Immune response to influenza infection and vaccination

Resposta immunitària a la infecció i vacunació enfront al virus influença

Júlia Vergara i Alert

PhD Thesis

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Tesi doctoral presentada per na **JÚLIA VERGARA i ALERT** per optar al grau de Doctora 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 del Dr. **AYUB DARJI** i la tutoria de la Dra. **NATÀLIA MAJÓ i MASFERRER**

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Salvador Dali: *La persistencia de la memoria, 1931*

El Dr. **AYUB DARJI**, investigador del Centre de Recerca en Sanitat Animal (CReSA) i la Dra. **NATÀLIA MAJÓ i MASFERRER**, professora titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigadora adscrita al CReSA,

Certifiquen:

Que la memòria titulada "*Immune response to influenza infection and vaccination*" presentada per na **JÚLIA VERGARA i ALERT** per a l'obtenció del grau de Doctora en Veterinària, s'ha realitzat sota la seva direcció i supervisió i, considerant-la acabada, n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra (Barcelona), a 18 de juliol de 2012.

Dr. Ayub Darji	Dra. Natàlia Majó i Masferrer	Júlia Vergara i Alert
Director	Tutora	Doctoranda

Al pilar de la meva vida,
a la meva mare,

i al David,
l'altre part de mi.

Hi ha vegades que el nostre destí s'assembla a un arbre fruiter a l'hivern. Qui pot pensar que aquestes branques tornaran a rebrotar i florir? Però esperem que així sigui, i sabem que així serà"

[Johann Wolfgang von Goethe]

...i quan ets a ple hivern i sembla que la primavera no arribarà mai, quan penses que les branques del teu arbre no tornaran a florir, és llavors que el suport dels "teus" es fa del tot imprescindible. Sense la vostra ajuda avui no recolliria aquests fruits. He tingut la immensa sort d'estar envoltada de gent que m'ha ajudat a creure en la "primavera" i, el que és més important, a creure en mi. Espero que aquestes línies serveixin per a mostrar-vos l'afecte i gratitud que us tinc. Aquesta tesi és més vostra que meva!

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PART II: STUDIES

List of abbreviations

AIV	avian influenza virus(es)
BAL	broncho alveolar lavages
BALT	bronchial-associated lymphoid tissue
BIP	broncho-interstitial pneumonia
BSL-3	biosafety level 3
CALT	conjunctival-associated lymphoid tissue
CNS	central nervous system
CPDF30	cleavage and polyadenylation specificity factor
CTL	cytotoxic T cells
cRNA	complementary RNA
CS	cloacal swab
DC	dendritic cell
DIVA	differentiating infected from vaccinated animals
EID_{50}	median embryo infectious dose
ELD_{50}	median embryo lethal dose
ELISA	enzyme-linked immunosorbent assay
GALT	gut-associated lymphoid tissue
GMT	geometric mean titer
HA	hemagglutinin
HA1	hemagglutinin subunit 1
HA2	hemagglutinin subunit 2
HI	hemagglutination inhibition
HPAI	highly pathogenic avian influenza
HPAIV	highly pathogenic avian influenza virus(es)
IAV	influenza A virus(es)
IF	immunofluorescence
IFN	interferon

Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
ISM	informational spectrum method
IV	influenza virus
LPAI	low pathogenic avian influenza
LPAIV	low pathogenic avian influenza virus(es)
M1	matrix protein
M2	membrane ion cannel protein
MDCK	Madin-Darby canine kidney cells
MHC	main hisocompatibility complex
mRNA	messenger ribonucleic acid
NA	neuraminidase
NEP	nuclear export protein
NES	nuclear export signal
NLS1	nuclear localization sequence 1
NLS2	nuclear localization sequence 2
NP	nucleoprotein
NS1	non-structural protein 1
NS2	non-structural protein 2
OAS	2'-5'-oligoadenylate synthetase
OD	optical density
OPD	o-Phenylenediamine dihydrochloride
ORF	open reading frame
OS	oropharyngeal swab
PA	polymerase acid protein
PABPII	poly(A)-binding protein I
PB1	polymerase basic protein 1
PB1-F2	proapoptotic polymerase basic protein 1

PB2	polymerase basic protein 1
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PI3K	phosphoionositide 3-kinase
PKR	protein kinase
RBC	red blood cells
RBD	receptor binding domain
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
RNP	viral ribonucleoprotein
RT	room temperature
RTD	recognition and targeting domain
SA	sialic acid
SIV	swine influenza virus(es)
SLA	swine leukocyte antigen
SPF	specific pathogen free
SNT	seroneutralization assay
ssRNA	single-stranded ribonucleic acid
svRNA	small viral ribonucleic acid
$TCID_{50}$	median tissue culture infectious dose
TMB	3,3',5,5'-tetramethylbenzidine
vRNA	viral ribonucleic acid

Abstract

Influenza A viruses (IAV) are zoonotic pathogens that can replicate in a wide range of hosts, including birds, pigs and humans, among others. Millions of human infections caused by seasonal influenza virus are reported annually. Influenza pandemics have also a significant health and economic repercussions. Although certain subtypes of IAV are better selected in avian species than in humans, there are reports that evidence cases of human infections with avian influenza viruses (AIV). The susceptibility of pigs to infection with influenza viruses of both avian and human origins is also important for public health.

The genome of influenza virus is segmented and consists of eight single-stranded negative-sense ribonucleic acid (RNA) molecules encoding 11 or 12 proteins. Thus, if a single cell is simultaneously infected by two distinct influenza viruses, a reassortment can occur resulting in the generation of a novel virus strain. Moreover, mutations in the surface glycoproteins (mainly in the hemagglutinin, HA) are the responsible of the high variability of IAV.

Influenza vaccines against seasonal epidemics, although have good efficacy do not elicit immune response against a wide variety of IAV. Thus, seasonal vaccines only confer protection against the circulating viral strains. This, together with the risk of potential pandemics, has highlighted the importance of developing a universal vaccine able to elicit heterosubtypic immunity against multiple viral subtypes.

In this thesis the immune response to IAV infection and vaccination was evaluated in the light of the risk of highly pathogenic AIV (HPAIV) A/H5N1 and A/H7N1, and the pandemic IAV A/H1N1. The work is divided into three parts and each one is further divided into chapters.

Part I (chapters 1 and 2) contains the general introduction and the objectives of the thesis. The aim of this first part is to give a global overview and to introduce

information to understand (i) the influenza infection, (ii) the immune responses elicited after IAV infection and (iii) a brief summary of current vaccines against influenza. Afterwards, the initial objectives to be achieved are exposed.

Part II is the body of the thesis and it contains four studies (from chapter 3 to 6) developed during the four-year period comprising the PhD program. All the chapters are published or submitted to publish in international peer-reviewed journals. Thus, each study contains an abstract, a specific introduction, the materials and methods section, the obtained results and a discussion.

To study the role of IAV determinants and to characterize the influenza infection in different hosts could be of great importance to direct the efforts to the formulation of more efficient vaccines. The non structural 1 (NS1) protein is known to be a major determinant of virulence in mammals but little is known about its role in avian species. In chapter 3, the involvement of NS1 in viral pathogenicity was evaluated in chickens. Birds were challenged with two reassortant AIV carrying the NS-segment of H5N1 HPAIV in the genetic background of an H7N1 HPAIV. The pathological manifestations, together with the immunological outcome were evaluated.

The role of pre-existing immunity during an outbreak is also important and can determine whether the animals succumbed to infection or not. In chapter 4, chickens pre-exposed to H7N2 low pathogenic AIV (LPAIV) were challenged with H7N1 HPAIV and subsequently infected with H5N1 HPAIV. Pre-exposed animals were protected against the lethal H7N1-challenge whereas naïve animals succumbed. However, pre-existing immunity did not provide protection against HA-heterosubtypic virus (H5N1 HPAIV). The presence or absence of H7- and H5-inhibitory antibodies correlate with the protection (or lack of it) afforded.

The control of current vaccination programs and their efficacy is useful to plan and design better vaccines. It is well known that wildfowl are the reservoirs of IAV; thus they are extremely important concerning the ecology of the virus. Sera from several avian species from Spanish zoos and wildlife centers were collected during two successive vaccination programs and were tested to evaluate the vaccine-elicited

humoral response (chapter 5). The main objective of this work was to determine the efficacy of current vaccines (inactivated water-in-oil) in several avian species and to compare the differences inter- and intra-specie.

Finally, and taking into account the potential risk that IAV represent to our society, the efforts were focused on developing a broadly protective influenza vaccine. The 2009 human H1N1 pandemic (pH1N1) is a clear example that pigs can act as a vehicle for mixing and generating new assortments of viruses. In chapter 6 pigs were immunized with HA-derived peptides and subsequently infected with pH1N1 virus. Although the HA-peptides induced broad humoral and cellular responses no neutralization activity was detected and only a partial effect on virus clearance was observed.

Part III (chapters 7 and 8) is where the implications of all the findings from the studies are discussed and the major conclusions are listed.

A list of all the references used to develop the thesis is listed after the three parts, in an independent section. An appendix section is also included to give further information.

Resum *(en català)*

"Hi ha gent a qui no li agrada que es parle, s'escriga o es pense en català. És la mateixa gent a qui no li agrada que es parle, s'escriga o es pense" [Ovidi Montillor]

Els virus de la influença tipus A (VIA) són patògens zoonòtics que poden infectar un ampli nombre d'hostes incloent-hi les aus, els porcs i els homes, entre altres. Anualment es documenten milions d'infeccions en humans causades per virus de la influença estacionals. Les pandemies causades pel virus influença també tenen una elevada repercussió pel que fa a la sanitat i l'economia. Tot i que determinats subtipus de VIA s'adapten millor en espècies d'aus que en humans, hi ha hagut casos d'infeccions en humans per virus de la influença de tipus aviars. La susceptibilitat dels porcs per infectar-se amb virus de la influença tant d'origen aviar com humà és també important pel que fa a la salut pública.

El genoma del virus influença és segmentat in consta de vuit molècules de ARN de cadena senzilla i sentit negatiu que codifiquen per 11 o 12 proteïnes. Per tant, si una cèl·lula s'infecta simultàniament per dos VIA diferents, pot succeir un reagrupament amb la conseqüent generació d'una nova soca de virus. A més, mutacions a les glicoproteïnes de superfícies (sobretot a l'hemaglutinina, HA) són les responsables de l'elevada variabilitat de VIA.

Tot i que les vacunes front a les epidèmies estacionals són eficaces, no produeixen resposta immunològica front una amplia varietat de VIA. És a dir, les vacunes estacionals només protegeixen front a les soques virals circulants durant una determinada estació. Aquest fet, junt amb el risc de possibles pandèmies, han fet encara més important i urgent el desenvolupament d'una vacuna universal capaç de produir immunitat front a múltiples subtipus virals.

En la present tesis s'ha estudiat la resposta immunitària front a la infecció i vacunació del VIA en el context de VIA d'alta patogenicitat (vIAAP) A/H5N1 i A/H7N1 i el virus pandèmic A/H1N1 (pH1N1). El treball s'ha dividit en tres parts i cada part s'ha subdividit en capítols.

Part I (capítols 1 i 2), conté la introducció general i els objectius de la tesi doctoral. L'objectiu d'aquesta primera part és donar una visió global i introduir informació per entendre (i) la infecció pel virus de la influença, (ii) la resposta immunològica provocada després de la infecció per VIA i (iii) un breu resum de les vacunes actuals front a influença. A continuació, s'exposen els objectius a aconseguir.

Part II, és el cos de la tesis i conté els quatre treballs (del capítol 3 al 6) duts a terme durant els quatre anys que ha durat el programa de doctorat. Tots els capítols presentats han estat publicats o sotmesos a publicació en revistes indexades internacionals. Per tant, cada estudi manté l'estructura estàndard de: resum, introducció específica, materials i mètodes, resultats i breu discussió.

Estudiar el paper dels determinants virals i caracteritzar la infecció pel VIA en diversos hostes pot ser de gran interès a l'hora de dissenyar vacunes òptimes. S'ha descrit la proteïna NS1 com a un dels principals determinants de virulència en mamífers, però no s'ha estudiat gaire el paper d'aquesta en aus. En el capítol 3 es va avaluar la implicació de la proteïna NS1 en la patogenicitat viral en pollets. Es van infectar pollets amb vIAAP H7N1 que contenien el segment NS de vIAAP H5N1. Les manifestacions patològiques i la resposta immunològica conseqüència de la infecció amb cada un dels virus van ser avaluades.

També és molt important el paper de la immunitat prèvia durant un brot perquè pot ser determinant de la mort o supervivència de l'animal. En el capítol 4 es van exposar pollets a un virus H7N2 de baixa patogenicitat (vIABP) i a continuació es van infectar amb un vIAAP H7N1. Posteriorment es van infectar amb un vIAAP H5N1. Els animals que havien estat infectats prèviament amb vIABP quedaven protegits a la posterior infecció letal amb el vIAAP H7N1. No obstant, la resposta immunitària produïda no era suficient per a protegir els pollets front a la infecció amb

un virus heterosubtípic (vIAAP H5N1). La presència o absència d'anticossos inhibitoris front a H7- i H5- correlacionaven amb la presència o absència de protecció, respectivament.

Conèixer els programes de vacunació actuals i la seva eficàcia és útil per a planificar i dissenyar futures estratègies de vacunació. Les aus aquàtiques són el reservori dels VIA; per tant, són extremadament importants pel que fa a l'ecologia del virus. Aprofitant els programes de vacunació es va testar el sèrum de diverses espècies d'aus de zoològics i centres de recuperació d'Espanya (capítol 5). Els sèrums es van utilitzar per a l'avaluació de la resposta humoral deguda a la vacuna. El principal objectiu del treball era determinar l'eficàcia de vacunes disponibles (inactivades en suspensió oliosa) en diverses espècies d'aus i comparar la variabilitat inter- i intra-espècie.

Finalment, i tenint en compte el potencial risc del VIA, els esforços es van focalitzar en desenvolupar una vacuna capaç de protegir a un ampli nombre de subtipus de VIA. La pandèmia de 2009 amb el virus H1N1 (pH1N1) és un clar exemple que els porcs poden actuar com a "coctelera" i generar nous virus. En el capítol 6 es van immunitzar porcs amb pèptids derivats de l'HA i a continuació es van infectar amb el virus pH1N1. Tot i que els pèptids-HA produïen una molt bona resposta humoral i cel·lular, no es va detectar activitat neutralitzant i només es va obtenir un efecte parcial en l'eliminació del virus.

Part III (*Capítols 7 i 8*), és la secció on es discuteixen les implicacions dels resultats obtinguts en els diferents estudis i on s'enumeren les conclusions principals. En una secció a part, s'han inclòs totes les referències bibliogràfiques utilitzades per a l'elaboració de la tesi. S'ha inclòs també un apèndix per afegir informació addicional.

The results presented in this thesis have been published or submitted for publication in international scientific peer-reviewed journals:

Vergara-Alert J; Busquets N; Ballester M, Chaves AJ; Rivas R; Dolz R; Pleschka S; Majó N; Rodríguez F; Darji A. *The NS1Protein of H5N1 Avian Influenza Viruses (AIV) Enhances the Virulence of an H7N1 AIV in Chickens.* Submitted for publication

Vergara-Alert J; Moreno A; Costa TP; González JP; Bertran K; Cordón I; Rivas R; Majó N; Busquets N; Cordioli P; Rodríguez F; Darji A. *Exposure to a Low Pathogenic A/H7N2 Virus in Chickens Protects against Highly Pathogenic A/H7N1 Virus but not against Subsequent Infection with A/H5N1.* Submitted for publication

Vergara-Alert J; Fernández-Bellon H; Busquets N; Alcántara G; Delclaux M; Pizarro B; Sánchez C; Sánchez A; Majó N; Darji A. *Comprehensive Serological Analysis of Two Successive Heterologous Vaccines against H5N1 Avian Influenza Virus in Exotic Birds in Zoos.* Clinical and Vaccine Immunology, 2011 May; 18(5):697-706.

Vergara-Alert J; **Argilaguet JM; Busquets N; Ballester M; Martín-Valls GE; Rivas R; López-Soria S; Solanes D; Majó N; Segalés J; Veljkovic V; Rodríguez F; Darji A.** *Conserved Synthetic Peptides from the Hemagglutinin of Influenza Viruses Induce Broad Humoral and T-Cell Responses in a Pig Model.* PLoS ONE, 2012; 7(7):e40524.

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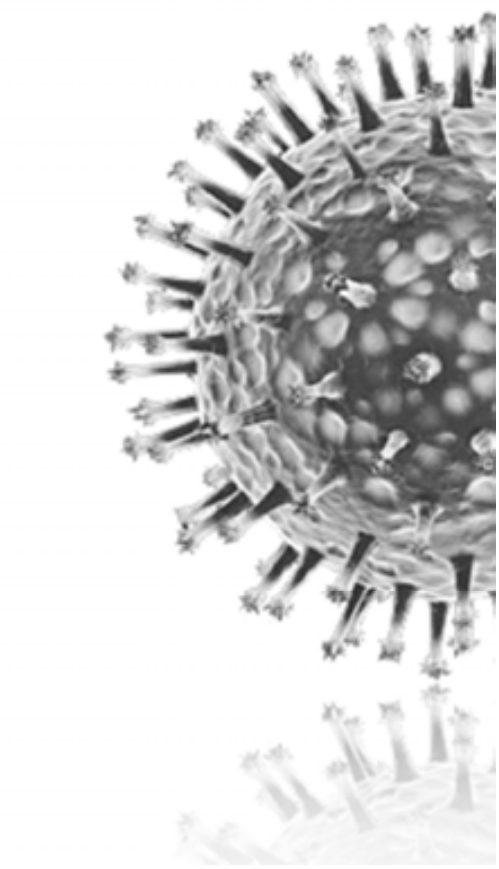
[Confucius]

PART I:

General Introduction and Objectives



"Ningú ens va dir que ho intentéssim, ningú ens va dir que seria fàcil…

algú va dir que som els nostres somnis, que sinó somniem, estem morts"

[Kilian Jornet Burgada]

Chapter 1

General Introduction

1.1. Influenza infection

1.1.1. Etiological agent: influenza virus

1.1.1.1. Classification and nomenclature

Influenza viruses belong to the *Orthomyxoviridae* family which includes five genera of RNA viruses: Influenzavirus A, B and C, Isavirus and Thogotovirus (ICTV, 2005; Suarez, 2008). The classification of influenza viruses into genera is based on serological reactions based on their nucleocapsid and matrix protein antigens (Alexander, 2008). Avian influenza viruses (AIV) are classified within the type A or influenza A viruses (IAV) and they are further subdivided into subtypes according to their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). At present, 17 subtypes of the HA and 9 subtypes of the NA have been described (Fouchier *et al.*, 2005; Wright *et al.,* 2007; Alexander, 2008; Tong *et al.*, 2012).

Influenzavirus B mainly affect humans, but it has been described that the seal (Osterhaus *et al.,* 2000) and the ferret (Jakeman *et al.,* 1994) are also susceptible to infection with this genus. Influenza C virus can infect humans, dogs and pigs, but is less common that the other genera [Guo *et al.,* 1983; Ohwada *et al.,* 1986; Manuguerra *et al.,* 1992; Kimura *et al.,* 1997; Matsuzaki *et al.,* 2006].

The current nomenclature for the designation of influenza viruses includes: the antigenic type or genera, the host of origin (except humans), geographic localization of isolation, strain reference number and year of isolation (WHO, 1980; Suarez, 2008). The HA and NA subtype of influenza viruses is indicated in parentheses; e.g. A/Goose/Guangdong/1/96 (H5N1) and A/South Carolina/1/18 (H1N1).

1.1.1.2. Morphology and genome organization

Influenza viruses are enveloped and spherical to pleomorphic, with a diameter ranging from 80-120 nm (Fujiyoshi *et al.*, 1994; Lamb *et al.*, 2001). The genome of influenza is

single-stranded, negative sense RNA, ssRNA(-), containing eight segments of viral RNA (vRNA) coding for 11 or 12 proteins (*Figure 1-1*) (Palese *et al.*, 2007; Wright *et al.*, 2007; Wise *et al.,* 2009).

On the lipid-bilayer membrane there are two major surface glycoproteins (HA and NA) which are projected from the viral envelope and expressed from their own segment (4 and 6, respectively) and a minor ion channel protein M2 encoded by the M segment (segment 7). The internal proteins that form IAV are: the nuclear export protein (NEP or NS2) and the non-structural protein 1 (NS1), which are encoded by the NS segment (segment 8); the nucleoprotein (NP) and the polymerase complex, expressed from their corresponding segments (5 and 1-3, respectively); the matrix protein M1, which is encoded by the M segment (segment 7); and the newly discovered N40 protein, expressed from the second segment (Wise *et al.*, 2009).

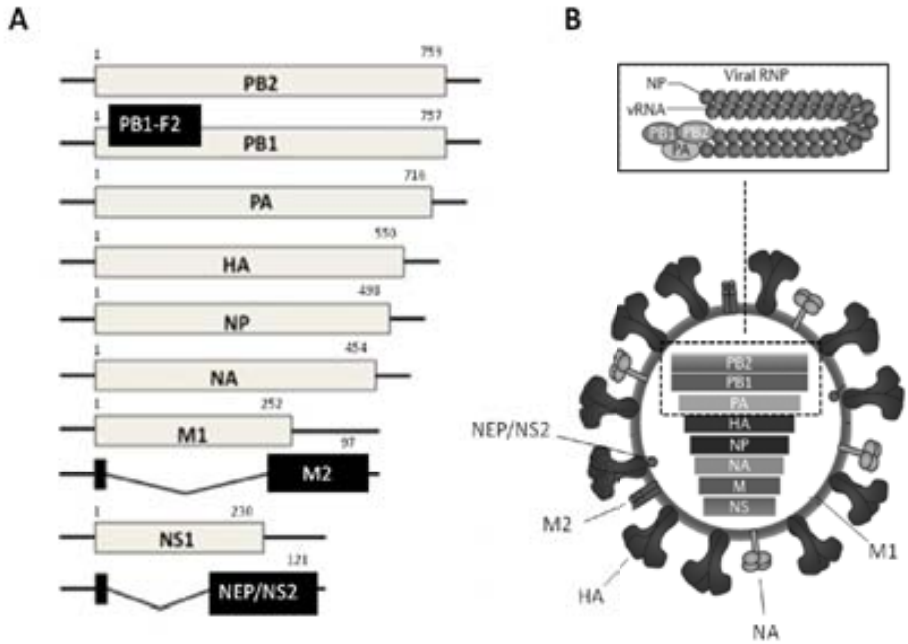


Figure 1-1. Schematic representation of the Influenza A virus genome and particle. **a|** Genome structure of IAV: RNA segments (in nucleotides) and their encoded proteins. **b|** Diagram of and IAV particle: HA, NA and M2 are inserted into the host-lipid membrane; the M1 underlies this lipid envelope and NEP/NS2 is also associated with the virus. The viral RNP is shown in detail (modified from Palese *et al.,* 2007; Medina and García-Sastre, 2011).

