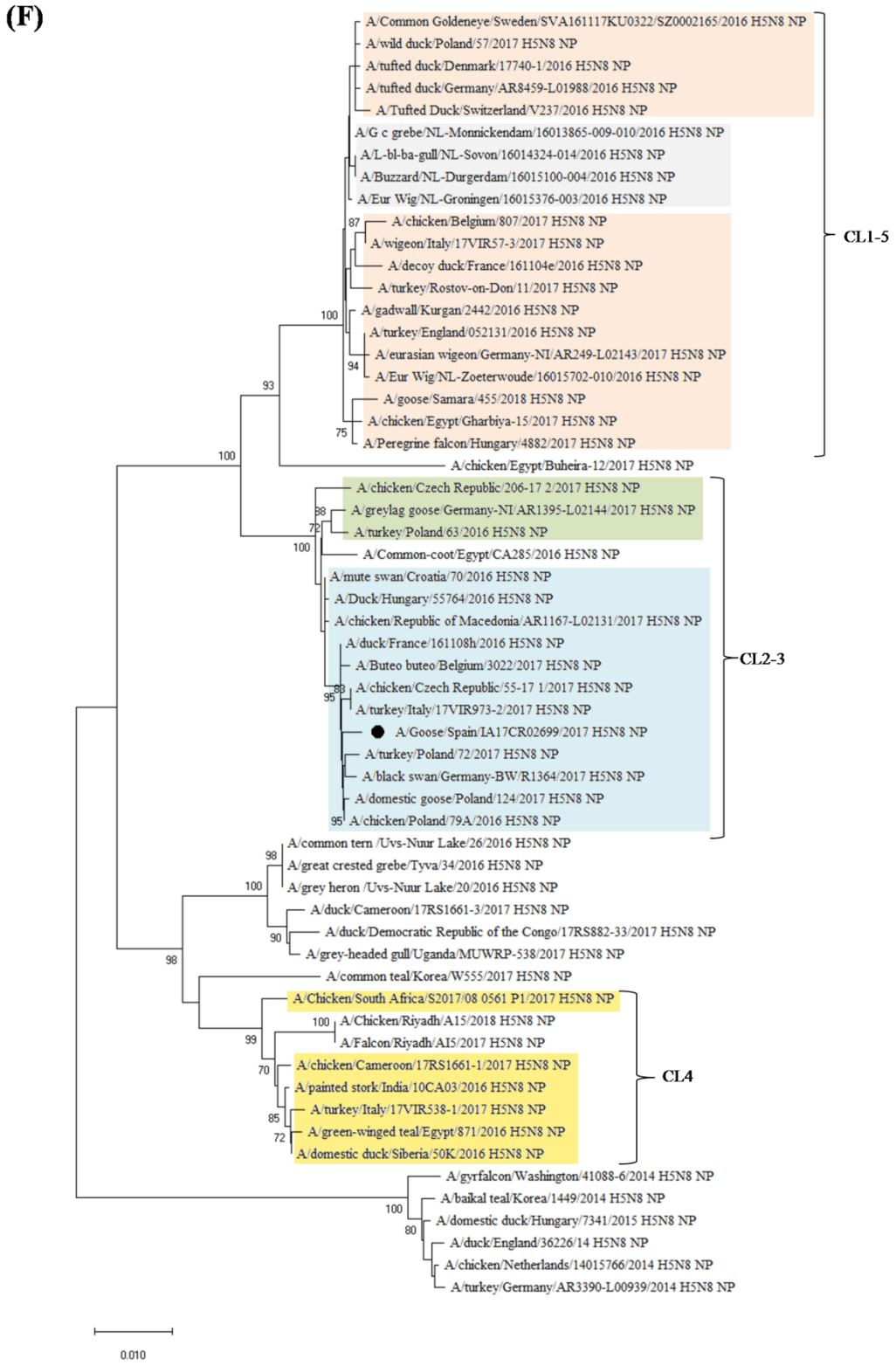

Supp. Figure 1. Neighbor-joining phylogenetic trees of M (A), NS (B), PB2 (C), PB1 (D), PA (E) and NP (F) gene segments. The Spanish H5N8 is highlighted with a black dot. Bootstrap values ≥70% (700/1000 replicates) are shown. The different genetic groups are presented: cluster 1 (orange), cluster 2 (blue), cluster 3 (green), cluster 4 (yellow), cluster 5 (grey). Scale bar indicates nucleotide substitution per site.

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