The polymerase complex is formed by polymerase acid protein (PA) and the polymerase basic protein 1 (PB1) and 2 (PB2) (Detjen *et al.,* 1987; Area *et al.,* 2004). In addition, some viruses express the protein PB1-F2, encoded in an alternative open reading frame (ORF) near de 5' terminal of the PB1 gene (segment 2) (Chen *et al.,* 2001). The genome structure of IAV is represented in *Figure 1-1a*. Within the virion, each of the viral segments form a viral ribonucleoprotein (RNP) complex, which consists in vRNA coated with NP and bound to the viral polymerase complex (*Figure 1-1b*) (Noda *et al.,* 2006).

Functions of viral proteins and their implications in viral pathogenicity are discussed below (section *1.1.1.3.* and *1.1.3.2*).

1.1.1.3. Replication cycle and viral proteins

During virus replication (*Figure 1-2)* the HA attaches to either, α-2,6- or α-2,3- linked sialic acids (α-2,6-SA or α-2,3-SA) on the host cell surface. Human influenza viruses show preference for the α-2,6-SA, whereas AIV better binds to α-2,3-SA (Rogers *et al.,* 1983; Connor *et al.,* 1994; Yu *et al.,* 2011; Costa *et al.,* 2012). Once attached, the virus can enter the cell by receptor-mediated endocytosis (Patterson *et al.,* 1979).

The precursor protein HA0 is cleaved by a trypsin-like protease (cellular proteases) into HA subunit 1 and 2 (HA1 and HA2) (Kawaoka and Webster, 1988; Chen *et al.,* 1998]. HA1 contains the receptor binding and antigenic domains while HA2 mediates the fusion of both membranes (Steinhauer, 1999). Thus, the HA cleavage is necessary for the fusion between the viral envelope and the endosomal membrane. A decrease in the pH inside the endocytic vesicle is triggered by the M2 ion channel (Bullough *et al.,* 1994; Pinto and Lamb, 2007). The acidification is required to uncoat the RNP complexes containing the viral genome and also to release these complexes in the cytoplasm. Afterwards, vRNA is transported to the nucleus where replication takes place (O'Neill *et al.,* 1998).

In the nucleus, the RNA-dependent RNA-polymerase is the responsible of the transcription and replication of the viral RNA ((-) vRNA) resulting in three types of RNA: the complementary RNA ((+) cRNA), which serve as a template to generate more vRNA; small viral RNAs (svRNAs), which is suggested to regulate the switch from transcription to regulation; and the viral messenger RNAs (mRNAs), which are exported to the cytoplasm for translation (Bouvier and Palese, 2008; Perez *et al.,* 2010; Umbach *et al.,* 2010). Those proteins required in replication and transcription processes are transferred back to the nucleus, and RNPs are exported to the cytoplasm for packaging, supported by M1 and NEP/NS2 (O'Neill *et al.,* 1998). The synthesized proteins (HA, NA, M2) arrive at the plasma membrane transported by the *trans*-Golgi pathway, where M1 protein helps in the formation of virus particles. After budding, the release of the virion from the host cell is mediated by the NA, which destroys the SA of both, the viral and cellular glycoproteins (Lamb *et al.*, 2001; Medina *et al.,* 2011).

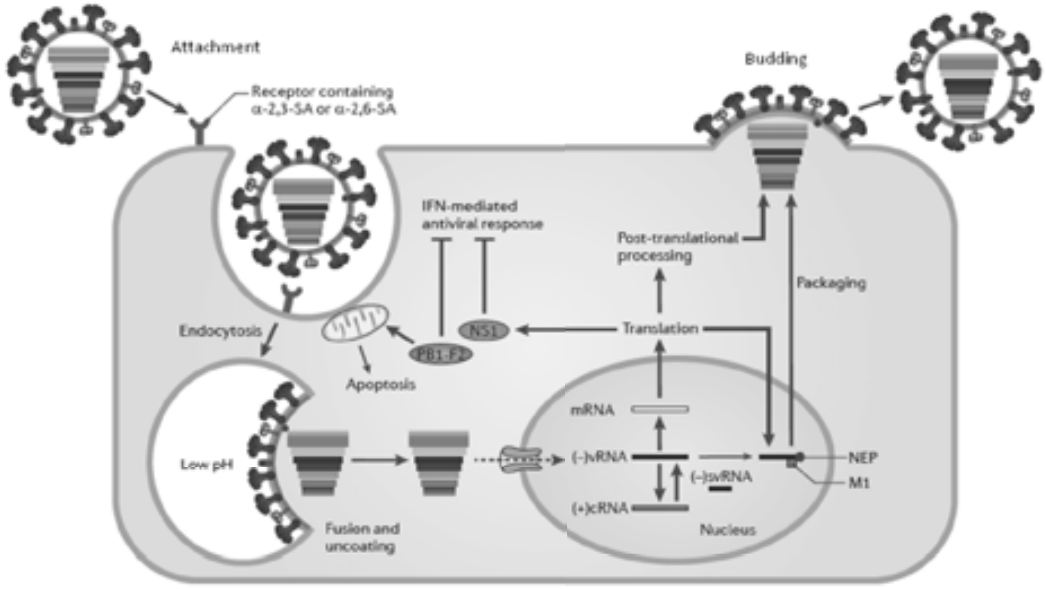


Figure 1-2. Representation of the replication cycle of Influenza A virus (modified from Medina *et al.,* 2011).

1.1.1.4. Genetic and antigenic variability

Influenza A viruses can suffer changes in their genome by two mechanisms: *antigenic drift* (punctual mutations) or *antigenic shift* (genetic reassortment). These phenomena mainly happen due to the segmented genome of the IAV, but also because of the lack of a proofreading mechanism of replication (error-prone RNA-dependent RNA-polymerase) (Palese *et al.,* 2007; Suarez, 2008).

Antigenic drift is caused by the immunological pressure over HA and NA as a result of vaccination (e.g. seasonal vaccination in humans) and also in situations where viruses are circulating during a large period in the field (e.g. AIV circulation in poultry industry); thus, increasing their adaptation by mutations, mainly in the HA (Plotkin *et al.,* 2003). The antigenic changes result in a virus strain that cannot be neutralized by pre-existing antibodies (Bouvier and Palese*,* 2008); consequently, the viruses can replicate and transmit more efficiently (Suarez, 2008).

On the other hand, the reassortment of genes from different influenza virus subtypes or *antigenic shift* can happen when a single cell or host is infected by different virus subtypes (Bouvier and Palese*,* 2008). The transmission of an entire virus from one species to another is also considered an antigenic shift. In the following section 1.1.2 (see *1.1.2.2.*, *"Influenza A in humans"*) an example of a recent IAV reassortment (an outbreak of swine-origin H1N1 viruses started in February 2009) is mentioned (Vincent *et al.,* 2008; Fraser *et al.*, 2009; Neumann *et al.,* 2009; Smith *et al.,* 2009; Medina and García-Sastre, 2011).

1.1.2. Epidemiology and importance

1.1.2.1. Host range

Type A influenza affects a wide range of birds and mammal species, including humans, pigs, horses and dogs (Webster *et al.,* 1992; Kalthoff *et al.,* 2010). Most IAV circulate in waterfowl, the natural reservoir (Munster *et al.,* 2006; Stallknecht and Brown*,* 2008), except the recent described HA-subtype (H17) which, so far, was only isolated from fruit bats and which is very divergent from other IAV (Tong *et al.,* 2012).

From wild aquatic birds the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls, terns and waders) are considered the major natural reservoir for AIV (Stallknecht and Brown, 2008; Suarez, 2008). In these populations the transmission of AIV is fecal/oral dependent via contaminated water (Hinshaw *et al.,* 1979; Brown *et al.,* 2007). From poultry, although chickens and turkeys are the most commonly IAV-affected species, IAV are demonstrated to infect other gallinaceous birds such as Japanese and Bobwhite quail, guinea fowl, pheasants and partridges (Perkins and Swayne *et al.,* 2001).

1.1.2.2. Epidemics and pandemics

Influenza A in birds

In avian species, AIV cause a wide range of clinical manifestations, from asymptomatic to a severe acute disease with mortality rates reaching 100% (Swayne and Pantin-Jackwood, 2008). Thus, AIV can be classified on the basis of their virulence in chickens into low and high pathogenic avian influenza (LPAI and HPAI) viruses (LPAIV and HPAIV) (Webster *et al.*, 1992; Swayne and Suarez, 2000; Perkins and Swayne, 2001; Capua and Alexander, 2004; Suarez, 2010).

Although HPAI was traditionally restricted to domestic poultry, it was considered a rare disease, with only 17 episodes being reported worldwide from 1959 to 1998 (Alexander, 2000). However, there were evidences that HPAI could also affect wild birds, as demonstrated in 1961 in terns (Becker, 1966). Since 1999, several epidemics involving HPAIV have occurred in poultry (affecting mainly chickens and turkeys) and farmed ostriches (Capua *et al.,* 2000; ProMed-mail, 2004). Moreover, during the lasts years, an increased number of LPAI incidences caused by H5 and H7 HA-subtypes have also been noted. Current evidences strongly support the hypothesis that HPAIV arise as a result of H5 or H7 LPAIV mutations (Li *et al.,* 1990; Capua and Alexander, 2004; Kalthoff *et al.,* 2010; Suarez, 2010) or as a consequence of reassortments between LPAIV subtypes that co-infect birds (Sharp *et al.,* 1997; Dugan *et al.,* 2008].

In the last 15 years, the number of outbreaks has been unprecedented, affecting a wide range of avian species worldwide: Hong Kong in 1997 (H5N1) (Shortridge *et al.,* 1998), Italy in 1999 (H7N1) (Capua and Marangon, 2000), Chile in 2002 (H7N3) (Suarez *et al.,* 2004), the Netherlands in 2003 (H7N7) (Stegeman *et al.,* 2004), Canada 2004 (H7N3) (Bowes *et al.,* 2004) and Southeast Asia, since 2003 (H5N1) (Li *et al.,* 2004). Although wild birds were not suggested to be implicated in the initial HPAI cases, in 2002, an outbreak of H5N1 HPAIV affected a wide range of wild birds in Hong Kong (Ellis *et al.,* 2004). Since then, the H5N1 HPAIV has spread round the world affecting a huge number of avian species with, not only ecological and economic consequences, but also with zoonotic risk (Xu *et al.,* 1999; Lin *et al.,* 2000].

Influenza A in pigs

Swine influenza viruses (SIV) can infect pigs and humans, but also wild boar (Saliki *et al.,* 1998) and avian species, such as domestic turkey (Hinshaw *et al.,* 1983; Olsen *et al.,* 2006) and, less common, waterfowl (Ramakrishnan *et al.,* 2010).

Although pigs can be experimentally infected with several IAV subtypes (Kida *et al.,* 1994), only H1N1, H1N2 and H3N2 SIV subtypes circulate widely among them. Naturally occurring infections of pigs with several subtypes of AIV have also been documented (Kida *et al.,* 1988; Brown, 2000; Choi *et al.,* 2005), but without maintenance in the swine population. SIV is widespread in farms of many European countries (Van Reeth *et al.,* 2008; Simon-Grifé *et al.,* 2011) with prevalences being higher in sows than in fattening pigs (Poljak *et al.,* 2008; Simon-Grifé *et al.,* 2011). However, the potential risk of IAV infections in pigs is that they are suggested to act as *mixing vessel* hosts to generate new assortments of influenza viruses potentially pathogenic (Van Reeth, 2007) (see *section 1.1.3.1*).

Influenza A in humans

Influenza A virus is the responsible of recurrent epidemics and global pandemics. In humans, seasonal influenza result in millions of infections worldwide with significant

health and economic burdens (Molinari *et al.,* 2007). Currently, together with influenza B virus, there are strains from two IAV subtypes circulating in human population: H1N1 and H3N2; but from 1957 to 1968, viruses from the H2N2 subtype were also present in humans (Schäffer *et al.,* 1993).

Pandemics can also have devastating effects resulting in millions of deaths (Johnson *et al.,* 2002). Human population has experienced four pandemics since the beginning of the 20th century: "Spanish influenza" in 1918-1919 (H1N1), "Asian influenza" in 1957 (H2N2), "Hong Kong influenza" in 1968 (H3N2), and "Russian influenza" in 1977 (H1N1). These pandemics were the consequence of: direct IAV infection and adaptation in humans (1918), reassortments between human and avian viruses (1957 and 1968) and re-emergence of the H1N1 virus (1977) (Horimoto and Kawaoka, 2005; Taubenberger e*t al*., 2005; Taubenberger, 2006). Recently, the influenza pandemic (pH1N1) emerged in 2009 by reassortment producing a new virus containing genes from avian virus (PB2 and PA), PB1 from a human virus, and the other gene segments from two distinct lineages of swine viruses (Vincent *et al.,* 2008; Fraser *et al*., 2009; Neumann *et al.,* 2009; Smith *et al.,* 2009; Medina and García-Sastre, 2011]. This was considered the first pandemic of the 21st century.

Thus, as historically demonstrated, the introduction of fully AIVs (H5-, H7- and H9-subtypes) have been reported to be transmitted from birds to humans with severe consequences (Banks e*t al.,* 1998; Subbarao *et al.,* 1998; Fouchier *et al.,* 2004]. Moreover, by genetic reassortment of human influenza virus with SIV and/or AIV, new viruses can be introduced into humans.

1.1.3. Viral pathogenesis

To be efficiently transmitted and cause disease, IAV have to be shed, find the appropriate attachment region and replicate into the new host. Therefore, the pathogenesis depends on both, the viral strain and the receptive host. In the current section the dissemination in the different hosts and the viral determinants are

introduced, with special focus to those related with the studies presented in this thesis: the HA and NS1 proteins.

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1.1.3.1. Dissemination in the host

The upper respiratory tract is the main initial site of IAV-replication. Influenza virus requires binding of viral HA to host glycans or gangliosides that terminate in sialic acids (SA) to start the infection (see section *1.1.1.3*). In the epithelial cells of the nasal cavity, the virus replicates causing inflammation and afterwards, it is released infecting other cells. To initiate the dissemination of IAV, the macrophages and heterophils recruited in the inflammation site are required (Swayne and Pantin-Jackwood, 2008). Various host factors which affect the viral life cycle have been described (Suzuki, 2005; Hatta *et al.,* 2007; Watanabe *et al.,* 2010; Brotz *et al.,* 2011) leading to evolution of species-specific virus lineage (Parrish and Kawaoka, 2005).

In poultry species, after replication, LPAIV can infect other cells in the respiratory and in the intestinal tracts (Swayne, 2007) were α-2,3-SA receptors are found abundantly. As a consequence, the infected birds can show respiratory signs and shed the virus in their feces. Thus, virus transmission occurs primarily by the fecal-oral route (Webster *et al.,* 1992). Occasionally, LPAIV replicate in kidney, pancreas and other organs containing trypsin-like proteases (Swayne and Halvorson, 2008). Contrarily, after HPAI infection and replication, the virus spreads through the vascular system causing viremia and extensive visceral damage, with severe clinical signs and a multiorgan failure responsible of animal death. Therefore, HPAIV are more readily transmitted by nasal and oral routes (Swayne and Halvorson, 2008). Interestingly, receptors from both types (α-2,3-SA and α-2,6-SA) have been found in other avian species, such as pheasants, turkeys, quail and guinea fowl (Wan *et al.,* 2006; Kimble *et al.,* 2010), suggesting these species as potential vehicle to generate new assortments of influenza viruses .

SIV infection is normally restricted to the respiratory tract and viral replication has been demonstrated in epithelial cells of the nasal mucosa, tonsils, trachea, lungs

and tracheobronchial lymph nodes (Brown *et al.,* 1993; Heinen *et al.,* 2000). As previously mentioned, pigs have receptors containing both α-2,3-SA and α-2,6-SA in their trachea that allow for binding of both avian and human viruses (Ito e*t al.,* 1998).

In humans, IAV infects the epithelial cells of the larynx, trachea and bronchi (containing α-2,6-SA receptors) and can also infect type I and II pneumocytes, were α-2,3-SA receptors are found (Guarner *et al.,* 2000). Although AIV have been also isolated in humans, most AIV transfers to primate species have resulted in limited spread (Parrish and Kawaoka, 2005; Kalthoff *et al.,* 2010).

1.1.3.2. Viral determinants

Among the first lines of defense against influenza virus infection, type I interferon (IFN) response plays a major role. Many viruses have developed strategies to evade host innate immune responses, e.g. Newcaslte disease virus (Park *et al.,* 2003) and bovine respiratory syncytial virus (Bossert and Conzelmann, 2002) which counteract the host type I IFN antiviral- response. To survive in nature and to combat against the antiviral response mounted by the infected cells (Randall and Goodbourn, 2008), IAV have also evolved multiple mechanisms.

Several studies indicate the relevance of certain amino acid positions of the PB2 protein in relation to the host range of the virus strain and the viral efficiency in replication and pathogenicity (Subbarao *et al.,* 1993; Hatta *et al.,* 2001; Yao *et al.,* 2001; Hatta *et al.,* 2007). More recently, PB2 has been shown to inhibit IFN-β production (Iwai *et al.,* 2010). The proapoptotic PB1-F2 protein is described to act as modulator of polymerase activity by interaction with the PB1 protein (Mazur *et al.,* 2008) and it also has synergistic effect on the function of PA and PB2 (Conenello *et al.,* 2011). Moreover, PB1-F2 is suggested to increases secondary pneumonia infections (Chen *et al.,* 2001; McAuley *et al.*, 2007). More recently, Gannage and collaborators have described that M2 protein interferes with cellular autophagy (Gannage *et al.,* 2009) and that NP can also interplay in the innate immunity mediating the role of cellular inhibitors (Sharma *et al.,* 2011).

Despite the mentioned inhibitory activities of different influenza virus proteins in the IFN response, the NS1 protein seems to play a main role on it, not only concerning innate immune responses but also the adaptive ones.

Role of NS1 in virulence

Some strategies developed by IAV against the immune responses are strain-specific (Grimm *et al.,* 2007; Dittmann *et al.,* 2008). Although mechanisms by which NS1 acts may be also specific of each viral strain (Hayman *et al.,* 2006; Kochs *et al.,* 2007), the viral NS1 protein is widely regarded as factor by which all IAV antagonize immune responses (Egorov *et al.,* 1998; García-Sastre e*t al.,* 1998; Hale *et al.,* 2008; Keiner *et al.,* 2010].

The NS segment (segment 8) encodes the NS1 protein, which is translated from unspliced mRNA; and the NS2/NEP, which is translated from spliced mRNA transcripts (*Figure 1-1a*). The NS1 protein can contain one or two nuclear localization sequences (NLS) which mediate the active nuclear import of NS1 (Greenspan *et al.,* 1988; Melen *et al.,* 2007): NLS1 is highly conserved and involves three residues (Arginine (Arg)-35, Arg-38 and Lysine(Lys)-41), whereas NLS2 is not present in a large number of virus strains and comprises specific amino acids (Lys-219, Arg-220, Arg-231 and Arg-232). The NS1 cytomplasmatic localization can be regulated by a latent nuclear export signal (NES) within residues 138-147 (Li *et al.,* 1998) as well as by a competition between the NLS and NES (Garaigorta *et al.,* 2005).

Concerning its structure, NS1 is divided into two functional domains: N-terminal homodimeric RNA-binding domain (residues 1-73) (Yin *et al.,* 2007) and C-terminal "effector" domain (residues 74-230). Although naturally occurring NS1 proteins with C-terminal truncations (Suarez and Perdue, 1998), NS1 has a length of 230-237 amino acids, depending on the viral strain, and a molecular mass of 26 kDa (Palese and Shaw, 2007). Not only truncations, but also several amino acid extensions, can happen in the C-terminal domain.

The main function of NS1 is to antagonize type I IFN-α/-β -antiviral responses of infected cells by both pre-transcriptional (cytoplasmic) or post-transcriptional (nuclear) processes (*Figure 1-3*). It has been described that the generation of IAV unable to express NS1 (delNS1), or that naturally express truncated forms of NS1, induce large amounts of IFN in infected cells and consequently, delNS1 viruses are attenuated (Egorov *et al.,* 1998; García-Sastre *et al.,* 1998). It is also possible to find virus strains which have lost one of the mentioned mechanisms; e.g. only being able to limit pre-transcriptional events but not post-transcriptional ones (Hayman *et al.,* 2006; Kochs *et al.,* 2007).

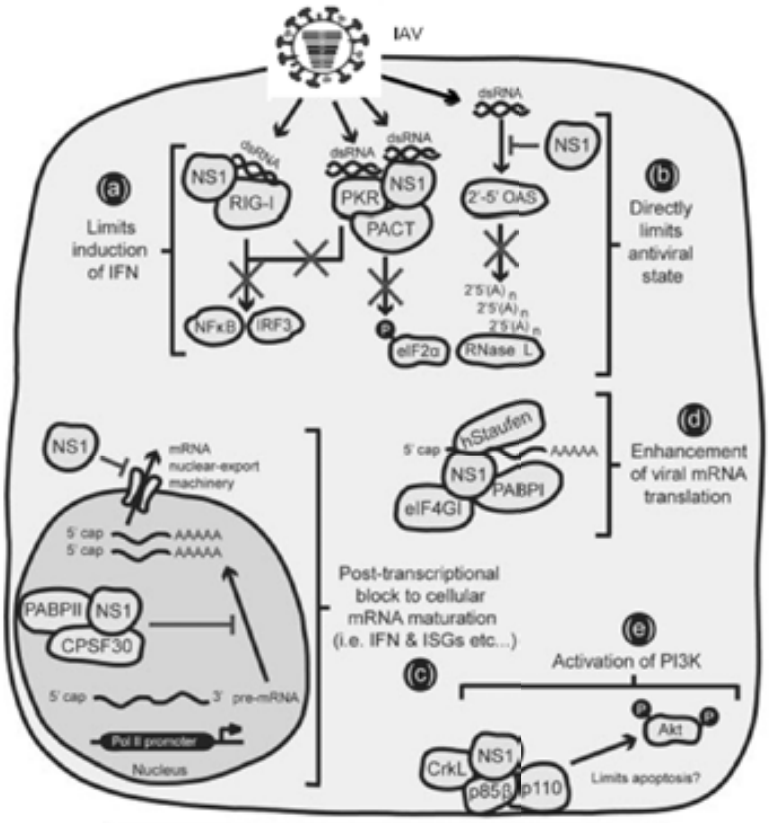


Figure 1-3. Schematic representation of the multiple functions of NS1 protein within an infected cell (modified from Hale *et al.,* 2008).

More in detail, NS1 can bind to double-stranded RNA (dsRNA); therefore suppressing the activation of two antiviral proteins: dsRNA-activated protein kinase (PKR) and 2'-5'-oligoadenylate synthetase (OAS) which are known to be stimulators of type I IFN (*Figure 1-3b)* (Tan and Katze, 1998; Bergmann *et al.,* 2000; Min and Krug, 2006; Min *et al.,* 2007). Moreover, it can also block the induction of IFN by inhibiting the retinoic acid-inducible gene I (RIG-I); thus, preventing activation of IRF-3, NF-κB and c-Jun/ATF-2 transcription factors (*Figure 1-3a)* (Talon *et al.,* 2000; Wang *et al.,* 2000; Ludwig *et al.,* 2002). There are mainly two amino acid residues involved in RNA-binding: Arg-38 and Lys-41 (Talon *et al.,* 2000), and therefore, implicated in the inhibition of OAS and RIG-I. NS1 contains binding sites for a plethora of host-cell proteins, including poly(A)-binding protein I (PABPII), hStaufen and elF4GI; thus, being responsible of the enhancement of viral mRNA translation *(Figure 1-3d)* (Burgui *et al.,* 2003). The C-terminal domain of NS1 also binds to the p85β-regulatory subunit of phosphoionositide 3-kinase (PI3K) (Hale *et al.,* 2006) *(Figure 1-3e),* which is thought to limit the cell apoptosis, cell proliferation and cytokine production (reviewed in Ehrhardt and Ludwig, 2009).

Although some of the described processes are strain-specific, the role of NS1 to prevent the nuclear post-transcriptional processing of RNA polymerase II transcripts seems to be a shared strategy to limit IFN-production between IAV (Nemeroff *et al.,* 1998; Hayman *et al.,* 2006; Kochs *et al.,* 2007; Twu *et al.,* 2007). The effector domain (C-terminal) of NS1 binds directly to the subunit of cleavage and polyadenylation specificity factor (CPDF30) and interacts with PABPII (Nemeroff *et al.,* 1998; Chen *et al.,* 1999; Twu *et al.,* 2006).

It is therefore evident that NS1 protein plays an important role in viral pathogenicity and replication in mammals, but it is still unclear its role in avian hosts. The NS1 genes of AIV differ from those of viruses adapted for replication in humans at different positions (Shaw *et al.,* 2002).

Role of HA in virulence

The pre-existing immunity against a particular IAV strain can be additionally skipped by the virus due to the high genetic variability, mainly of the HA protein. Influenza virus HA is the main target for the humoral response and escape variants of this protein are originated due to the immune pressure it suffers (Plotkin *et al.,* 2003). The antigenic properties of influenza HA are one of the main determinants of viral pathogenesis. HA is a trimer which requires the cleavage of its single precursor to activate the infectivity (Steinhauer, 1999). The site of cleavage for most HAs is a single Arg residue which is only recognized by specific extracellular trypsin-like proteases (present only in the intestinal and respiratory surfaces). Contrarily, some IAV of the H5 and H7 subtypes have acquired multiple basic amino acids at the site of cleavage site which are recognized by intracellular ubiquitous proteases (Perdue *et al.,* 1997).

Although each HA monomer is comprised by a receptor-binding domain (RBD) with conserved amino acids (Tyrosine (Tyr)-98, Tryptophan (Trp)-153, Histidine (His)-183 and Tyr-195) and conserved elements of secondary structure (Skehel and Wiley, 2000), many amino acid changes occur near the RBD during antigenic variation. As previously mentioned, HA is formed by two subunits: HA1 and HA2; and both the N- and C- terminal parts of HA1 together with HA2 comprise the stalk of the molecule (Wilson *et al.,* 1981). This characteristic is very important when developing vaccines able to protect against future infections (Steel *et al.,* 2010).

This viral surface polypeptide (HA) mediates both, the binding of IAV to the host membrane and the fusion of viral and endosomal membranes (Neumann *et al.,* 2009). The role of HAs in SA-receptor binding is species-specific (reviewed in Gamblin and Skehel, 2010). The affinity of different IAV to either α2,3-SA (avian, equine and swine viruses) or α2,6-SA (human and swine viruses) reflects the abundance of SA on tissues at the sites of infection (see *section 1.1.3.1*).

1.2. Immunity

1.2.1. Particularities of immunity in birds

Birds and mammals evolved from a common reptilian ancestor more than 200 million years ago and their basic mechanisms involved in the immune responses do not differ significantly. However, birds have developed some different strategies concerning their immunological system which are discussed in this section (Davison, 2008; Swayne and Kapczynski, 2008).

Concerning anatomy and physiology, the immune system of birds differs from that of mammals in certain basic respects, most notably in the absence of organized lymph nodes (Davison, 2008), although they have been described in ducks (Sugimura, 1977; White, 1981; Payne, 1984). In chickens, the bursa of Fabricius (cloacal bursa) and the thymus are the two major primary lymphoid organs which are located at anatomically diverse locations: in the cervical area and near cloaca, respectively. Consequently, the development of humoral- [bursa (B)-dependent lymphocytes] and cellular- [thymus (T)-dependent lymphocytes] compartments of the immune system is separated (Pastoeret *et al.,* 1998). Secondary lymphoid organs include spleen, Harderian glands (paraocular), bone marrow, bronchial-associated lymphoid tissue or BALT, gut-associated lymphoid tissue or GALT and conjunctival-associated lymphoid tissue or CALT.

Birds respond to antigenic stimuli by generating both, antibodies and cellular immunity. There are three main classes of antibodies in birds: immunoglobulin (Ig)-M, IgY and IgA (Sharma, 1997). The Harderian gland is the main production site for antiviral IgA antibody-forming cells; therefore, is thought to be critical for initiating local immune responses (Russell, 1993; Khan, 2007). Chickens possess IgY which, although is the equivalent of IgG, there is no IgG class switching as described in mammalian species (Higgins, 1996; Sharma, 1997).

Despite differences in structure between avian and mammalian species, functional aspects of lymphoid cells are very similar. Like mammals, cell-mediated

immunity has been described as an important factor of protection against viral diseases in vaccinated-chickens (Sharma, 1999; Seo and Webster, 2001). As in mammals, avian T cells engage in helper and cytotoxic functions that are MHC restricted. Subsets of T cells described in avian species include $CD3^+$, $CD4^+$ (T helper cells) and $CD8^+$ (cytotoxic T cells) (Davidson, 2008).

Although there are still lots of gaps concerning avian immunology, mainly due to the lack of existing reagents; nowadays, since the publication of the chicken (*Gallus gallus*) genome IN the International Chicken Genome Sequencing Committee (Hillier e*t al.,* 2004), it is possible to develop new tools and reagents to study immune responses in this specie.

1.2.2. Immune control of influenza virus

Hosts organisms have developed sophisticated antiviral responses to fight against IAV, by neutralizing them or limit their replication. Since 1939, immunity to IAV infection has been a research topic (Andrewes, 1939). In this section the innate and adaptive immune mechanisms involved in host defense against IAV infection are exposed (reviewed in Kreijtz *et al.,* 2011).

1.2.2.1. Innate immune response

The innate immune system is the first barrier against pathogens. It consists of physical barrier (epithelial surfaces) and rapid innate cellular responses.

The pattern recognition receptors (PRRs) recognize viral RNA which is the main pathogen-associated molecular patterns (PAMPs) of IAV. There are three families of PRRs: toll like receptors (TLR), retinoic acid inducible gene-I (RIG-I) and NOD-like receptor family pryin domain containing 3 (NLRP3) (Pang & Iwasaki, 2011). Signaling of receptors generates a fast and broadly response that results in: (i) secretion of proinflammatory cytokines and type I IFNs; (ii) secretion of chemokines that attract immune cells; and (iii) apoptosis of infected cells.

The production of type I IFN (IFNα and IFNβ) triggers an antiviral state contributing to regulate the infection. IFNα/β play an important role in initiating the adaptive immune response, resulting in enhancement of antigen presentation (by stimulating dendritic cells, DCs) to $CD4^+$T cells and $CD8^+$cytotoxic T cells (CTL) (Theofilopoulos *et al.,* 2005). The expansion of specific $CD4^+$T cells is also mediated by NLRP3-activation after IAV infection and M2 activity, which lead to activate IL-1β, a pro-inflammatory cytokine (Ben-Sasson *et al.,* 2009).

Alveolar macrophages, dendritic cells (DCs) and natural killer cells (NK) also help to limit viral spread. The macrophages are activated after infection of the alveoli and phagocytose IAV-infected cells (Tumepy *et al.,* 2005). Moreover, they can also regulate the development of antigen-specific T cell immunity (Wijburg *et al.,* 1997). However, once they are activated during the infection, macrophages also contribute to IAV-pathology by producing nitric oxide synthase 2 (NOS2) (Jayasekera *et al.,* 2006) and tumor necrosis factor alpha (TNF-α) (Peper and Van Campen, 1995).
DCs present the virus-derived antigens to T cells and activate them. After degrading viral proteins, DCs present the resultant peptides by Major Histocompatibility Complex (MHC)- class I or class II molecules. MHC class I/peptides are recognized by specific $CD8^+$ cytotoxic T cells (CTL). Resulting MHC class II/peptides are recognized by $CD4^+$ T helper (Th) cells. Finally, NK cells recognize antibody-bound influenza virus infected cell and lyse these cells.

1.2.2.2. Adaptive immune response

The adaptive immune response is virus-specific and is based on humoral (antibodies) and cellular immunity (T cells) (*Figure 1-3.*).

Humoral response

Influenza A infection results in the induction of virus-specific antibodies (Potter and Oxford, 1979) which prevent infection of the host. Antibodies against the viral HA

(and to lesser extend to NA) described to be neutralizing, conferring protection against IAV infection (Gerhard, 2001; Nayak *et al.,* 2009).

The HA-specific antibodies prevent the binding and entry of the virus to the host cell. The NA-specific antibodies do not directly neutralize the virus but limits the release of virus particles from the cell surfaces. Although NP is an important target for T cells, NP-antibodies may also contribute to protection against IAV (Lamere *et al.,* 2011). Furthermore, three types of antibody isotypes (IgA, IgM and IgG) are related to specific humoral response. Early after IAV infection, the presence of IgA antibodies (mucosal) is demonstrated to confer local protection (Voeten *et al.,* 1998). IgM antibodies activate the neutralization of IAV, and IgG present in the serum afford long-live protection.

The major problem of antibody-mediated immunity is that, although it can last for a long time, the breadth of protection is limited to the specific subtype (reviewed in Schmolke and García-Sastre, 2010). Some reports demonstrate that specific antibodies against NP may contribute to protection against IAV infection as well (Carragher *et al.,* 2008).

Cellular response

Upon infection with IAV, $CD4^+$T lymphocytes and $CD8^+$T cells are induced. $CD4^+$T cells or T helper (Th) cells recognize virus peptides in association MHC class II molecules. The main role of Th2 cells is to produce IL-4 and IL-13 cytokines to stimulate the proliferation and differentiation of B-lymphocytes (Wright *et al.,* 2007), whereas Th1 cells are involved in cellular immune responses and produce INF-γ and IL-2. The virus-specific $CD8^+$ T cells response is essential to eliminate infected-cell, which present virus-peptides by MHC class I molecules. Thus, they are also referred as cytotoxic T lymphocytes (CTL). CTL responses are mainly directed to internal and conserved IAV proteins (NP, M1 and polymerases), meaning that responses are cross-reactive; therefore, contributing to heterosubtypic immunity (Subbarao and Joseph, 2007).

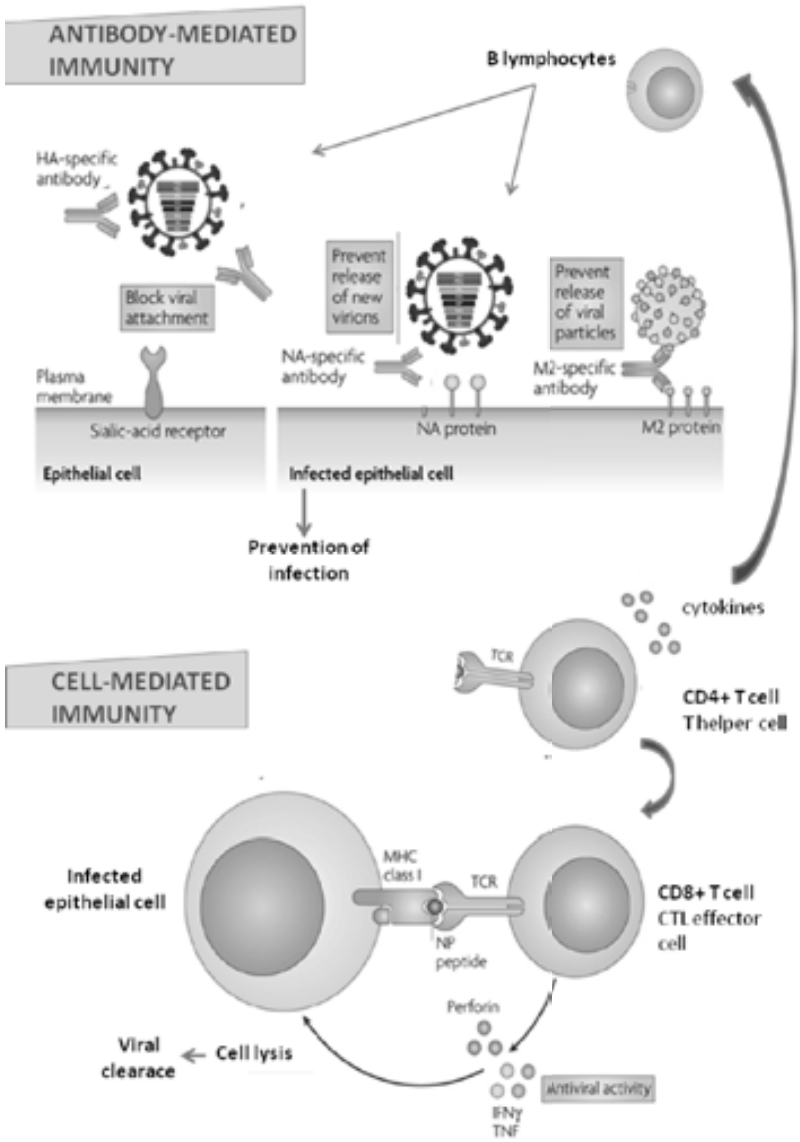


Figure 1-3. The humoral and cell-mediated immune response to influenza virus infection. **a|** The humoral branch of the immune system comprises B-lymphocytes, which after interaction with influenza differentiate into antibody-secreting plasma cells. Antibodies specific for HA block virus attachment preventing infection of cells or fusion. Antibodies specific for either NA or M2, prevent the release of virions attaching the virus to the cell. **b|** The cellular response starts with antigen presentation via MHC I and II molecules by dendritic cells, which then leads to activation, proliferation and differentiation of antigen-specific T cells ($CD4^+$T or $CD8^+$T). These cells gain effector cell function to either help directly, release cytokines, or mediate cytotoxicity following recognition of antigen (modified from Subbarao and Joseph, 2007).

Contrarily to the study of IAV in mouse model, very little research has focused on the role of helper ($CD4^+$) and cytotoxic ($CD8^+$) T-lymphocytes in avian influenza pathogenesis.

1.3. Prevention and control of influenza: vaccine development

The aim of vaccination is to mimic the development of naturally acquired immunity to prevent animals and humans from possible infections. The first to describe the term "vaccine" (from Latin *vacca*) was Edward Jenner when in 1796 demonstrated that experimental vaccination in humans with cow-pox virus conferred immunity against the lethal smallpox virus (Baxby, 1996).

As described in section *1.2*, the immune system responses after influenza infection are mainly characterized by the production of neutralizing antibodies directed against HA and NA (Gerhard, 2001; Nayak *et al.,* 2009). However, due to the high antigenic variability typical of IAV, every year a new vaccine is developed to protect human population to the strains predicted to circulate in the next season. Moreover, the emergence of new IAV is becoming a risk of increasing potential to human health (Peiris *et al.,* 2007; Neumann *et al.,* 2009; Watanabe *et al.,* 2012).

Vaccination keeps being the primary strategy for the prevention and control of influenza (Cox *et al.,* 1999; Nichol *et al.,* 2006) and, although difficulties in finding a universal formulation, lots of efforts are focusing on finding an optimal design and vaccination. However, the criteria for successful veterinary vaccines can be very different from those for human vaccines. Thus, while the main requisite of livestock vaccines is that they should be cost-benefit, vaccination against viral strains of potential zoonotic should reduce or eliminate the risk of animal-to-human transmission (Meeusen *et al.,* 2007).

1.3.1. Conventional vaccines against influenza

Several vaccine formulations are available to control influenza infection but still have some limitations, and the protection they confer varies widely depending on the antigenic match between the circulating viruses and those present in the vaccine (Lambert and Fauci, 2010). Vaccine efficacy also depends on the specie receiving the vaccine and the age and health status (related to immunological status) of the individual. In the following section the importance of vaccination as well as current vaccines available for birds, pigs and humans are discussed.

Current vaccines in birds

Since 1959, twenty-seven outbreaks or epidemics of HPAI in poultry and other birds around the world have been reported (Alexander and Capua, 2008; Swayne, 2008). Four of the epidemics used a combination of depopulation and vaccination to eliminate the clinical disease and maintain the economic viability of poultry production. Vaccination in poultry is now considered to be a preventive control measure in several countries (Peiris *et al.,* 2007; Swayne and Kapczynski, 2008).

In poultry, the most common vaccine preparation used is the inactivated whole-virus water-in-oil emulsion vaccine. Inactivated vaccines have been used in a variety of avian species and, although their effectiveness is well documented, protection is virus subtype-specific. Poultry vaccines are not filtered and purified like human vaccines because these processes are very expensive. Moreover, the use of mineral oil as adjuvant, although induces strong immune responses, can cause inflammation and/or abscesses. Recently, alternatives containing an H5 gene insert in combined recombinant fowlpox vaccines (Swayne *et al.,* 2000), recombinant infectious laryngotracheitis virus (Lüschow *et al.,* 2001) and Newcastle disease virus (Veits *et al.,* 2006), have been developed. In *Table 1-1* a list of some of the vaccines formulated to poultry species is provided.

Table 1-1. Avian influenza vaccines for poultry. This list provides information on commercialized influenza vaccines for poultry.

Manufacturer/ Distributor	Strain(s) and subtype	Commercial name
Monovalent inactivated vaccines		
Boehringer Ingelheim	A/Ch/Mexico/232/94/CPA (H5N2)	Volvac AI KV
Ceva	A/Ch/Mexico/232/94/CPA (H5N2)	FLU-KEM
Fort Dodge Animal Health	A/TY/California/20902/2002 /H5N2) A/Ch/Italy/22ª/H5N9/1998	Avian Influenza Vaccine, H5N2 Poulvac Flufend i-AI H5N9
Intervet	A/duck/Postdam/1402/86 (H5N2) A/Ch/Mexico/232/94/CPA (H5N2) Influenza H5N2 + ND	Nobilis Influenza H5N2 Nobilis Influenza H5 Nobilis®IA+ND INAC
Laprovet	A/Ch/Mexico/232/94/CPA (H5N2)	ITA-FLU
Merial	A/Th/Wisconsin/68 (H5N9) A/Ch/Italy/22A/98	Gallimune Flu H5N9 Gallimune Flu H5N9
Monovalent reverse genetics H5 vaccines		
Fort Dodge Animal Health	Rg-A/ck/VN/C58/04 with N3 gene from H2N3 and six internal genes from PR8	Poulvac Flufend I AI H5N3 RG
Recombinant vaccines with H5 component		
Merial	Fowlpox virus-vectored H5 gene from A/Tk/Ireland/83	Trovac AIV-H5
Bivalent inactivated AI vaccines		
Fort Dodge Animal Health	A/Ch/Italy/22A/1998 (H5N9) A/Ch/Italy/1067/1999 (H7N1)	Poulvac Flufend i-AI H5N9 H7N1
Merial	A/Ch/Italy/1067/99 (H7N1) A/Ch/Italy/22°/98 (H5N9)	BioFlu H7N1 and H5N9
Monovalent inactivated vaccines		
Bioimmune vaccines-Ceva	A/Ch/NY/273874/03 (H7N2) A/Tk/Utah/24721-10/95 (H7N3)	Layermune AIV H7N2 Layermune AIV H7N3
Intervet	A/Chicken/Italy/473/99 (H7N1) A/duck/Postdam/15/80 (H7N7) A/Ch/UAE/415/99	Nobilis influenza H7N1 Nobilis influenza H7N7 Nobilis influenza H9N2
Fort Dodge Animal Health	A/Ch/Italy/1067/1999 (H7N1)	Poulvac Flufend i-IA H7N1

Current vaccines in pigs

Commercial vaccines currently available in swine are either inactivated whole- or split- virus and are adjuvanted. Most manufactures include an H1N1 and H3N2 swine origin influenza virus strains to vaccine. However, they do not confer cross-protection against new viral subtypes. Although recent studies report their efficacy in providing heterosubtypic immunity, modified live-influenza virus vaccines are no available for swine. In *Table 1-2* a list of some of the vaccines formulated to swine species is provided.

Table 1-2. Swine influenza vaccines for pigs. This list provides information on commercialized influenza vaccines for pigs.

Manufacturer/ Distributor	Strain(s) and subtype	Commercial name	Formulation
Fort Dodge Animal Health - *Pfizer*	A/Sw/Netherlands/25/80 (H1N1) A/Port Chalmers/1/73 (H3N2)	Suvaxvn flu®	Whole virus
Hipra	A/Sw/Olost/84 (H1N1) Port Chalmers/1/73 (H3N2)	Gripork®	Whole virus
IDT Biologika	A/Sw/Belgium/230/92 (H1N1) A/Sw/Belgium/220/92 (H3N2)	Respiporc Flu®	Whole virus
Merial	A/New Jersey/8/76 (H1N1) A/Port Chalmers/1/73 (H3N2)	Gripovac®	Split
	Sw/Haselünne/IDT2617/03 (H1N1) Sw/Bakum/1832/00 (H1N2) Sw/Bakum/IDT1769/03 (H3N2)	Gripovac 3®	

Current vaccines in humans

There are different vaccine formulations available for IAV in humans: inactivated-virus vaccines (whole-, split- and subunit-formulations) and live attenuated-virus vaccines.

Inactivated vaccines work mainly through the generation of antibodies to HA. Although immunogenic, inactivated whole-virus vaccines showed reactogenicity, particularly in children (Gross *et al.,* 1977). Consequently, this drove the development

of the split (Bresson *et al.,* 2006) and subunit (Treanor *et al.,* 2006) vaccines, which were proven to be safe. Unfortunately, they are not able to induce a strong immunity (mainly split-formulation); thus, being necessary to provide at least two doses of vaccine to generate protective immune response (Stepehson *et al.,* 2003). Inactivated-vaccine production is a tedious and long-lasting process which starts with the generation of vaccine reference strains.

Seasonal influenza vaccines are trivalent and contain strains considered to be the most likely to circulate in the upcoming influenza season: three viruses (or their HA proteins) representing the influenza A/H3N2, A/H1N1 and influenza B strains (Lambert and Fauci, 2010).

There are several issues that limit the utility of conventional vaccines. The reliance of the production system, the amount of time required to select correct vaccine strains (matching the epidemic strains antigenically) and some times, the lack of optimal efficacy are some of the problems when using these formulations (Ellebedy and Webby, 2009).

1.3.2. Next generation of vaccines

Searching for a universal vaccine is a must and a lot of effort is invested in improving the vaccines design and the whole production process, including timelines. Briefly, some of the most recent strategies are mentioned (*Table 1.3.)* (reviewed in Lambert and Fauci, 2010) paying particularly attention in the design of peptide candidates as efficacious alternative.

A recombinant trivalent HA-based influenza vaccine (FluBlok®) produced in insect cell culture using the baculovirus expression system has been developed (Treanor *et al.,* 2006; Cox *et al.,* 2008). It consists of three full-length recombinant HAs (derived from H1, H3 and B viral strains) and provides an alternative to the egg-based trivalent inactivating vaccine (TIV).

Other promising formulation of influenza vaccines is the use of noninfectious virus-like particles (VLP) which is demonstrated to be efficient (Galarza *et al.,* 2005; Quan *et al.,* 2007). Bright *et al.* described a cross-clade protective immune response obtained to proteins from H5N1 influenza isolates (Bright *et al.,* 2008). Recently, a trivalent VLP vaccine showed to elicit broad immunity and protection in mice and ferrets (Ross *et al.,* 2009).

A new way of introducing IAV proteins into the immune system are viruses which do not replicate or cause disease ("carrier viruses"). HA genes from influenza have been cloned into viral vectors, including *vaccinia* virus (Kreijtz *et al.,* 2009a; Kreijtz *et al.* 2009b; Kreijtz *et al.* 2010; Hessel *et al.,* 2011), adenoviruses, Newcastle disease virus and baculoviruses; among others.

DNA-based vaccines have been tested experimentally and are less risky than live vaccines. Moreover, as recombinant DNA vaccines induce both humoral and cellular immunity they may provide higher cross-protection than conventional killed vaccines. New developments have succeeded in eliciting neutralizing antibodies to conserved regions of the HA (Gao *et al.,* 2006).

Major targets in the search for a "universal" vaccine (Du *et al.,* 2010) have been highly conserved epitopes or proteins of the influenza M2, NP, M1 (Tompkins *et al.,* 2007; Kitikoon *et al.,* 2009; El Bakkouri e*t al.,* 2011) and HA proteins (Vergara-Alert *et al.,* 2012; Wang e*t al.,* 2010). Vaccines that target the extracellular portion of the M2 protein (M2e) are one of the most developed (Schotsaert, *et al.,* 2009). Although some studies in mice demonstrate protection against a range of influenza strains conferred by M2e, results reported in other animal model are less satisfactory and other also question the immunity afforded in mice.

Table 1-3. New generation of influenza vaccines. A summary of vaccine formulations, few recent examples of each formulation, the animal model where have been tested and the main result obtained is given.

Vaccine formulation	Example	Model	Main result	References
Recombinant HA (trivalent HA-based)	FluBlok®	CD-1 outbred mice (pre-clinical tests) Human	Safe, immunogenic and effective Higher seroconversion rate than TIV	[Treanor *et al.,* 2011 & 2006; Baxter *et al.,* 2011; Cox *et al.,* 2008]
Virus-like particles (VLP)	H5N1 VLP	Mice	Cross-clade protective immunity against H5N1 Homo- and heterlogous protective immunity	[Bright *et al.,* 2008] [Quan e*t al.,* 2007]
	Tri-VLP	Mice and ferrets	Broad immunity and protection	[Ross *et al.,* 2009]
Viral vectors	MVA-based H5N1/H1N1	Mice, macaques and ferrets	One immunization confers cross-clade protection Two immunizations induced Ab responses and protection	[Kreijtz *et al.,* 2009a, 2009b and 2010]
DNA-based	Expressing PB1	Mice	Protective immunity	[Košík *et al.,* 2012]
Universal vaccines	M2e HA-peptides	Mice Mice, pigs	Humoral and cellular immunity. Partial viral clearance	[Wolf *et al.,* 2012] Chapter 6 [Vergara-Alert *et al.,* 2012]

Interest in bioinformatics is increasing because it has become an essential tool to identify functional protein domain representing candidate targets for vaccines. One example is the informational spectrum method (ISM), which is a virtual method to analyze the relation between structure and function of proteins and nucleotide sequences (Veljkovic *et al.,* 2008). Briefly, ISM consists in three steps: (i) to assign a numerical value (representing the electron-ion interaction potential, EIIP) to each amino acid/nucleotide and to transform the alphabetic code of the primary structure into numbers; (ii) to convert the numbers into informational spectrum (by a mathematical model); and finally, (iii) to identify those frequency components in the IS of molecules which are important for biological functions. The EIIP is described to be one essential parameter determining properties of biological molecules (Veljkovic *et al.,* 1980). This method was used in *Chapter 6* to design the vaccine used.

1.3.3. Treatment

Currently, there are two families of antiviral drugs available for the treatment of human influenza infections. One family (amantadine and rimantadine) inhibit the function of the viral ion channel protein M2. Therefore, the exchange of H^+ in order to decrease the pH inside the virus and viral uncoating cannot take place. The second family comprises those drugs which function relies in inhibiting the NA function (oseltamivir and zanamivir) and prevents the cleavage of SA-residues; thus, blocking the release of newly virions from infected cells (Medina and García-Sastre, 2011).

The major problem of the mentioned drugs is that, although they are demonstrated to be efficacious against current IAV, their wide use can result in selection of resistant viruses (Beard *et al.,* 1987; Le *et al.,* 2005).

As a consequence, and although amantadine has been showed to be effective in decreasing mortality in poultry (Dolin *et al.,* 1982; Webster *et al.,* 1985) it is not approved for food animals. Thus, only supportive care and administration of antibiotics (to reduce or prevent from bacterial infections) are the allowed treatments. In pigs, as swine influenza is rarely fatal, treatment is also focused on supportive care. But to prevent the virus spread of the virus throughout the farm or to other farms, vaccination, in combination with optimal management, is the most important tool

More recently, another drug belonging to the second family has been used (peramivir) but it is only authorized for emergency use in the treatment of certain patients with pH1N1 infection (Antonelli and Turriziani, 2012; Louie *et al.,* 2012).

Chapter 2

Objectives

The economic losses caused by influenza A virus (IAV) in the poultry industry, the importance to protect endangered species against an infection by HPAIV as well as the capability of IAV to infect humans, are the reasons why the scientific community is focused on finding a universal IAV-vaccine.

The present thesis is a multidisciplinary research involved in national and international projects. The general objective of this work is to increase the knowledge concerning the relation between the immune response and the pathological outcome of IAV infection, with the subsequent goal of finding a universal vaccine against influenza infection.
To accomplish the general purpose four specific objectives were established:

1. To determine the contribution of the NS1 protein to the virulence of AIV in chickens and to determine the relation between the pathological manifestations and the elicited immune responses (*Chapter 3*).

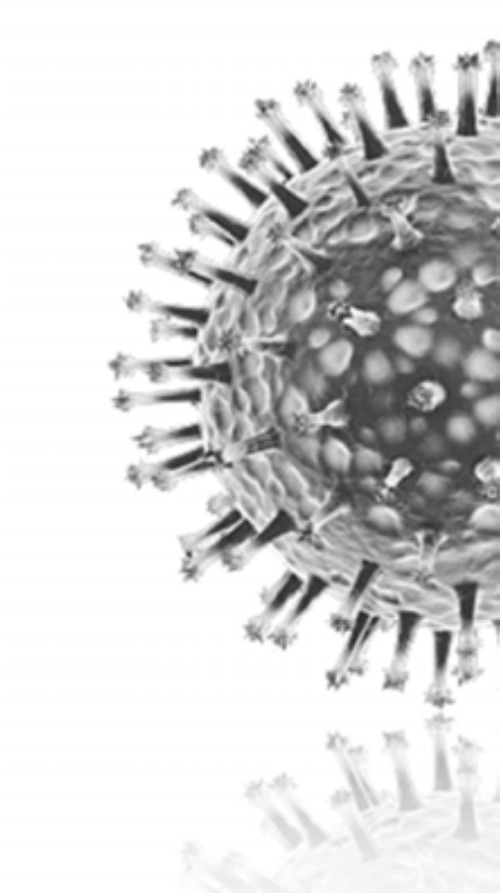
2. To study the impact of pre-existing immunity on the pathogenicity of AIV in chickens and to characterize the role of the induced humoral response in protection (*Chapter 4*).

3. To evaluate the efficacy and safety of two inactivated H5 vaccines in a wide selection of exotic avian species from Spanish zoos and to determine whether vaccination would be a suitable tool, together with other measures, during future outbreaks of HPAIV (*Chapter 5*).

4. To test a newly designed vaccine based on HA1-peptides in a pig model and to investigate its potential to elicit a broadly protective immunity (*Chapter 6*).

PART II:

Studies



"The roots of science are bitter, but the fruit is sweet"

[Aristotle]

Chapter 3

Study I: The NS1 protein of H5N1 Avian Influenza Viruses (AIV) enhances the Virulence of an H7N1 AIV in Chickens

3.1. Abstract

Numerous outbreaks involving highly pathogenic avian influenza viruses (HPAIV) have been reported during lasts years. Some of these outbreaks were responsible of avian-to-human transmissions, causing symptoms ranging from moderate signs to even death. One determinant of virulence by which influenza virus adapts to mammals and gain pathogenicity is the multifunctional NS1 protein. In a previous work, two reassortant-avian influenza viruses (designated FPV NS GD and FPV NS VN) carrying the NS-segment of the HPAIV strains A/Goose/Guangdong/1/96 (GD; H5N1) or A/Viet nam/1203/2004 (VN; H5N1), were engineered in the genetic background of the HPAIV strain A/FPV/Rostock/34 (FPV; H7N1) by reverse genetics. Although it was demonstrated that the FPV NS GD replicated more efficiently than FPV in different mammalian cell lines, and that it was able to cause disease and death in mice, further studies are still needed to understand the role of the NS1 in the pathogenicity of the influenza infection in avian species, their natural host.

To test this hypothesis, the pathogenicity of the two NS-reassortant viruses was determined using specific pathogen free (SPF) chickens as the avian model system. The viruses containing NS1 sequences from the H5-HPAIV demonstrated increased virulence in infected chickens compared with the wild type FPV virus, as characterized by higher viral loads, higher percentage of mortality and/or earlier presence of clinical lesions. Although some of the infected chickens suffered lymphopenia compared to the control group, the commonest event among NS-reassortant infected chickens was the increase of peripheral monocyte/macrophage-like cells expressing high levels of IL-1β, as determined by flow cytometry.

In summary, our results clearly confirm NS1 as a virulence factor, directly involved in triggering the typical cytokine storm and apoptosis found during HPAIV pathogenesis.

3.2. Introduction

During the last decades highly pathogenic avian influenza viruses (HPAIV) belonging to H5- and H7- subtypes were reported to cause major outbreaks in birds worldwide (Capua *et al.,* 2004; WHO, 2012a). The overall impact in the poultry industry has dramatically increased in few years, from 23 million birds affected between 1959 and 1998 to more than 200 million, between 1999 and 2004 (Capua *et al.,* 2004). Besides their economic importance, influenza viruses are currently considered one of the most important threats to human health because of their pandemic potential; underlining the importance of avian reservoirs for influenza A viruses (IAV). Since 2003, as confirmed by the World Health Organisation, transmissions of the H5N1 HPAIV to humans have caused approximately 600 disease cases and 350 deaths (WHO, 2012b). Therefore, it is important to increase our knowledge concerning the role of viral determinants in virulence to design better vaccines and therapies against IAV.

The innate immune response is the first unspecific barrier of the host against pathogens, and the induction of type I interferon (IFN) expression, mainly IFNα/β, is one of the earliest anti-viral cytokines expressed upon IAV infection (Randall *et al.,* 2008). Although hosts develop antiviral responses in order to control the infection, IAV have evolved multiple strategies to avoid these responses (Schmolke *et al.,* 2010). By expressing the non structural 1 protein (NS1), IAV antagonize the immune response of infected cells, especially limiting the production of type I IFN, as well as that of other immunomodulators (Haye *et al.,* 2009). IFN-β induction can be limited by NS1 protein by both pre-transcriptional (cytoplasmatic) and post-transcriptional (nuclear) processes (Kochs *et al.,* 2007; Hale *et al.,* 2008). However, the mechanisms and targets for NS1 depend on virus strain (Hayman *et al.,* 2007; Kochs *et al.,* 2007). Furthermore, it has been demonstrated that NS1 protein prevents virus-mediated activation of the following transcription factors: IRF-3 (Talon *et al.,* 2000), NFκB (Wang *et al.,* 2000) and c-Jun/ATF-2 (Ludwig *et al.,* 2002), which are essential for IFN-β induction. The contribution of the NS1 protein to the pathogenicity of IAV

has been demonstrated in mammalian models, such as mice and pigs (Quinlivan *et al.,* 2005; Solorzano *et al.,* 2005; Ma *et al.,* 2010). Although less is known in avian species, Li *et al.* reported that, as in mammals, in both chickens and geese the NS1 has an important role in viral virulence (Li *et al.,* 2006).

Pro-inflammatory cytokines secreted during IAV infection have also been described to have an important role in mammalian species (van Reeth *et al.* 2000; Lipatove *et al.* 2005). In a mouse model, lymphocyte apoptosis and high-level inductions of cytokines, including interleukin 1 (IL-1), have been proposed to contribute to the severity of IAV (Perrone *et al.* 2010). However, little is known about the role of IL-1 in chickens, the natural host of IAV. Further studies about the role of pro-inflammatory cytokines in disease severity and outcomes in chickens infected with HPAIV are needed.

With the objective to increase the knowledge for all the gaps mentioned above, in the present study recombinant viruses have been used to determine the impact that NS1 protein of H5-HPAIV would have on viral pathogenicity of an H7-HPAIV strain in chickens.

3.3. Materials and methods

3.3.1. Cell culture and viruses

Madin-Darby Canine Kidney (MDCK) cells were purchased from ATCC (CCL-34) and cultured according to manufacturer's instructions in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, S.A.) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin) at 37ºC in a 5% CO_2 humidified atmosphere.

Viruses used in this study were the A/FPV/Rostock/34 (FPV; H7N1) generated by recombinant technology, and two reassortants carrying the NS-segment of either A/Goose/Guangdong/1/96 (GD; H5N1) or A/Viet nam/1203/2004 (VN;

H5N1) in the genetic background of FPV. The reassortants are named FPV NS GD and FPV NS VN, respectively. All these viruses were generated in the facilities of the Institute of Medical Virology at Justus-Liebig-University in Giessen (Germany), as previously described (Ma *et al.,* 2010; Wang *et al.,* 2010). Virus stocks were propagated in the allantoic cavity of 11-day-old embryonated chicken specific-pathogen-free (SPF) eggs (Lohmann Tierzucht GmbH) at 37ºC for 72 h. The allantoic fluids were harvested, aliquoted and stored at -80ºC until use. Virus titer was determined in both, 11-day-old embryonated chicken SPF eggs and MDCK cells and measured as either egg lethal doses 50% (ELD_{50}) or tissue culture infectious doses 50% ($TCID_{50}$), respectively, by following the Reed and Muench method (Reed and Muench, 1938; Villegas, 2008).

3.3.2. Computer prediction of NS1 protein cellular localization

In silico analysis of NS1 viral protein cellular localization was assessed by the PSORT II program (http://psort.hgc.jp/) (Nakai and Horton, 1999). Conservation of described motifs and predicted domains were studied by the alignment of amino acids of NS1 full-length available genomes in GenBank of FPV (CY077424.1), GD (AF144307.1) and VN (EF541456.1) using Clustal W program.

3.3.3. Animal experiments

The present study was performed in strict accordance with the Guidelines of the Good Experimental Practices. Animal procedures were approved by the Ethical and Animal Welfare Committee of *Universitat Autònoma de Barcelona* (UAB) (Protocol #DMAH-5767). Chicken experiments were conducted at Biosafety Level 3 (BSL-3) facilities of the *Centre de Recerca en Sanitat Animal* (CReSA-Barcelona).

Ninety-five SPF-eggs (Lohmann Tierzucht GmbH) were hatched under BSL-3 containment conditions at CReSA. At 2-week-old, chickens were divided into four

groups and placed in independent negative pressure isolators. Animals were inoculated intranasally with $10^{5.5}$ ELD_{50} of either FPV (Group 1, n=25), FPV NS GD (Group 2, n=25) or FPV NS VN (Group 3, n=25) in a volume of 50 µl. One group of twenty chickens were mock-infected with 1ml PBS 1x in a volume of 50 µl and used as a negative control (Group 4). Chickens were monitored for flu-like signs and the mean clinical score, as well as mortality rate, were recorded. Clinical signs intensity was assessed by a semi-quantitative scoring (0 to 2 indicating lack of, moderate or severe signs). According to ethical procedures, animals were euthanized with intravenous administration of sodium pentobarbital (100 mg/kg) if severe clinical symptoms became apparent. Ten animals per group were kept to describe the clinical outcome and the mortality rate. The other chickens (n=15/group) were kept to obtain samples and to perform sequentially necropsy. From these animals, blood samples were obtained from three chickens from each group at 6 hours post infection (p.i.) and 1, 2, 3 and 4 days p.i. Blood was collected from the heart after anaesthetize the animals with Zoletil® (Virbac). Two to four ml of blood were collected in tubs contained 2 ml of Alsever's anticoagulant (Sigma-Aldrich). Oropharyngeal and cloacal swab samples (OS and CS, respectively) were collected at the same times p.i. in DMEM and antimicrobial drugs (100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin). Two animals from Group 4 (negative control) were also sampled at the same time points as the other groups.

3.3.4. Histopathology and AIV-nucleoprotein antigen determined by immunohistochemistry (IHC)

Necropsies and tissue sampling were performed according to a standard protocol. For histopathological analysis, collected tissues were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Tissue sections were processed routinely for hematoxylin/eosin (H/E) staining. The following tissues were examined:

central nervous system (CNS), lung, heart, kidney, pancreas, liver, spleen, thymus and bursa of Fabricius.

An IHC technique based on avidin-biotin complex immunoperoxidase system was performed as previously reported (Haines and Chelack, 1991; Rimmelzwaan *et al.,* 2011). Briefly, a mouse-derived monoclonal commercial antibody against the nucleoprotein (NP) of influenza A virus (IgG2a, Hb65, ATCC) was used as a primary antibody. As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Denmark) was used. Negative controls were those tissues from sham-inoculated animals (Group 4) and also tissues incubated without the primary antibody. Tissues from previous experiments demonstrated to be positive against NP by IHC were used as positive controls. To measure the extension of the staining in tissues a semi-quantitative scoring was assessed: no positive cells (-), single positive cells (+), scattered groups of positive cells (++), and widespread positivity (+++).

3.3.5. Virus quantification by real time RT-PCR (RRT-qPCR)

Viral RNA quantification using one step RRT-qPCR was performed in blood and OS and CS. Viral RNA was first extracted with Trizol (Life Technologies, S.A.) obtaining 60 µl of eluted viral RNA, as described by the manufacturer. Briefly, after 2-3 min incubation with 0.2 ml of chloroform, samples were centrifuged at 12,000 x g for 15 min at 4ºC. When the aqueous phase was removed and placed into a new tube, 0.5 ml of 100% isopropanol were added and incubated 10 min at RT. After centrifugation at 12,000 x g for 10 min at 4ºC, the RNA was washed with 1 ml of 75% ethanol, centrifuged at 7,500 x g for 5 min at 4ºC and air dried for 10 min. The RNA was re-suspended in DEPC-water and stored at -80ºC until use.

Amplification of a matrix (*M*) gene fragment was carried out using primers, probe, One-Step RT-PCR Master Mix Reagents (Life Technologies, S.A) and amplification conditions as described previously by Busquets *et al.* 2010 (Busquets, *et*

al. 2010) in Fast7500 equipment (Life Technologies, S.A) using 5 µl of eluted RNA in a total volume of 25 µl.

3.3.6. Cytokine quantification by real-time RT-PCR (RRT-qPCR)

Total RNA from blood from three chickens per group and per time point (6, 24 and 48 hours p.i.) was isolated using Trizol (Life Technologies, S.A.), as described in the previous section. The isolated RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies, S.A) following the manufacturer's instructions

Primers and probes for IL-1β and for the housekeeping gene 28S, designed by Kaiser *et al.* were used (Kaiser *et al.,* 2000). For IFN-β, primers and probe were designed for the IFN-β gene sequence (NM_001024836) available at the GenBank. The probe was labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5'-end and with the quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3'-end. Details of all probes and primers are given in *Table 3-1*.

Amplification and detection of specific products were performed using the TaqMan Universal PCR Master Mix (Life Technologies, S.A) with the following cycle profile: one cycle of 50ºC for 2 min, 95ºC for 10 min and 40 cycles of 95ºC for 15 sec, 60ºC for 1 min, in Fast7500 equipment (Life Technologies, S.A). The mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Results are expressed as fold change in comparison to a calibrator sample (samples from Group 4).

3.3.7. Isolation of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Histopaque®-1077 (Sigma-Aldrich). The instructions from the manufacturer were followed with some modifications. Briefly, at RT, 4 ml aliquots of Histopaque$_s$-

1077 were overlaid with 4 ml whole anticoagulated-blood and centrifuged for 20 min at 400 x g. Following centrifugation, the opaque interface was collected and washed twice with 1 ml PBS solution and centrifuged again for 10 min at 250 x g. Cell numbers were calculated using a dye solution and the cell concentration was adjusted to 10^7 cells/ml.

Table 3-1. Real time quantitative RT-qPCR primers and probes used.

RNA target		Sequence	Accession nº
28S	Probe	5'-(FAM*)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3'	X59733
	Fw[a]	5'-GGCGAAGCCAGAGGAAACT-3'	
	Rv[b]	5'-GACGACCGATTGCACGTC-3'	
IFN-β	Probe	5'-(FAM*)-CGCATCCTCCAACACCTCTT-(TAMRA)-3'	NM_001024836
	Fw[a]	5'-CCATTTCCAGAAACCCTTCTG-3'	
	Rv[b]	5'-TCCAGTGTTTTGGAGTGTGTGG-3'	
Il-1 β	Probe	5'-(FAM*)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3'	AJ245728
	Fw[a]	5'-GCTCTACATGTCGTGTGTGATGAG-3'	
	Rv[b]	5'-TGTCGATGTCCCGCATGA-3'	

*5-carboxyfluorescein
[a]Forward
[b]Reverse

3.3.8. Flow cytometric analysis

Flow cytometry studies allowed separating the blood subpopulations by size and complexity (Forward and side scatter, FSC and SSC); therefore distinguishing lymphocytes, monocytes/macrophages from histyocytes and heterophyles (Radcliff and Jaroszeski, 1998).

An Allophycocyanin (APC) Antibody Conjugation Kit (Bionova Cientifica) was used to conjugate both IL-1β and IFN- β purified mAb, following manufacturer's instructions. Approximately 10^6 PCMCs per well were added in V-bottomed 96-well plates. Cells were fixed (PBS + 2% PFA) during 15 min, washed with FACS diluent (PBS +2% FCS) and finally, cells were permeabilized with 150 µl of diluted detergent.

Following two washes with FACS diluents, 50 µl of either mAb IL-1β APC-conjugated or IFN-β APC-conjugated diluted with FACS diluents were added and incubated for 20 min. Then, the cells were washed and resuspended with FACS diluents. All procedures were carried out at 4ºC.

3.3.9. Statistical analysis

The results correspond to the Mean ± Standard Error of the Mean (SEM) of the indicated experiments. Differences between groups were tested for significance by using Student's t test. Differences were considered statistically significant at $P < 0.05$.

3.4. Results

3.4.1. H5-NS1 proteins increases the virulence and the shedding of H7N1 HPAIV in chickens

In order to better understand the impact of two different H5N1 HPAIV-NS1 proteins on FPV pathogenesis in their natural host, groups of twenty-five SPF-chickens were inoculated intranasally with $10^{5.5}$ ELD_{50}/50 µl per chicken of either, FPV, FPV NS GD or FPV NS VN. Another group receiving saline solution was used as control group (n=10).

Although FPV is not pathogenic in mice (Bonin and Scholtissek, 1983; Reinacher *et al.,* 1983), in chicken is considered a highly pathogenic virus (Feldmann *et al.,* 2000), as confirmed in the present study. FPV infection resulted in the manifestation of severe clinical signs between 5 and 7 days p.i. (*Figure 3-1a*), a 50% mortality by day 5 p.i. and in 70% of mortality rate by the end of the experiment, at day 10 p.i. (*Figure 3-1b*). The clinical signs found in FPV NS GD- infected chickens did not increase dramatically at the early time-points, showing very similar disease outcome and death kinetics when compared with FPV (*Figure 3-1a and b*), albeit the final mortality rate was higher, with 90% of the infected animals being death by day 10

p.i. A more dramatic effect was observed for the FPV NS VN-reassortant virus, causing an earlier and more severe disease from 2-3 days p.i. than the other two viruses (*Figure 3-1a*), with 90% of deaths recorded 5 days p.i. and a 100% mortality by day 10 p.i. (*Figure 3-1b*).

Clinical signs shown in all groups were non-specific and consisted of depression, apathy and ruffled feathers. More severe clinical signs as torticollis and lack of coordination were also monitored. Gross lesions were observed as soon as 2 days p.i. in FPV NS VN and started between 3 and 4 days p.i. in FPV and FPV NS GD groups. The lesions were similar between groups and consisted of mucous nasal discharge, conjunctivitis, multifocal to diffuse haemorrhages and cyanosis of the comb and diffuse oedema. At necropsy, petechial haemorrhages on leg muscles, breast and serosa of the proventriculum were detected in all the infected groups. From 3 to 7 days p.i., splenomegaly and atrophy of both thymus and bursa of Fabricius were observed in FPV NS GD and FPV NS VN groups.

Tissue samples were fixed with 10% formalin and embedded in paraffin for histopathological analysis. Tissue sections were stained with H/E and AIV-NP was detected by IHC (*Table 3-2*). Evaluation of H/E sections from chickens sacrificed both at 6 hours p.i. and 1 day p.i. revealed no tissue damage in comparison to the non-infected group. Nervous system lesions were detected as early as 3 days p.i. in all groups, being more extensive in both the reassortant-infected groups. The lesions in CNS consisted of multifocal areas of malacia associated to immunostaining on neurons and glial cells. Multifocal areas of myocardial necrosis were observed in birds mainly from FPV NS GD and FPV NS VN groups from 3 days p.i. Positivity to AIV antigen observed in myocytes correlates with this finding. In FPV-infected animals this lesions were first observed at day 4 p.i. and only in one animal. However, one bird showed AIV-positive myocytes 3 days p.i. In all groups, slight lesions were observed in the liver with Kupffer's cells showing positivity for viral antigen. Moderate lesions were observed in kidney of FPV-infected birds, while more severe lesions were observed in the reassortant-groups, consisting of areas of tubular necrosis associated

to the presence of viral antigen. Infiltration of heterophils and macrophages were found in the necrotic areas. Severe lesions were also observed in pancreas, which from 3 days p.i. showed diffuse areas of necrosis and stained for the presence of associated viral antigen. Concerning the studied lymphohematopoietic organs (thymus, spleen and bursa of Fabricius) we observed moderate to severe lymphoid depletion from day 2 p.i.

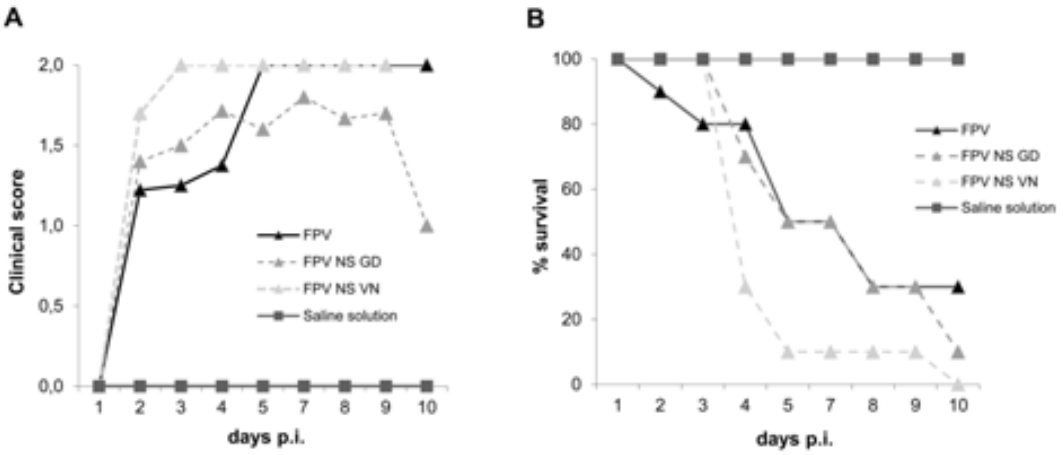


Figure 3-1. Clinical score and survival rate of SPF-chickens after infection with FPV, FPV NS GD or FPV NS VN. Chickens were intra-nasally infected with $10^{5.5}$ ELD_{50} of either, FPV (▲), FPV NS GD (▲) or FPV NS VN (▲). **a|** Average clinical signs of the surviving chickens and **b|** survival rate from each group measured at the indicated time points.

The viremia and the shedding of the three viruses was characterized by determining the amount of viral RNA present in blood (*Figure 3-2a*) and in OS (*Figure 3-2b*) and CS (*Figure 3-2c*) at early time points post infection. A quantitative real time RT PCR was performed from 6 hours p.i. until day 3 p.i. Animals infected with FPV NS VN showed significantly higher presence of viral RNA in blood and in OS at 2 days p.i. compared with the FPV-group ($P<0.05$). In FPV NS GD group, viremia and shedding was also higher compared with the FPV-group, but the differences were not

statistically significant when compared between groups. No differences were observed between both NS-reassortant groups in any of the time points.

Table 3-2. Average distribution of AIV-NP antigen determined by immunohistochemistry (IHC) in tissue samples from chickens inoculated with FPV, FPV NS GD or FPV NS VN at different time-points. The extension of the staining in tissues was measured by a semi-quantitative score: no positive cells (-), single positive cells (+), scattered groups of positive cells (++), and widespread positivity (+++).

VIRUS STRAIN / Tissue	Time of AIV NP antigen detection				Main localization
	1 dpi	2 dpi	3 dpi	4 dpi	
FPV					
CNS	-	-	+	+	Neurons, glial cells
Heart	-	+	+	+	Myocytes
Kidney	-	-	+	+	Epithelial tubular cells
Pancreas	-	-	+	+	Exocrine acinar cells
Liver	-	-	+	+	Kupffer's cells
Spleen	-	-	+	+	Macrophages
Thymus	-	+	+	+	Macrophages
Bursa of Fabricius	-	-	+	+	Macrophages
FPV NS GD					
CNS	-	-	++	+	Neurons, glial cells, ependymal cells
Heart	-	++	+	+	Myocytes, macrophages
Kidney	-	+++	++	++	Epithelial tubular cells
Pancreas	-	-	++	+	Exocrine acinar cells
Liver	-	-	+	+	Kupffer's cells
Spleen	-	+	+	+	Macrophages, Endothelial cells
Thymus	-	+	+	+	Macrophages, Endothelial cells
Bursa of Fabricius	-	-	+	+	Macrophages, Endothelial cells
FPV NS VN					
CNS	-	-	++	+	Neurons, glial cells, ependymal cells
Heart	-	++	+	+	Myocytes, macrophages
Kidney	-	-	++	++	Epithelial tubular cells
Pancreas	-	-	++	+	Exocrine acinar cells
Liver	-	-	+	+	Kupffer's cells
Spleen	-	-	+	+	Macrophages, Endothelial cells
Thymus	-	-	+	+	Macrophages, Endothelial cells
Bursa of Fabricius	-	-	+	+	Macrophages, Endothelial cells
Saline Solution					
CNS	-	-	-	-	-
Heart	-	-	-	-	-
Kidney	-	-	-	-	-
Pancreas	-	-	-	-	-
Liver	-	-	-	-	-
Spleen	-	-	-	-	-
Thymus	-	-	-	-	-
Bursa of Fabricius	-	-	-	-	-

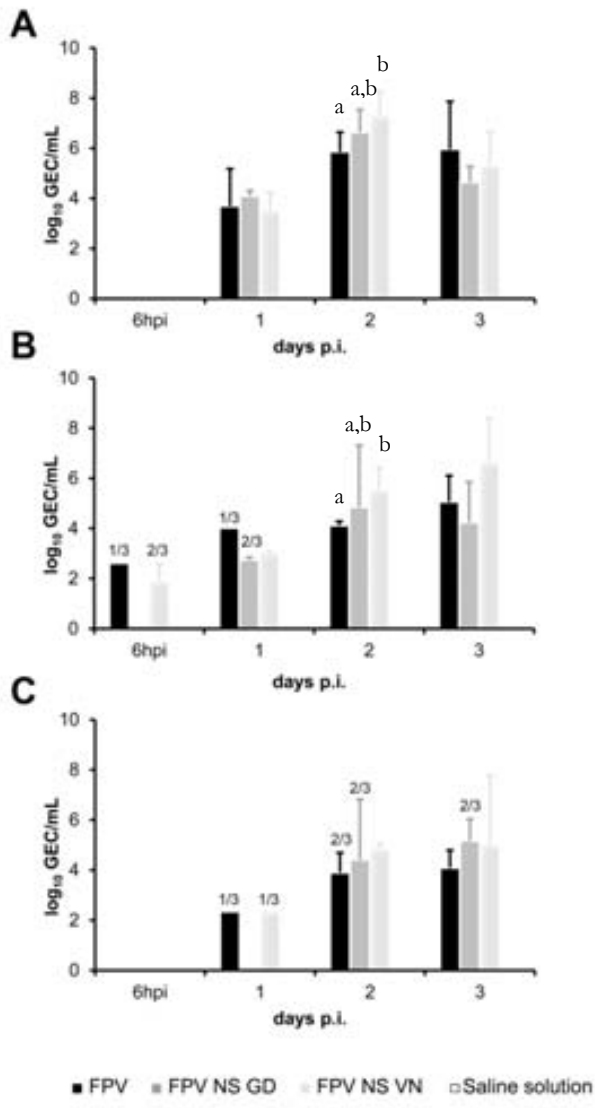


Figure 3-2. Viral shedding of SPF-chickens after infection with FPV, FPV NS GD or FPV NS VN. Viral RNA shedding measured by RRT-PCR in **a|** blood **b|** oropharyngeal swab and **c|** cloacal swab at the indicated time points. Results are expressed as log copies of genome and shown as means ± SD. Statistical significant difference ($P<0.05$) are indicated with letters.

3.4.2. Comparison of the transcription and expression of IL-1β and IFN-β genes in infected-chickens

In the present work, a comparison between animals infected with FPV virus and its recombinants carrying H5-NS1 protein, was done. Antiviral and pro-inflammatory cytokine expressions (IFN-β and IL-1β) in blood were sequentially studied through the infection. We performed a relative RT-qPCR to study the different amount of IFN-β and IL-1β mRNA produced after the infection with IAV, using the 28S gene as house-keeping gene.

At 48 hours p.i, different levels of IFN-β gene expression were observed between groups (*Figure 3-3a*). At this time-point, the levels of IFN-β mRNA were higher in FPV NS VN-infected animals, coinciding with the disease exacerbation observed. At the earlier time-point no evident differences were detected between groups. A significant differential IL-1β gene expression was also observed between inoculated and non-inoculated groups at 48 hours p.i. ($P<0.05$; *Figure 3-3b*), showing higher IL-1β mRNA levels in the formers. In contrast with that observed for IFN-β, FPV-infected animals showed a higher up-regulation of IL-1β compared with both those infected with the NS-recombinants. Interestingly, slight down-regulations of IL-1β were observed in FPV- and FPV NS GD-blood at 24 and 6 hours p.i., respectively, albeit these differences were not statistically significant (*Figure 3-3b*).

3.4.3. IL-1β up-regulation correlates with an increase in monocytes/macrophage-like cells

To test the amount of synthesized protein, as well as the cell population distribution after the infection, a flow cytometry analysis was performed to separate the blood populations by size and complexity (*Figure 3-4*).

Independently of the individual variability, blood from reassortant-infected chickens clearly showed an evident lymphocyte reduction that paralleled with an increase of the number of macrophage/monocyte-like cells that picked at 48h p.i.

Interestingly, while no evident up-regulation of IFN-β was observed independently of the group and infection time, a dramatic increase of IL-1β expression was found in both reassortant-infected groups at 48h p.i., being higher in those animals infected with FPV NS GD (23.6% cells expressing IL-1β) (*Figure 3-4*).

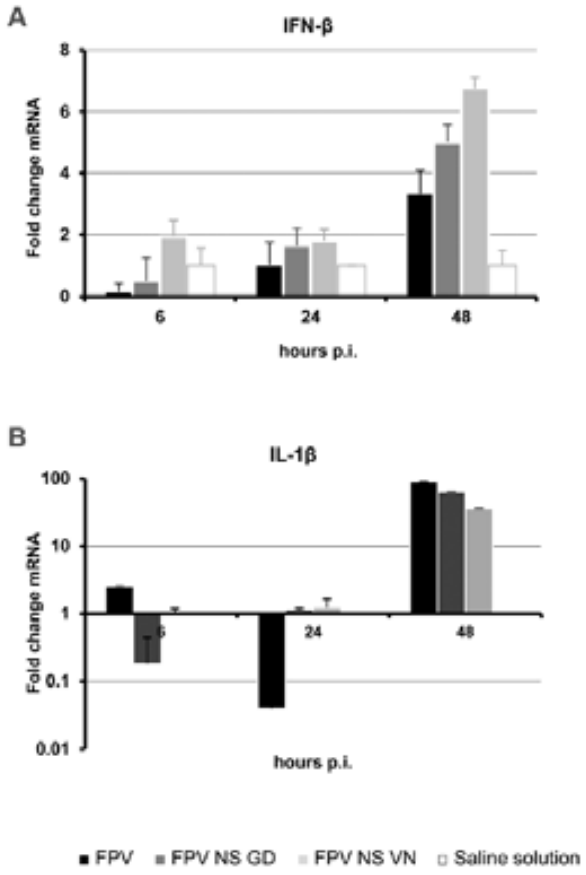


Figure 3-3. Quantification of IFN-β and IL-1β induced following infection with FPV, FPV NS GD or FPV NS VN in PBMC. a| IFN-β mRNA levels **b|** IL-1β mRNA levels. Results are expressed as means ± SD and as fold change in mRNA levels.

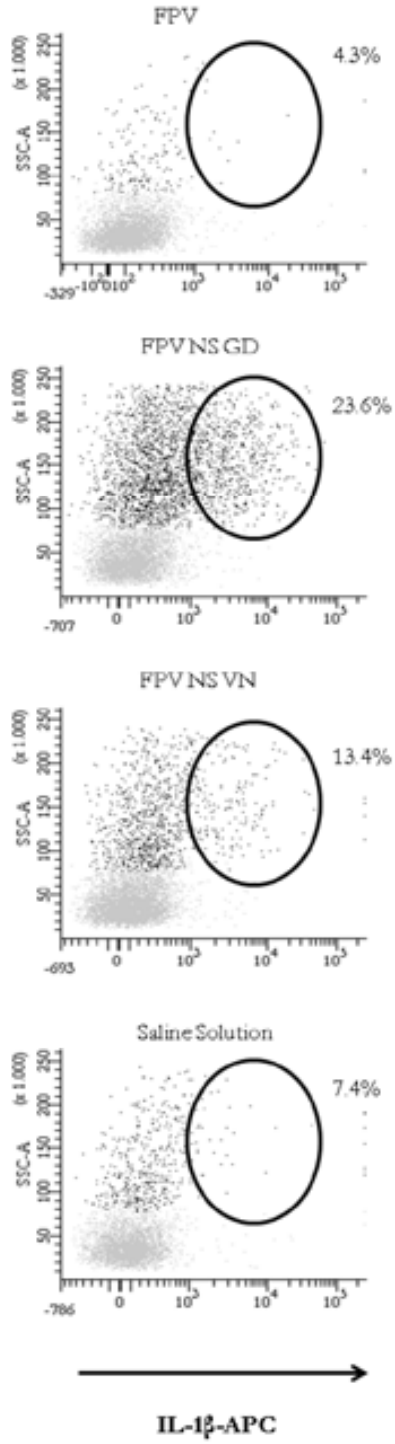


Figure 3-4. Expression of IL-1β protein induced following infection with FPV, FPV NS GD or FPV NS VN in PBMC measured by flow cytometry.

Black population represents monocytes/ macrophages-like cells and in light grey, the lymphocytes population is showed.

In contrast, at 48 h p.i., chickens infected with FPV exhibited a slight decrease (4.3%) in the total proportion of IL-1β single expressing cells compared to the non-infected group (*Figure 3-4*). These results, correlate with the presence of clinical manifestations. Thus, NS-reassortant infected-chickens showed clinical signs from day 2 p.i., whereas in FPV-infected ones the severe clinical signs (score=2) were observed from day 5 p.i. (*Figure 3-1*).

3.5. Discussion

The pathogenic potential of some strains of IAV has been related with multiple factors, including viral determinants and an excessive host immune response. As most of these descriptions have been done in mammals, in the present study we tried to extend these previous works to natural hosts of AIV. More specifically, we focused our studies aiming to understand the role that the AIV-NS1 of two different H5N1 HPAIV strains plays during pathogenesis. To this end, an experimental infection in SPF-chickens was designed with an H7N1 HPAIV (FPV) and two reassortants carrying the NS-segment of H5N1 HPAIV from either GD (FPV NS GD) or VN (FPV NS VN).

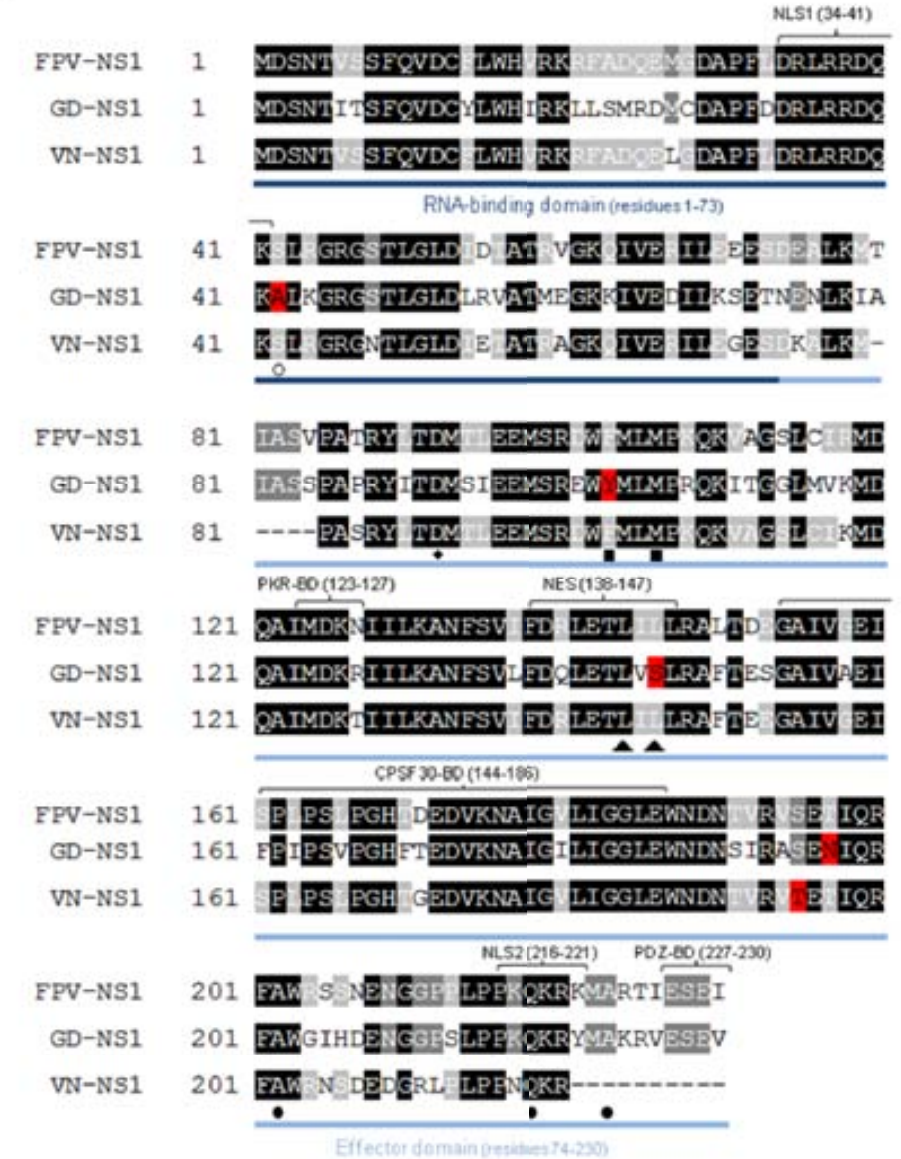
Amino acid changes in the viral hemagglutinin and polymorphisms in the polymerase subunit have been demonstrated to contribute to the virulence of AIV (*revised in* Salomon and Webster, 2009). However, among other viral proteins, the NS1 is one of the major pathogenicity factors and it mainly acts by suppressing type I IFN-activities (Hale, *et al.,* 2008) which are the first line of the host defense against viral infections. The role of AIV-NS1 in pathogenesis has been investigated for decades, albeit most of this work has been focused to understand its role in mammalian species. Treanor and collaborators reported that mammalian cells can be efficiently infected by FPV, depending on the NS1 allele incorporated within its (Treanor, *et al.,* 1989); a fact more recently confirmed by our collaborators which demonstrated that

the H5N1 NS1-reassortants increase the viral replication in mice and host range of the FPV (Ma, *et al.,* 2010; Wang, e*t al., 2*010). These studies have been here extended to SPF-chickens.

In our study, both reassortants (FPV NS GD and FPV NS VN) which differ from the FPV only in the NS-segment, resulted also more pathogenic in chickens. However, due to the fact that FPV is a HPAIV strain in chickens, the differences observed in this animal model were not as dramatic as that shown by Ma and collaborators in mice, where FPV is non-pathogenic (Ma et al; 2010). Therefore, we could conclude that apart from its role to break specie-specific barriers, AIV-NS1 also accomplish a very important role as a virulent factor for avian species. Differences in the NS1 primary and/or secondary structure might account for the differences observed in the pathogenesis of the FPV, FPV NS GD and FPV NS VN.

Amino acid differences or substituions in the NS1 protein are described to alter its subcellular localization (Han *et al.,* 2010; Li *et al.,* 2011). This fact was confirmed recently for the NS1-sequences for the viruses used in the present work (Ma *et al.,* 2010; Wang *et al.,* 2010). Thus, the comparison of the amino acid sequences of the viruses used in this study (*Figure 3-5*) showed that the residues 217 and 221 in the NLS2 of FPV NS VN are different and deleted, respectively, compared to FPV and FPV NS GD-NLS2. Moreover, FPV NS GD-NES present some amino acid differences compared to the other two viruses. The differences found, affecting to one of the nuclear localization signals (NLS2), to the nuclear export signal (NES) or to both, perfectly explain the differential localization of each NS1 within the infected cell. Therefore, FPV-NS1 is being found in the nucleous of the cell, the GD-NS1 being localized within the cytoplasm and with the VN-NS1 ocupaying both intracellular locations (Greenspan, *et al.* 1988; Qian, *et al.*1994; Wang, *et al.* 2010). This differential localization could also explain the different modulation observed for both the IFN-β and IL-1β cytokines, at the transcrptional (mRNA) and post-transcriptional (protein) level.

Figure 3-5. Comparison of the NS1 of FPV (H7N1) and GD (H5N1) and VN (H5N1)
Identical amino acids are boxed in black. The regions of the RNA-binding domain and of the effectors domain are underlined by dark blue and light blue bar, respectively.



Interestingly, a very strong overexpression of IL-1β was observed after the infection with the GD- and VN-NS1 reassortants, detectable even in the absence of any *in vitro* re-stimulation. The over-expression of IL-1β detected after the infection with the NS-reassortants might obey to an increase on the virus replication in monocytes and macrophages, rather than a direct effect of the reassortant NS1-viruses; since previous studies made *in vitro* with the NS1 and HA proteins clearly demonstrated that the latter was the only one susceptible to directly stimulate the IL-1β expression (Vongsakul *et al.,* 2011).

Independently of the differences found at the RNA level, no significant differences were observed regarding the expression of IFN-β, while significant differences were found for IL-1β, mainly at 48 h p.i. Thus, the presence of the GD- or VN-NS1 protein seemed to enhance the induction of IL-1β expression, mainly by monocytes and macrophage-like cells (*Figure 3-4*). IL-1β plays a dual role for host immunity. Together with IL-18, form what is named as imflamosome, an essential innate mechanism that has to be activated in order to prime the immune system for future memory adaptive specific responses. On the other hand, IL-1β by itself has been directly linked to autoimmune disorders and also to immunopathogenesis after infection with all kind of pathogens including viruses.

IL-1β is a key immunomodulator cytokine that plays multifactorial role including two apparently opposite functions: (i) IL-1β plays an essential role forming part of what has been called as inflammasome (as mentioned), an innate immune machinery that plays an essential role to mount adaptive immune responses against pathogens, including influenza (Ichinohe *et al.,* 2009) and (ii) on the other hand, as antipyretic. IL-1β can be elevated immediately after the infection with virulent avian virus strains at it has been demonstrated for reovirus (Wu *et al.,* 2008) or Marek infections (Abdul-Careem *et al*; 2009) and also as a consequence of bacterial and virus co-infections, likely contributing to exacerbate lesions (Loving *et al.*, 2010).

As main conclusions, our work clearly demonstrate: i) that the NS1 from the HPAIV H5N1 have a dramatic impact on the FPV pathogenesis in chickens and ii) that this increase in the pathogenesis was coincident with an early over-expression of IL-1β from monocyte/macrophages-like cells and with an increase in the specific apoptosis of peripheral lymphocytes. These studies could be of utility to better understand the pathogenesis of HPAIV and to develop future anti viral strategies. Thus, FPV NS GD or FPV NS VN could be additionally modified by deleting the RNA-binding motif of the NS1 protein, obtaining replication-deficient and much more immunogenic influenza virus vaccines that those previously described in the literature by using this method (Ferko *et al.*, 2004).

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Chapter 4

Study II: Exposure to a Low Pathogenic A/H7N2 Virus in Chickens Protects against Highly Pathogenic A/H7N1 Virus but not against Subsequent Infection with A/H5N1

4.1. Abstract

Recent evidence has demonstrated that the presence of low pathogenic avian influenza viruses (LPAIV) may play an important role in the host. While some authors have clearly demonstrated that LPAIV can mutate to render highly pathogenic avian influenza viruses (HPAIV), others have shown that their presence could provide the host with enough immunological memory to resist re-infections with HPAIV. In order to experimentally study the role of pre-existing host immunity on the pathogenicity of avian influenza viruses (AIV), chickens previously infected with H7N2 LPAIV were subsequently challenged with H7N1 HPAIV. Pre-infection of chickens with H7N2 LAPIV conferred protection against the lethal challenge with H7N1 HPAIV, dramatically reducing the viral shedding, the clinical signs and the pathogenic outcome. Correlating with the protection afforded, sera from chickens primed with H7N2 LPAIV reacted with the H7-AIV subtype in hemagglutination inhibition assay and specifically with the N2-neuraminidase. Conversely, subsequent exposure to H5N1 HPAIV resulted in a two days-delay on the onset of disease but all chickens died by 7 days post-challenge. Lack of protection correlated with the absence of H5-hemagglutining inhibitory antibodies prior to H5N1 HPAIV challenge.

Our data suggest that in natural outbreaks of HPAIV, birds with pre-existing immunity to LPAIV could survive lethal infections with HA-homologous HPAIV but not subsequent re-infections with HA-heterologous HPAIV. These results could be useful to better understand the AIV dynamics in chickens and might help in future vaccine formulations.

4.2. Introduction

Avian influenza viruses (AIV) can be classified into low and high pathogenic avian influenza viruses (LPAIV and HPAIV, respectively) depending on the severity of the disease that they cause in chickens, which ranges from asymptomatic infection to acute systemic disease and even death (Swayne and Pantin-Jackwood, 2008). Although the virulence can be linked to the presence of multiple basic amino acids in the hemagglutinin (HA) cleavage site, the acquisition of a multibasic cleavage site alone can be insufficient to increase viral pathogenicity (Schrauwen *et al.,* 2011).

During the last decades, HPAIV have been involved in several outbreaks in poultry and wild birds around the world. The disease has had a severe economic impact because millions of birds died or have been destroyed to prevent viral spread (Lupiani and Reddy, 2009). Seventeen HA and 9 NA subtypes have been identified so far (Fouchier *et al.,* 2005; Tong *et al.,* 2012) but HPAIV have been most commonly described for the H5 and H7 subtypes. It is well known that LPAIV can mutate into HPAIV. An example occurred during the outbreak in 1999-2000 in Italy. The isolated virus was first characterized as an H7N1 LPAIV; however, some months later, an H7N1 HPAIV causing 100% of mortality was isolated in a turkey flock (Capua *et al.,* 2002). On the other hand, HPAIV could also appear as a consequence of reassortments between different LPAIV subtypes that co-infect wild birds, their natural reservoirs (Dugan *et al.,* 2008; Sharp *et al.,* 2008). Therefore, it seems important that surveillance programs should focus on the control of LPAIV, mainly those caused by viruses of the H5 or H7 subtypes, to prevent future emergences of HPAIV (Garamszegi and Moller, 2007).

Conversely to the inherent risks of their presence, pre-existing immunity due to LPAIV have also been demonstrated to confer a certain degree of protection against subsequent challenges with LPAIV and HPAIV in different species (Kida *et al.,* 1980; Kalthoff *et al.,* 2008; Fereidouni *et al.,* 2009; Berhane *et al.,* 2010; Jourdain *et al.,* 2010; Costa *et al.,* 2011). To characterize the impact of pre-existing immunity on the

pathogenicity of AIV, chickens were experimentally infected to assess whether the pre-exposure to H7N2 LPAIV can confer protection against H7N1 HPAIV and also, against a subsequent challenge with H5N1 HPAIV.

4.3. Materials and Methods

4.3.1. Ethics statement

The present study was performed in strict accordance with the Guidelines of the Good Experimental Practices. Animal procedures were approved by the Ethical and Animal Welfare Committee of *Universitat Autònoma de Barcelona* (UAB) (Protocol #DMAH-5767). Chicken experiments were conducted at Bisafety Level 3 (BSL-3) facilities of the *Centre de Recerca en Sanitat Animal (CReSA*-Barcelona).

4.3.2. Influenza viruses

The viruses used in this study were the LPAIV A/*Anas plathyrhynchos*/Spain/1877/2009 (H7N2), the HPAIV A/FPV/Rostock/34 (H7N1) and the HPAIV A/Great crested grebe/Basque Country/06.03249/2006 (H5N1). The H7N2 LPAIV strain was obtained from the ongoing surveillance program carried out in Catalonia, north-east Spain. The H7N1 HPAIV was generated by reverse genetics, as reported previously (Ma *et al.,* 2010) and the H5N1 HPAIV virus was isolated from a surveillance program in north-Spain (Barral *et al.,* 2008).

Virus stocks were propagated in the allantoic fluid of 11-day-old specific pathogen free (SPF) embryonating chicken eggs at 37ºC for 48 h (H5N1 HPAIV) and for 72 h (H7N2 LPAIV and H7N1 HPAIV). The allantoic fluids were harvested, aliquoted and stored at -80ºC until use. The infectious virus titer was determined in SPF embryonating chicken eggs and titers were measured as median embryo infectious dose (EID_{50}) for H7N2 LPAIV and median embryo lethal dose (ELD_{50}) for

H7N1 and H5N1 HPAIV by following the Reed and Muench method (Villegas, 2008).

4.3.3. Animals and experimental design

Thirty SPF chicken eggs (Lohmann Tierzucht GmbH, Germany) were hatched under BSL-3 containment conditions at CReSA. At 2-week-old, chicks were divided into three groups (*Table 4-1*). Each group was housed in independent biocontainment isolation units ventilated under negative pressure with high efficiency particulate air filters. Birds on group 1 (G1; n=10) were initially inoculated with H7N2 LPAIV ($10^{5.5}$ EID_{50}/50µl) and challenged 15 days later with H7N1 HPAIV ($10^{5.5}$ ELD_{50}/50µl). Two weeks after the H7N1 HPAIV challenge, six animals from group 1 were inoculated with H5N1 HPAIV ($10^{4.5}$ ELD_{50}/50µl). Birds on group 2 (G2; n=10) were inoculated with saline solution and challenged two weeks later with H7N1 HPAIV ($10^{5.5}$ ELD_{50}/50µl): this group served as positive control of H7N1 HPAIV infection. Finally, birds on group 3 (G3; n=10) were inoculated with saline solution twice at a 15-day interval; two weeks later, six animals from this group were inoculated with H5N1 HPAIV ($10^{4.5}$ ELD_{50}/50µl). This group served as a positive control of H5N1 HPAIV infection. All animals received the inoculums intranasally with a volume of 50 µl.

Chickens were monitored for the development of any flu-like clinical signs, and the mean clinical score and mortality rate (MDT) were recorded. The intensity of the clinical signs was assessed by a semi-quantitative scoring: healthy (0), sick (1), severely sick (2), moribund or dead (3). According to ethical procedures, animals presenting severe clinical symptoms (score 2) were euthanized with intravenous administration of sodium pentobarbital (100 mg/kg, Dolethal®, Vétoquiunol, France).

For the serological analysis, blood was collected from all birds 15 days post-H7N2 LPAIV inoculation and 10 days after H7N1 HPAIV challenge. In addition, cloacal (CS) and oropharyngeal (OS) swabs were collected for virus isolation at 1, 4, 7,

and 12 days post-H7N2 LPAIV inoculation, and at 1, 4, 7, and 12 days after H7N1 HPAIV challenge. The experiment was terminated 10 days after H5N1 HPAIV inoculation, time at which all the remaining birds were euthanized as described above and full necropsies were performed. All samples were stored at -80ºC until tested.

Table 4-1. Experimental design. Thirty 2-week old SPF-chickens were randomly distributed into three groups. Animals received the first inoculum (day 0) and 2 weeks later (day 15), birds were challenged with the respective inoculum 2. Six birds from G1 and G2 were consecutively infected 2 weeks later (day 30) with the third inoculum

Group	Nºanimals (n)	Inoculum 1 Day 0	Inoculum 2 Day 15	Nºanimals (n)	Inoculum 3 Day 30
G1	10	H7N2 LPAIV[a]	H7N1 HPAIV[b]	6	H5N1 HPAIV[c]
G2	10	Saline solution	H7N1 HPAIV	--	--
G3	10	Saline solution	Saline Solution	6	H5N1 HPAIV

Abbreviations: LPAIV= low pathogenic avian influenza virus; HPAIV= highly pathogenic avian influenza virus

[a]Chickens from G1 were inoculated intranasally with LPAIV A/*Anas plathyrhynchos*/Spain/1877/ 2009 (H7N2) ($10^{5.5}$ ELD_{50}). Birds from G2 and G3 received saline solution.

[b]Chickens from G1 and G2 were intranasally challenged with HPAIV A/FPV/Rostock/34 (H7N1) ($10^{5.5}$ ELD_{50}) 15 days after the pre-exposure to H7N2 LPAIV. Birds from G3 received saline solution.

[c]Chickens from G1 and G3 were inoculated intranasally with $10^{4.5}$ ELD_{50} of A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) 15 days after the challenge with H7N1 HPAIV.

4.3.4. Histopathology

Necropsies and tissue sampling were performed according to a standard protocol. For histopathological analysis, samples of central nervous system, heart, kidney, spleen, thymus, bursa of Fabricius and liver were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Tissue sections were processed routinely for hematoxylin/eosin staining

4.3.5. Virus quantification by real time RT-PCR (RRT-PCR)

Viral RNA quantification using one step RRT-PCR was performed in OS and CS, which were collected in sterile Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, S.A., UK) with antimicrobial drugs (100 units ml^{-1} penicillin-streptomycin). Viral RNA was extracted with QIAamp Viral Mini kit (Qiagen, Inc., Germany). Amplification of a matrix gene fragment was carried out using primers, probe, One-Step RT-PCR Master Mix Reagents (Life Technologies, S.A, UK) as previously reported (Spackman *et al.,* 2002) and amplification conditions as described by Busquets *et al.* (Busquets *et al.,* 2010) in Fast7500 equipment (Life Technologies, S.A, UK) using 5 µl of eluted RNA in a total volume of 25 µl. The limit of the detection of the assay was six viral RNA copies of *in vitro*-transcribed RNA per reaction, which was equivalent to Ct=39.16.

4.3.6. Solid phase competitive ELISA for H7-antibody detection

A competitive ELISA was developed for the evaluation of the presence of specific H7-antibodies in serum samples as previously described (Sala *et al.,* 2003). Briefly, micro-plates (Nunc, MaxiSorp™ microplates, DK, US) were coated with 50 µl per well of H7 AIV antigen diluted 1:500 in coating buffer (sodium bicarbonate 0.1 M) overnight at 4ºC. The LPAIV [A/Turkey/Italy/2676/99 (H7N1)] used as antigen was previously clarified, inactivated with β-propiolactone and partially purified by ultracentrifugation through a 25% (w/w) sucrose cushion. Sera from individuals were added to the H7 AIV-coated plate with 10-fold dilutions (starting from 1:10) and 25 µl of anti-H7 horseradish peroxidise (HRP)-conjugated monoclonal antibody (MAb) (7A4) were immediately added. After 1 h incubation at 37ºC, the plates were washed three times (PBS 1x/0.1% Tween20) and 50 µl of activated o-Phenylenediamine dihydrochloride (OPD) substrate solution were added to the wells. After 10 min incubation at room temperature (RT) the optical density (OD) was measured at

492nm. Positive H7N1 anti-serum (HI titre: 8 log_2) and negative control serum were included in each plate.

4.3.7. Liquid-phase blocking ELISA (LPBE) for N1- and N2-antibody detection

Sera were analyzed for the presence of N1 and N2 antibodies as previously described (Moreno *et al.,* 2009). Briefly, 96-well plates (Nunc, MaxiSorp™ microplates, DK, US) were coated with 50 µl per well of N1- (5B2, diluted 1:500) or N2- (4C11, diluted 1:200) specific capture monoclonal antibodies (MAbs) in coating buffer (sodium bicarbonate 0.1 M) overnight at 4ºC. AIV used as antigens in the respective LPBE [A/goose/Italy/296426/03 (H1N1) LPAIV and A/Turkey/England/28/73 (H5N2) LPAIV] were previously inactivated with β-propiolactione and then disrupted by adding Triton X100 to a final concentration of 3%. Mixtures of antigen at a pre-determined dilution and test sera diluted 1/2 and 1/4 (1/4 and 1/8 final dilutions) were pre-incubated at 37ºC for 60 min in an auxiliary micro-plate, then 50 µl were transferred into the respective MAb-coated plate and further incubated at 37ºC for 60 min. Plates were washed three times with PBS 1x/0.1% Tween20 and 50 µl of the homologous anti-N1 (5B2) and anti-N2 (4C11) HRP-conjugated MAb was added to wells followed by 1 h incubation at 37ºC. After washing the plates three times (PBS 1x/0.1% Tween 20), 50 µl of OPD substrate solution were added to the wells and allowed to develop for 8-10 min at RT. The OD was measured at 492 nm. An H7N1 anti-serum (HI titre: 8 log_2) and H9N2 anti-serum (HI titre: 8 log_2) were used as positive controls in the N1- and N2-ELISA, respectively. Serum from SPF chickens was used as a negative control.

Results from both ELISAs were calculated by determining the absorbance value reduction and were expressed as percentage of inhibition with respect to the reference value (100% control wells).

4.3.8. Hemagglutination inhibition test

Serum samples were also analyzed for the presence of antibodies against specific H5- and H7-subtypes by hemagglutination inhibition (HI) test. The assay was performed according to the international standard procedure (OIE, 2011) for testing avian sera using chicken red blood cells and 4 hemagglutination units of either H5N1 or H7N2 AIV. To avoid nonspecific positive reactions, sera were pre-treated by adsorption with chicken red blood cells and heat-treated at 56ºC for 30 min. Known positive and negative sera were used as controls.

4.3.9. Statistical analysis

Data obtained from the evaluation of OS and CS by RRT-PCR were analyzed by Kruskal-Wallis test for significant differences ($P<0.05$) between groups. The statistical tests were performed using the Statistical Package for the Social Sciences (SPSS) for Windows Version 17.0.

4.4. Results

4.4.1. Pre-exposure to LPAIV protects against the infection with an HA-homosubtypic HPAIV

In order to assess the role of pre-existing immunity in subsequent HPAIV, SPF-chickens were experimentally inoculated with H7N2 LPAIV and 15 days later, challenged with H7N1 HPAIV (the same HA-subtype). No clinical signs or lesions were observed after H7N2 LPAIV inoculation (G1), whereas inoculation of naïve animals with H7N1 HPAIV (G2) induced severe clinical signs and mortality from day 2 after inoculation (*Figure 4-1a*). Clinical signs mainly consisted in depression, apathy and ruffled feathers. Impaired breathing was observed in some of the animals from G2. Mortality was recorded until 7 days post-inoculation (dpi) and MDT was 4.5 days (range 2-7 days). In clear contrast, chickens pre-infected with H7N2 LPAIV were effectively protected against H7N1 HPAIV challenge. Thus, nine out of ten chickens

from G1 survived, showing only ruffled feathers at 1 dpi and no additional clinical signs of disease. The only animal from G1 that died at 1 dpi did not show flu-like clinical signs or pathological lesions. Additionally, birds from this group gained weight normally, while G2-birds lost it (*Figure 4-1b*).

After H7N1 HPAIV-challenge, lesions related to influenza were observed only in G2 from 3 days post-challenge (dpc) onwards. At 3 dpc, petechial hemorrhages on the comb and edema in the articulations were present only in one bird. Hemorrhages on the comb, wattles and legs were present in almost all the animals (8/10) from 4 dpc onwards. Between day 4 and 6 after challenge, crop congestion and proventriculus lesions which were characterized by glandular patron and petechial hemorrhages were detected in almost all birds (7/10). No lesions were observed in G1 confirming the solid protection against H7N1 HPAIV by the pre-exposition to H7N2 LPAIV. Animals from G3 (sham inoculated group) did not show clinical signs or lesions during this period.

Histopathological evaluation of tissue sections (*Figure S4-1*) from H7N1 HPAIV-infected chickens (G2) revealed myocardial degeneration and necrosis, moderate tubular necrosis in kidney, and moderate to severe lymphoid depletion in thymus, spleen and bursa of Fabricius from 3 dpc; and neuropil vacuolation and gliosis in the CNS and lymphoplasmacytic hepatitis at 4 dpc. No lesions were observed on the three birds from G1, which were euthanized on the last day of the trial (10 dpc-H7N1 HPAIV).

4.4.2. Previous infections with LPAIV and HPAIV do no protect against subsequent challenge with an HA-heterosubtypic HPAIV

To further analyze the potential cross protection afforded by the successive infection, two weeks after H7N1 HPAIV challenge, six chickens from G1 were inoculated with H5N1 HPAIV. Six birds from G3 were used as H5N1 HPAIV-positive control.

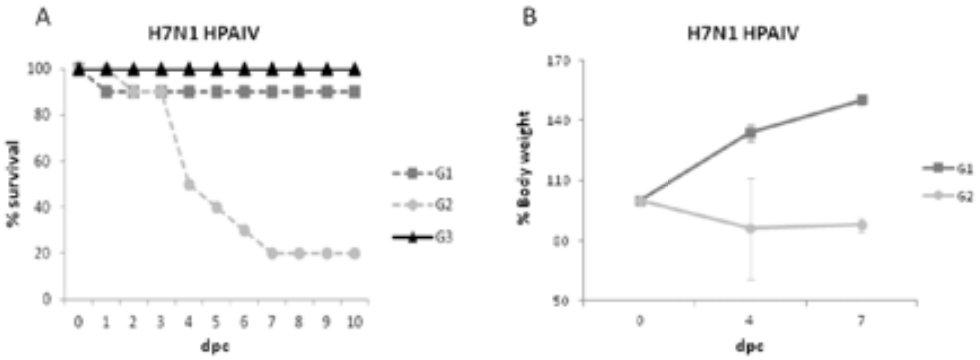


Figure 4-1. Lethality and weight loss in chickens after challenge with H7N1 HPAIV. a| Survival curves (in percentage) of SPF-chickens from G1 (pre-exposed to H7N2 LPAIV), G2 (positive control) and G3 (negative control) after H7N1 HPAIV-challenge. **b|** Weight loss curves of SPF-chickens from G1 and G2 after infection with H7N1 HPAIV. Mean %-body weight of animals normalized to initial weight ± SD is represented.

Three days after H5N1 HPAIV-challenge, one chicken (16.6%) from G1 died while five from G3 (83.3%) succumbed. In spite of this apparent delay of mortality rate, only one of the birds from G1 remained alive by 5 dpc and all were dead by 7 dpc (*Figure 4-2*). All animals lost weight and either exhibited neurologic signs prior to succumb or were found dead without previous clinical manifestations. The onset of morbidity ranged from 2 to 5 dpc in birds from G3 and from 3 to 6 dpc in G1. For the control group (G3) MDT was 3.7 days (range 3-6 days), while in G1 MDT was 4.5 days (range 3-7 days).

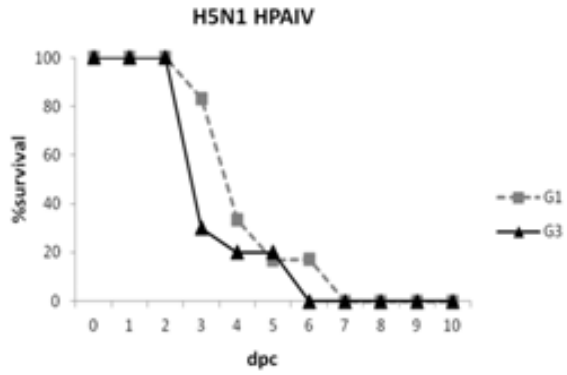


Figure 4-2. Lethality in chickens after challenge with H5N1 HPAIV. Survival curves (in percentages) of SPF-chickens from G1 (pre-exposed to H7N2 LPAIV and subsequently infected with H7N1 HPAIV) and G3 (positive control) after H5N1 HPAIV-challenge

4.4.3. Previous infection with LPAIV reduces HPAIV shedding

Oropharyngeal and cloacal shedding was assessed on days 1, 4, 7 and 12 after H7N2 LPAIV inoculation and H7N1 HPAIV challenge. After H7N2 LPAIV-inoculation, all chickens from G1 showed viral shedding at least once during the selected time-points as detected in either the OS, CS or both. No viral RNA was detected in G2 which, at this time-point, only received saline solution (*Figure 4-3a and b*). After H7N1 HPAIV-infection, all chickens from G2 (exposed only to H7N1 HPAIV) showed a consistent viral shedding from 1 to 7 dpc. No viral RNA was detected at 12 dpc in the two animals that survived.

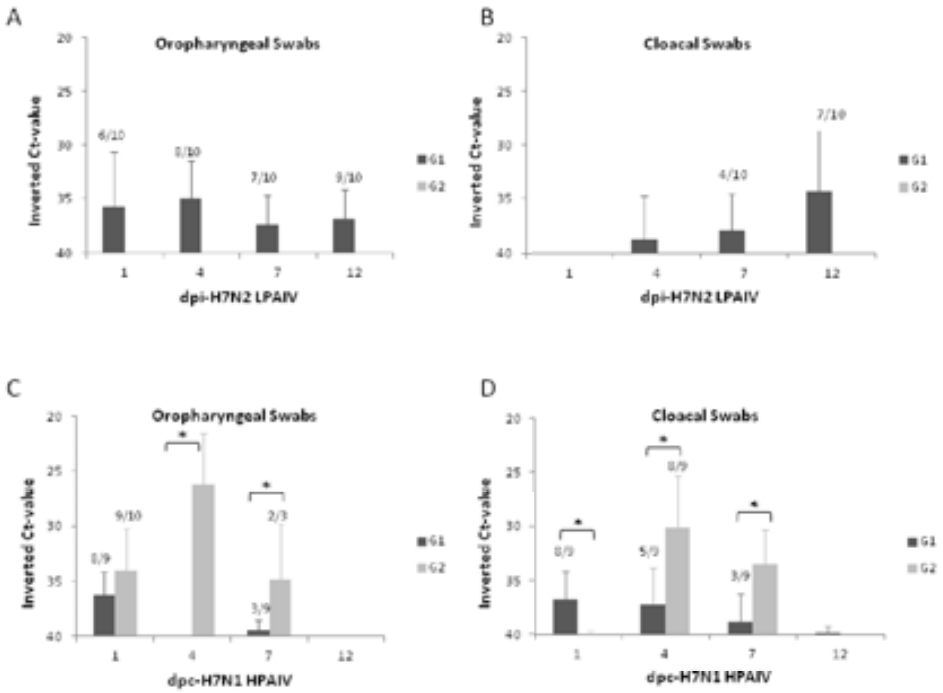


Figure 4-3. Viral shedding from experimental infected chickens with H7N2 LPAIV and to subsequent infection with H7N1 HPAIV. Viral RNA shedding measured by RRT-PCR in swab samples (oropharyngeal and cloacal) at 1, 4, 7 and 12 days after **a|**H7N2 LPAIV infection and **b|** H7N1 HPAIV challenge. Results are expressed as inverted Ct-value and shown as means ± SD. Ct, cycle of threshold. Asterisk (*) indicates statistical significant difference ($P<0.05$).

Conversely, pre-exposure to H7N2 LPAIV significantly ($P<0.05$) reduced shedding of H7N1 HPAIV from 4 dpc onwards, as compared to positive controls (G2) (*Figure 4-3c and d*). Although in G1 viral RNA was detected at 1 dpc (in both OS and CS), it is not possible to confirm whether the viral RNA detected is from H7N1 HPAIV or form the previous H7N2 LPAIV inoculation.

4.4.4. Pre-existing immunity to AIV has a role in the outcome of HPAI infection

Sera collected from chickens that were pre-exposed to H7N2 LPAIV (G1) inhibited hemagglutination by H7N3 antigen but did not elicit HI titers against H5N1 antigen (*Table 4-2*). Serum from only one animal from this group did not show any H7-hemagglutination inhibitory activity. However, it did not show clinical signs after H7N1 HPAIV infection. Sera collected 10 days after H7N1 HPAIV infection also inhibited the hemagglutination by H7N3 in all the birds from G1 and in the two birds from G2 that survived until the end of the experiment (*Table 4-2*).

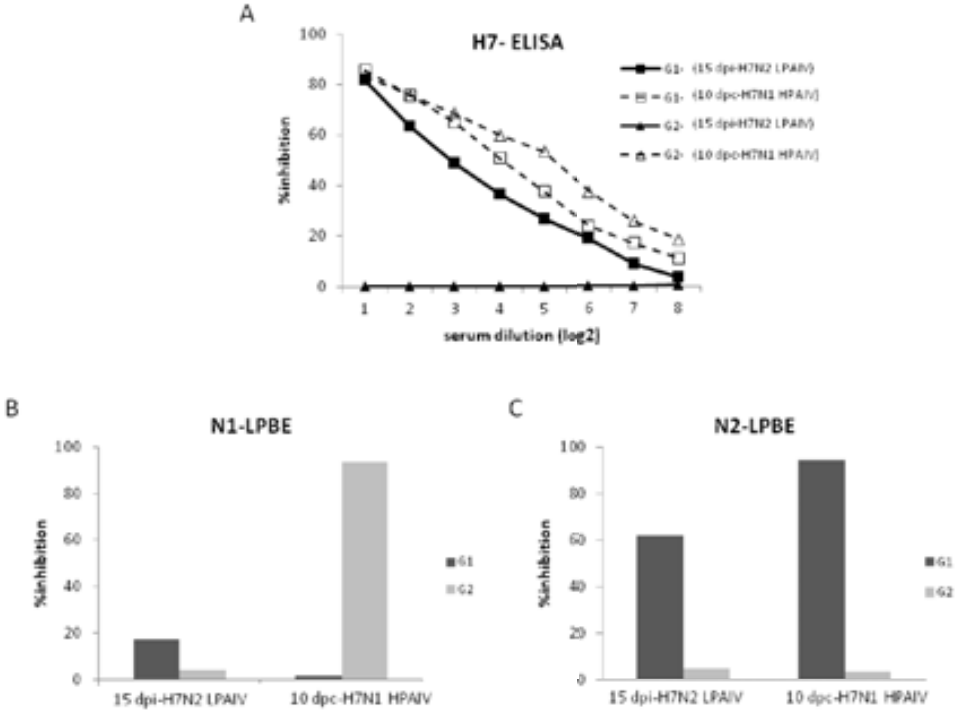
To further characterize the elicited humoral response, sera were also analyzed for the presence of antibodies against the specific hemagglutinin (H7) and neuraminidases (N2 or N1) by ELISA (*Figure 4-4*). As expected, the specific HA-ELISA yielded similar results than the HI assay (*Table 4-2*). Interestingly, no significant boosting effect was observed for the G1 group after H7N1 HPAIV challenge. Moreover, lower titers of antibodies against the H7-hemagglutinin seemed to exist for animals within this group than for the two survivors from the G2 at a given time (*Figure 4-4a*). In agreement with this data, sera from G1 elicited specific anti-N2 antibodies (*Figure 4-4c*) but did not elicit specific antibodies against N1, even after H7N1 HPAIV infection (*Figure 4-4b*). In contrast, sera from survivor chickens from the G2 showed antibodies against N1 but not against N2.

Table 4-2. Serological status, as determined by hemagglutination inhibition, of chickens 15 days after experimental pre-exposure to H7N2 LPAIV[a] and 10 days after challenge with H7N1 HPAIV[b]. Sera from the animals were tested against H7 and H5 antigens.

	HI Titer[c]			
Group Bird identification	**15 days post-H7N2/LP exposure (Day 15)**		**10 days post-H7N1/HP challenge (Day 25)**	
	H7[d]	**H5[e]**	**H7**	**H5**
G1				
1	16	<4	32	<4
2	32	<4	64	<4
3	8	<4	8	<4
4	<4	<4	32	<4
5	64	<4	128	<4
6	32	<4	64	<4
G2				
7	<4	<4	†	†
8	<4	<4	†	†
9	<4	<4	†	†
10	<4	<4	128	<4
11	<4	<4	†	†
12	<4	<4	128	<4
G3				
13	<4	<4	<4	<4
14	<4	<4	<4	<4
15	<4	<4	<4	<4
16	<4	<4	<4	<4

[a]Chickens were inoculated intranasally with A/*Anas plathyrhynchos*/Spain/1877/2009 (H7N2) ($10^{5.5}$ ELD_{50}). Serologic data from six randomly selected birds per group are presented.
Due to the lack of seroconversion, only four animals from the naïve group are represented in the table.
[b]Chickens were challenged intranasally with A/FPV/Rostock/34 (H7N1) ($10^{5.5}$ of ELD_{50}) 15 days after the pre-exposure to H7N2 LPAIV.
[c]HI titers ≥8 were considered positive
[d]HI using antigen against H7N3 subtype
[e]HI using antigen against H5N1 aubtype
†= succumbed to H7N1 HPAIV-infection

Figure 4-4. Presence of specific antibodies against H7-, N1- and N2- evaluated by ELISA. Pooled sera from chickens were taken 15 days post-H7N2 LPAIV exposure and 10 days post-H7N1 HPAIV challenge and were tested for binding to **a|** H7 hemagglutinin **b|** N1 or **c|** N2 neuraminidase by ELISA.



4.5. Discussion

The immune response induced by a pre-exposure to H7N2 LPAIV not only protected from H7N1 HPAIV mortality, clinical signs and viral shedding, but also blocked the incoming HPAIV to the point of not allowing enough antigen to prime for antibodies against the N1-neuraminidase, neither to boost the anti-H7 antibodies. These data could also have important implications for the host ecology because, in case of subsequent infections, the transmission of the virus between animals, although present, would be reduced. As described for other LPAIV strains in domestic and wild birds (Berhane *et al.,* 2010; Terregino *et al.,* 2010) this protection coincided with the

presence of specific hemagglutin inhibitory antibodies prior to challenge. Similarly, human vaccines only protected against closely related viruses and do not confer protection to all viruses sharing same HA-subtype. Thus, the protection afforded depends on the antigenic match between the viruses in the vaccine and those circulating (Fiore *et al.,* 2010).

The correlation between the presence of anti-H7 antibodies and protection seemed to have the exception with the presence of one animal that resulted protected in the absence of detectable antibodies against H7 prior to H7N1 HPAIV challenge. Several mechanisms could explain the protection afforded in this bird, including the induction of cross-reactive T-cells (Droebner *et al.,* 2008; Hillaire *et al.,* 2011; Kapczynski *et al.,* 2011). The presence of low, albeit undetectable levels of H7-inhibitory antibodies before HPAIV challenge in this animal should not be ruled out. This hypothesis seems to be confirmed by the fact that this single individual showed similar levels of H7-specific hemagglutinin inhibitory activity after HPAIV challenge, than the rest of the animals within the group, indicating the existence of some kind of previous priming (*Table 4-2*).

Lack of solid protection against H5N1 HPAIV challenge correlated with the absence of anti-H5 antibodies prior to challenge. The slight delay found on disease onset observed for the G1 animals could be related with the induction of cross-reactive T-cells or with the induction of cross-reactive antibodies against other viral determinants (Ding *et al.,* 2011). The fact that almost no anti-N1 antibodies were present in pre-immunized chickens seemed to demonstrate their implication in the protection observed, contrary to that observed in other studies in pigs (Van Reeth *et al.,* 2009). The absence of protection against H5N1 HPAIV was surprising taking into account recent published results using a similar experimental approach (Jourdain *et al.,* 2010), where mallard ducks infected with an H7N7 LPAIV were efficiently protected against heterosubtypic challenge with a H5N2 LPAIV. The degree of protection observed between each of these studies was extremely variable and might depend on multiple factors including: the host, the strain and virulence of the AIVs used during

the experimental procedure and the time-interval spanned between the infections (Kida *et al.,* 1980).

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Chapter 5

Study III: Comprehensive Serological Analysis of Two Successive Heterologous Vaccines against H5N1 Avian Influenza Virus in Exotic Birds in Zoos

5.1. Abstract

In 2005, European Commission directive 2005/744/EC allowed controlled vaccination against avian influenza (AI) virus of valuable avian species housed in zoos. In 2006, 15 Spanish zoos and wildlife centers began a vaccination program with a commercial inactivated H5N9 vaccine. Between November 2007 and May 2008, birds from 10 of these centers were vaccinated again with a commercial inactivated H5N3 vaccine. During these campaigns, pre- and post-vaccination samples from different bird orders were taken to study the response against AI virus H5 vaccines. Sera prior to vaccinations with both vaccines were examined for the presence of total antibodies against influenza A nucleoprotein (NP) by a commercial competitive enzyme-linked immunosorbent assay (cELISA). Humoral responses to vaccination were evaluated using a hemagglutination inhibition (HI) assay.

In some taxonomic orders, both vaccines elicited comparatively high titers of HI antibodies against H5. Interestingly, some orders, such as Psittaciformes, which did not develop HI antibodies to either vaccine formulation when used alone, triggered notable HI antibody production, albeit in low HI titers, when primed with H5N9 and during subsequent boosting with the H5N3 vaccine. Vaccination with successive heterologous vaccines may represent the best alternative to widely protect valuable and/or endangered bird species against highly pathogenic AI virus infection.

5.2. Introduction

Avian influenza (AI) is an infectious disease caused by type A influenza viruses of the *Orthomyxoviridae* family. AI virus subtypes are classified according to their surface glycoproteins: hemagglutinin (H1 to H16[1]) and neuraminidase (N1 to N9) (Fouchier *et al.,* 2005). To date, highly pathogenic avian influenza (HPAI) viruses are restricted mainly to infections with H5 and H7 subtype viruses, which have caused unprecedented morbidity and mortality in birds within the last few years (Capua *et al., 2006*). Aquatic wild birds, including Anatidae (ducks, geese, and swans) and Charadriidae (shorebirds), are widely considered to be the natural reservoir of AI virus (Munster *et al.,* 2006). Although wild birds were not known to be implicated in the initial HPAI outbreaks, in 2002, an outbreak of H5N1 HPAI virus in Hong Kong caused mortality in a wide range of avian species, including migratory birds and resident waterfowls (Ellis *et al.,* 2004). Since then, the H5N1 subtype of HPAI virus has spread throughout Asia and into Europe and Africa, affecting a large number of species. In 2005, an outbreak killed over 6,000 water birds (mainly bar-headed geese [*Anser indicus*], great cormorants [*Phalacrocorax carbo*], Pallas's gulls [*Larus ichthyaetus*], brown-headed gulls [*Larus brunnicephalus*], and ruddy shelducks [*Tadorna ferruginea*]) at the Qinghai Lake National Nature Reserve in northwest China (Chen *et al.,* 2006). Furthermore, several reports indicate direct bird-to-human transmission in some Asian countries (Xu *et al.,* 1999; Lin *et al.,* 2000). These zoonotic consequences and the ecologic value of protecting avian species have emphasized the need for effective control measures.

Due to unprecedented morbidity and mortality caused by H5N1 HPAI virus and given the value of birds kept in zoos, in 2005 the European Commission directive 2005/744/EC allowed vaccination against AI virus in such birds in zoos, under strict surveillance (European Commission, 2005). In the following years, different European

[1] As mentioned in Chapter 1 "*General Introduction"*; recently, a new HA (H17) has been described (Tong *et al.,* 2012). However, as the present study corresponds to an already published manuscript, the author decided not to modify the information.

countries established preventive vaccination campaigns in zoological institutions. In 2006, 15 Spanish zoos and wildlife centers underwent a vaccination program with a commercial inactivated H5N9 vaccine. Between November 2007 and May 2008, birds from 10 of these centers were vaccinated again with a commercial inactivated H5N3 vaccine, as decided by the Spanish government. The decision of changing the vaccine used in the first AI vaccination program (VP1) was based on experimental results showing that the H5N3 vaccine, a reverse genetics monovalent vaccine, was shown to elicit a strong immune response and protected chickens (Kumar *et al.,* 2007) and ducks (Middleton *et al.,* 2007) from experimental H5N1 infection, with no detection of viral shedding.

The goal of the present study was to compare the seroprotection elicited by inactivated H5N9 and H5N3 vaccines and evaluate the boost effect of H5N3 vaccine in inducing immune responses after priming a wide selection of avian species with H5N9 in Spanish zoos.

5.3. Materials and Methods

5.3.1. Vaccination

An inactivated, commercial, water-in-oil adjuvanted H5N9 (A/CK/Italy/22A/ H5N9/1998) vaccine (Poulvac i-AI H5N9-, Fort Dodge Animal Health, Weesp, Netherlands), containing at least 128 hemagglutination units (HAU) according to potency test, was used in zoos during the first AI vaccination program (VP1) in Spain. Vaccination against AI virus in some of the zoos began in March 2006, with the remaining zoos vaccinating up to September 2006. More than 2,600 birds were vaccinated in the 15 zoos participating in this study. The birds were vaccinated twice within a 3-week interval via the subcutaneous route. Eighteen months later, between November 2007 and May 2008, a second vaccination program (VP2) was carried out. At that time, an inactivated, commercial, water-in-oil adjuvanted H5N3 (strain rg-A/ck/VN/C58/04) vaccine (Poulvac i-AI H5N3-, Fort Dodge Animal Health,

Weesp, Netherlands), containing at least 256 HAU, was used. Ten out of the 15 zoos took part in the second vaccination program. More than 450 birds were vaccinated either once (if they had been previously vaccinated with the H5N9 vaccine) or twice (those being vaccinated for the first time). Most of the animals receiving the vaccine for the first time were born after VP1. Both vaccines are effective against the virus type in circulation and support the DIVA (differentiating infected from vaccinated animals) principle, as the N antigen differs from N1, which makes it possible to distinguish vaccinated birds from H5N1-infected birds while maintaining acceptable efficacy. Further details may be obtained from the manufacturer. In the two campaigns, the vaccine dose administrated was adapted to body weight. Thus, birds with a body weight of <2 kg were given 0.2 ml, those animals from 2 to 10 kg were given 0.5 ml, and those >10 kg were given 1 ml. Published mean body weights of the different species were used instead of using individual weights (Del Hoyo *et al.,* 2005).

5.3.2. Sampling

Blood was collected from the right jugular, brachial, or ulnar vein (left or right). In VP1, samples were obtained on the days of both first (*n*= 2,672 samples from 17 taxonomic orders) and second (*n*= 947 samples from 17 taxonomic orders) vaccinations, as well as 9 (*n*= 933 samples from 17 taxonomic orders) and 18 (*n*= 542 samples from 16 taxonomic orders) weeks following the first vaccination dose.

In VP2, blood was collected on the day of vaccination (*n*= 469 samples from 16 taxonomic orders) and 6 (*n*= 398 samples from 14 taxonomic orders) and 12 (*n*= 376 samples from 15 taxonomic orders) weeks after the first vaccination. In VP2, birds receiving an AI vaccine for the first time (107 out of 469) were revaccinated after 6 weeks (*Figure 5-1*). The official sampling protocol also included collecting cloacal swabs to detect the presence of AI virus by reverse transcription-PCR (RT-PCR), as described previously (Munster *et al.,* 2006).

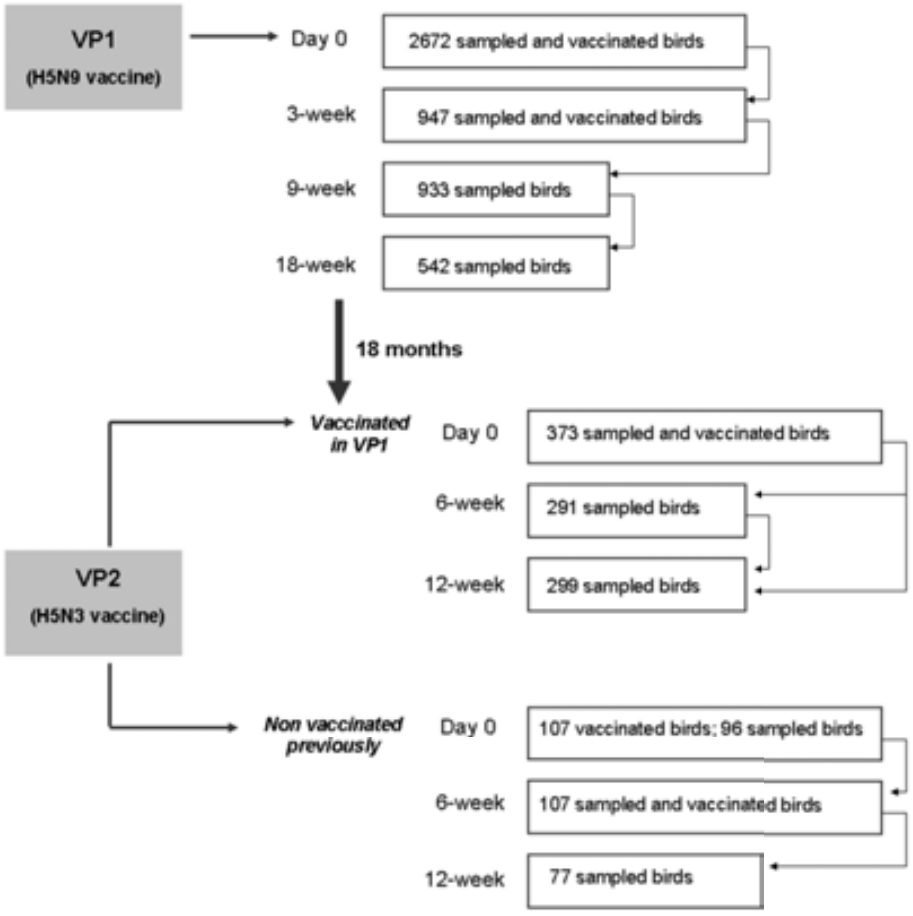

Figure 5-1. Vaccination and sampling schedule. In VP1, animals were vaccinated twice with an inactivated H5N9 vaccine, at day 0 and 3 weeks after the first dose. Eighteen months later, birds were vaccinated with an inactivated H5N3 vaccine (VP2). In VP2, two groups were differentiated, those being vaccinated for the first time and those that were previously vaccinated with H5N9. Serum samples were collected at all the time points indicated in the figure and tested by cELISA and HI. The numbers of animals tested are also indicated in the rectangles next to each time point.

5.3.3. Serology

Sera prior to vaccinations with H5N9 (A/CK/Italy/22A/H5N9/1998) and H5N3 (rg-A/ck/VN/C58/04) were examined for the presence of total antibodies against influenza A nucleoprotein (NP) by a commercial competitive enzyme-linked immunosorbent assay (cELISA) kit (ID VET, Montpellier, France). The cELISA is

based on recombinant AI virus NP as the antigen and a conjugated antibody directed against the NP of AI virus. The assay was performed according to manufacturer instructions.

To evaluate the humoral immune response induced after both vaccinations, homologous H5-specific antibody titers were determined by an HI test by following standard procedures (Palmer *et al.,* 1975). Briefly, chicken erythrocytes and 4 HAU of an H5 antigen (GD-Animal Health Service Deventer, Netherlands) were used for the test. Sera from some bird species may cause agglutination of the chicken erythrocytes used in the HI test, which may mask low levels of HI activity. For that reason, before doing the test, sera from all animals were pretreated with a 50% suspension of chicken erythrocytes for 1 h. Fifty microliters of pretreated serum was diluted by 2-fold serial dilution (1:2 to 1:4,096) in phosphate-buffered saline (PBS) solution in U-bottomed microwell plastic plates (Nunc, Copenhagen, Denmark), and 4 HAU of virus was added to each well. Following incubation at room temperature for 30 min, 50 µl of 0.6 to 0.75% chicken red blood cells (RBC) was added to each well, and the plates were incubated at room temperature for 30 to 45 min to allow RBC to settle. The HI titer was determined as the value of the highest dilution of serum causing complete inhibition of the 4 HAU. Vaccine-induced titers of≥32 were considered to be a measure of vaccine efficacy, and titers <16 were considered negative according to 92/40/EEC guidelines (European Commission, 1992). In poultry, HI titers of >16 were shown to indicate protection against infection when animals were challenged with HPAI H7N7 virus after vaccination with inactivated H7 AI vaccines (van der Goot *et al.,* 2005). Since performing challenge experiments in valuable zoo species is not possible and in accordance with the European Food Safety Authority (EFSA), we chose an HI titer of 32 as a threshold of protective vaccine efficacy, as vaccine manufacturers do (EFSA, 2007).

To evaluate the specific immune response against an HPAI H5N1 virus strain and to test the breadth of antibody response, post-vaccination serum was tested against A/Mallard/It/3401/05 (H5N1) and A/Tky/Eng/647/77 (H7N7).

No adverse reactions to vaccination were reported in any of the participating centers.

5.3.4. Statistical analysis

For each species and for each order, the geometric mean titer (GMT) and the percentage of animals with titers higher than 32, were calculated. Differences of GMT values between orders were tested with the Mann-Whitney test. Statistical analyses were performed using SPSS for Windows, version 17.0.

5.4. Results

5.4.1. Humoral response against H5N9 vaccination (VP1)

Detailed data concerning humoral immune response against an inactivated H5N9 vaccine from each order and species studied is provided in the appendix section (*Table S5-1*). Before receiving the vaccine, only 33 birds out of 2,672 (1.2%) showed antibodies against AI virus NP when tested by cELISA. Similarly, less than 1% of the animals were seropositive for H5 AI virus by an HI test using the homologous antigen. These 25 birds, presenting HI titers of 32 or higher, belonged to four orders (Phoenicopteriformes [*n*= 19 birds], Anseriformes [*n*= 3 birds], Ciconiiformes [*n*=2 birds], and Pelecaniformes [*n*= 1 bird]).

HI antibody titers 3 weeks after the first vaccination (at the time of the second vaccination) (*n*= 947 birds) and 9 (*n*= 933 birds) and 18 (*n*= 542 birds) weeks after the first dose were determined. After the first vaccine dose, the geometric mean titer (GMT) was 81, and 31.8% of birds reached a serum antibody titer of ≥32 against the H5 antigen. On average, after the booster vaccination, the GMT reached 103, and 51.4% had a titer of ≥32 against the H5 antigen.

To evaluate longer-lasting immunity, titers 15 weeks after the second vaccination were studied. More than 45% of the birds were considered positive, and the overall GMT

was 59. Of the 7 taxonomic orders for which more than 45 individuals were subjected to serological follow-up, 6 reached mean titers greater than 32 (*Figure 5-2*). Falconiformes, Pelecaniformes, Phoenicopteriformes, and Struthioniformes presented HI titers over 120. In contrast, Psittaciformes and Galliformes showed the lowest GMT values. However, only Phoenicopteriformes reached prevalences over 75% of antibody titers at 32 or higher. Over 50% of birds belonging to the orders of Galliformes, Falconiformes, and Anseriformes reached a serum antibody titer of ≥32.

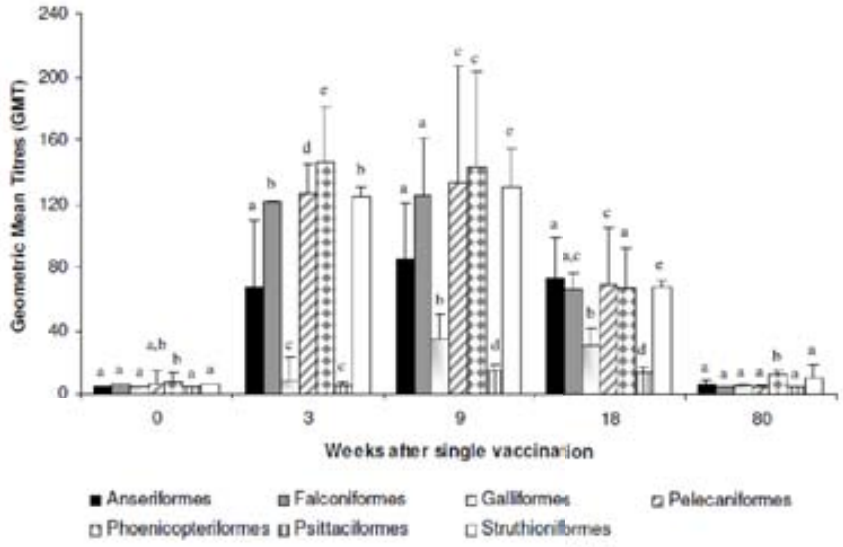

Figure 5-2. Humoral immune response following vaccination with an inactivated H5N9 vaccine (VP1). An inactivated H5N9 vaccine was used and administered twice within a 3-week interval. Bars represent the geometric mean titers (GMT) with standard errors (SE) of different taxonomic orders at different time points. The statistical significance of the difference (Mann-Whitney test) between taxonomic orders for each time point is indicated with a letter ($P < 0.05$).

5.4.2. Humoral response against H5N3 vaccination (VP2)

Detailed data concerning humoral immune response against an inactivated H5N3 vaccine from each order and species studied are provided in the appendix section (*Table S5-2*). Of 469 birds tested prior to VP2, 190 tested positive by the cELISA (40%). Most of the seropositive birds were from the following orders: Phoenicopteriformes (n= 74), Anseriformes (n= 51), Psittaciformes (n= 16), and

Ciconiiformes (*n*= 15). However, only 26 out of 190 animals were not vaccinated in the previous vaccination program (VP1). By HI test, 279 out of 469 (60%) birds were seronegative for H5 AIV.

In VP2, antibody titers at 6 (*n*= 398 samples) and 12 (*n*= 376 samples) weeks post-vaccination were studied. In both cases, the number of seropositive animals was around 40%, and the overall GMTs were different between those animals vaccinated in the previous vaccination program (VP1 with H5N9) and those vaccinated for the first time with H5N3 (*Figures 5-3 and 5-4*). Six weeks after the second dose of the H5N3 vaccine, Galliformes and Pelecaniformes orders (that were included in the VP2 with only the H5N3 vaccine) manifested a GMT higher than 150 (*Figure 5-3*). The Falconiformes order showed a weaker response, with a GMT of 50. The other birds that had not been vaccinated previously had a GMT of less than 32. Among animals vaccinated in VP1, Galliformes showed a very high response (GMT= 437) 12 weeks after receiving the H5N3 vaccine. The Psittaciformes and Struthioniformes orders reached seropositivity with a GMT of 58 and 128, respectively (*Figure 5-4*).

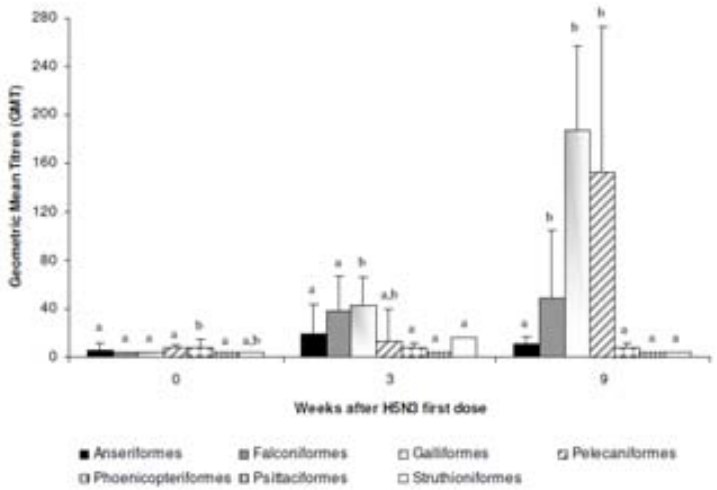

Figure 5-3. Humoral immune response following vaccination with an inactivated H5N9 vaccine (VP1). An inactivated H5N9 vaccine was used and administered twice within a 3-week interval. Bars represent the geometric mean titers (GMT) with standard errors (SE) of different taxonomic orders. The statistical significance of the difference (Mann-Whitney test) between taxonomic orders for each time point is indicated with a letter ($P<0.05$).

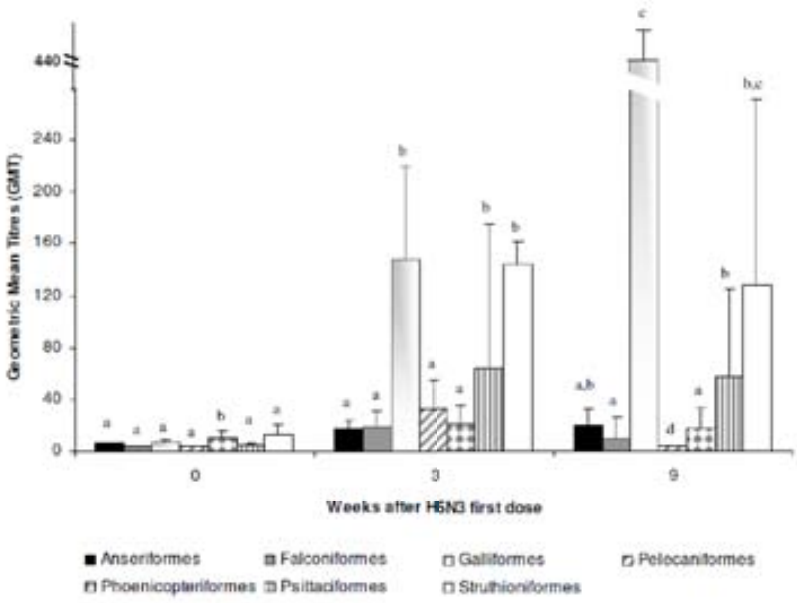

Figure 5-4. Humoral immune response in birds vaccinated with an inactivated H5N3 vaccine (VP2) and vaccinated previously with an inactivated H5N9 vaccine in VP1. An inactivated H5N3 vaccine was used and administered once. Bars represent the geometric mean titers (GMT) with standard errors (SE) of different taxonomic orders. The statistical significance of the difference (Mann-Whitney test) between taxonomic orders for each time point is indicated with a letter ($P<0.05$).

After H5N3 vaccination, 338 birds were evaluated for the presence of serum antibody titers against an HPAI H5N1 strain circulating in Europe (A/Mallard/It/3401/05) and for the presence of A/Tky/Eng/647/77 (H7N7)-specific antibodies. The response obtained against H5N1 was compared to those elicited against the H5N3 vaccine component. Moreover, two groups were differentiated between those being H5N9 and H5N3 vaccinated and those receiving only the H5N3 vaccine. The frequencies of birds reaching a seroprotective titer (≥32) are similar when testing antibody titers against H5N1 as well as for the vaccine compound in both the studied groups (*Figure 5-5*). No immune response against the H7N7 strain was detected in any of the studied animals.

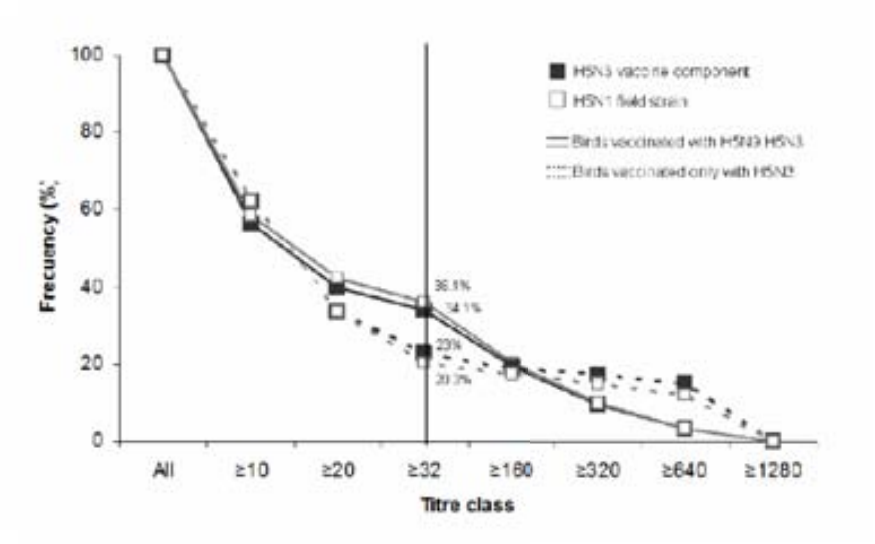

Figure 5-5. Comparison of serum hemagglutination inhibition (HI) antibody titers against the H5N3 vaccine and H5N1 field virus following vaccination with either a single vaccine (H5N3) or two successive heterologous vaccines (H5N9 and H5N3). HI titers against the vaccine component (A/ck/VN/C58/04; H5N3) and the field strain (A/Mallard/It/3401/05; H5N1) were determined in 338 birds 12 weeks after starting VP2.

5.4.3. Virus detection

No AIV antigen was detected in collected cloacal swabs in VP1. Prior to VP2, two animals that were RT-PCR positive were probably exposed to AI virus during this time interval. Both animals were from the Phoenicopteriformes order.

5.5. Discussion

In the present work, we demonstrate that carrying out two vaccination programs with successive heterologous vaccines in wild animals from Spanish zoos can be the key to widely protect species from taxonomic orders which did not develop HI antibody to a unique vaccine. In 2005, when the European Commission directive 2005/744/EC allowed vaccination against avian influenza (AI) in zoos (European Commission, 2005), other European countries also embarked on the mass vaccination program in zoo birds. Lately, results from some of the zoos, judging the efficacy of different vaccine formulations used, have been reported (Philippa *et al.,* 2005; Bertelsen *et al.,* 2007; Philippa *et al.,* 2007). Comparison of different vaccine formulations in eliciting a strong humoral response could be instrumental to decide future vaccination programs against AI virus.

In 2006, both Spain (data from present study, VP1) and Denmark (Bertelsen *et al.,* 2007) used inactivated H5N9 vaccines from different manufacturers in their vaccination programs in zoo birds. We observed that 51.4% of the H5N9-vaccinated birds in Spanish zoos had an HI titer of≥32 after booster vaccination, with an overall GMT of 103. The present data were comparatively lower than those previously reported by Bertelsen *et al.* (Bertelsen *et al.*, 2007), also using the H5N9 vaccine, where 76% of the zoo birds developed a titer of 32 with a GMT of 137. The differences in seroprotection efficacy between our results and those reported by Bertelsen et al. (Bertelsen *et al.*, 2007) may be due to different amounts of antigen or adjuvants used in the vaccine preparation, since the inactivated H5N9 vaccine studied by the Danish group was derived from a different manufacturer. Moreover, it should be noted that the present work is comprised of a large number of exotic birds (n= 933 after booster vaccination) from various orders, which may influence the amount of the overall GMT. This fact may also explain the heterogeneity in the antibody responses that we observed in serological analysis in vaccinated birds, which varied greatly, not only between taxonomic orders but also between species of a single order and even within

species. Similar observations with an inactivated H7N1 vaccine were published by Philippa *et al.* (Philippa *et al.,* 2005), who described a high seroprotection rate of 81.5% and an overall GMT of 190, with variations in HI titers among different bird orders examined. In general, based on the serological analysis from a huge number of H5N9-vaccinated Spanish zoo birds, we observed that more than 75% of birds from Phoenicopteriformes manifested a GMT of ≥32, and from the other 15 orders studied after booster vaccination, 12 had a protection rate less than 50%.

For the second vaccination program (VP2), the Spanish Ministry replaced the H5N9 vaccine with an H5N3 recombinant vaccine. The decision was based on the results given by the manufacturer, showing that H5N3 (a reverse genetics vaccine), besides protecting chickens (Kumar *et al.,* 2007) and ducks (Middleton *et al.,* 2007) from experimental AI infection, also prevented viral shedding. Masking disease signs while the bird continues to shed viruses may be a serious problem both for valuable exotic birds and humans. Thus, limiting virus shedding and further transmission is of extreme importance.

Vaccination with inactivated recombinant H5N3 vaccine was equally effective as VP1 in eliciting high titers of HI antibodies against H5 among most of the bird orders studied, except for birds belonging to Psittaciformes, which did not develop HI antibodies to either vaccination protocol. Interestingly, however, priming with H5N9 and subsequently boosting with the H5N3 vaccine induced a significant antibody response in Psittaciformes birds, albeit at lower titers than the others. Similarly, Galliformes and Struthioniformes birds responded to the H5N3 vaccine with much higher HI titers after booster vaccination. This strategy (prime-boost) could be used in some of the orders or species which do not respond to a unique vaccine. However, we also have to carefully pay attention to the antibody titer length. As shown in *Figure 5-2*, GMT after 18 months decreased drastically. Thus, some of the orders receiving H5N3 vaccine only once, because they were previously vaccinated with H5N9 (*Figure 5-4*), did not show high titers. Philippa *et al.* (Philippa *et al.,* 2007), based on previous

reports, have pointed to the need of a revaccination between 6 to 10 months after vaccination to maintain seroprotective titers among different wild species in zoos.
This was similar to the results we obtained in VP1 18 months after the single vaccination, where seroprotection titers started to decrease. The effect of a booster vaccination is seen clearly in VP2, in those animals nonvaccinated previously in VP1 (*Figure 5-3*), especially for the orders of Galliformes and Pelecaniformes, where GMT increased four times. These results are similar to those obtained by Philippa *et al.*, after booster vaccination increased the GMT by 30% (from 50.5% after single vaccination to 80.5% after booster vaccination) (Philippa *et al.,* 2007).

To design future vaccination strategies in exotic wild birds, it is important to evaluate both the response against the vaccine and the durability of HI antibodies. Sera 80 weeks after a single H5N9 dose were analyzed. On average, the birds had titers less than 20, meaning that, 1.5 years after vaccination, we cannot detect HI titers in serum samples. Antibody titers against HPAI H5N1 showed a similar trend as those against the homologous strain, with 34.1% of birds developing a titer of≥32 (animals vaccinated with successive vaccines, H5N9 and H5N3) and 20.3% of the animals receiving only the H5N3 vaccine showing seroprotective titers. However, both groups showed lower titers than the results reported by Philippa *et al.* (Philippa *et al.,* 2007), where 61.2% of the birds had a titer of≥40 against the HPAI strain tested, and more than 80% had a seroprotective titer against the homologous strain.

Taking into account that inactivated H5N3 vaccine induces strong immune responses and, more importantly, limits viral shedding (sterile immunity), a prime (H5N9)-boost (H5N3) vaccine strategy in future vaccination programs within exotic valuable zoo birds and in particular in the Psittaciformes, Galliformes, and Struthioniformes orders would be more adequate and advisable. Together with increased biosecurity measures and monitoring, vaccination may represent the best alternative to protect valuable and/or endangered bird species against HPAI virus infection. However, variations in elicited antibody responses among different bird orders and species must be carefully scrutinized in designing future vaccination

programs. This will not only protect vaccinated birds from infection but also restrict further dissemination of otherwise devastating HPAI virus.

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Chapter 6

Study IV: Conserved Synthetic Peptides from the Hemagglutinin of Influenza Viruses Induce Broad Humoral and T-Cell Responses in a Pig Model

6.1. Abstract

Outbreaks involving either H5N1 or H1N1 influenza viruses (IV) have recently become an increasing threat to cause potential pandemics. Pigs have an important role in this aspect. As reflected in the 2009 human H1N1 pandemic, they may act as a vehicle for mixing and generating new assortments of viruses potentially pathogenic to animals and humans. Lack of universal vaccines against the highly variable influenza virus forces scientists to continuously design vaccines *à la carte*, which is an expensive and risky practice overall when dealing with virulent strains. Therefore, we focused our efforts on developing a broadly protective influenza vaccine based on the Informational Spectrum Method (ISM). This theoretical prediction allows the selection of highly conserved peptide sequences from within the hemagglutinin subunit 1 protein (HA1) from either H5 or H1 viruses which are located in the flanking region of the HA binding site and with the potential to elicit broader immune responses than conventional vaccines.

Confirming the theoretical predictions, immunization of conventional farm pigs with the synthetic peptides induced humoral responses in every single pig. The fact that the induced antibodies were able to recognize *in vitro* heterologous influenza viruses such as the pandemic H1N1 virus (pH1N1), two swine influenza field isolates (SwH1N1 and SwH3N2) and a H5N1 highly pathogenic avian virus, confirm the broad recognition of the antibodies induced. Unexpectedly, all pigs also showed T-cell responses that not only recognized the specific peptides, but also the pH1N1 virus. Finally, a partial effect on the kinetics of virus clearance was observed after the intranasal infection with the pH1N1 virus, setting forth the groundwork for the design of peptide-based vaccines against influenza viruses. Further insights into the understanding of the mechanisms involved in the protection afforded will be necessary to optimize future vaccine formulations.

6.2. Introduction

In the last decades, several cases of human infection with the highly pathogenic avian influenza virus (HPAIV) H5N1 have been reported by the World Health Organization (http://www.who.int/influenza/human_animal_interface/avian_ influenza/en/). It is a common assumption that the pig may act as *mixing vessel* to generate new reassortant influenza viruses due to the presence of receptors for both avian and mammalian influenza viruses in the epithelial cells of their respiratory tract (Ito *et al.,* 1998). A recent example of the latter caused the first pandemic of the 21st century, starting in 2009 as a consequence of the global spread of a swine-origin influenza virus A H1N1 (pH1N1). This was a virus that contained genes from avian, pig and human origin (Vincent *et al.,* 2008). Although the virus was not as pathogenic to humans as expected, severe disease cases associated with pH1N1 have been more recently reported in England (http://www.who.int/influenza/surveillance_monitoring/ updates/2010_12_30_GIP_surveillance/en/). The future evolution of this or any emergent influenza virus (IV) is uncertain. This is a distressing matter particularly because available vaccines and therapies are strictly restricted to phylogenetically closely related circulating viruses. Therefore, finding universal and effective vaccines and therapeutic measures to fight against future IV is a must for public health.

IV hemagglutinin (HA) is a viral surface polypeptide that mediates both, the binding of IV to the host cell surface and the fusion of viral and endosomal membranes (Neumann *et al.,* 2009). HA is formed by subunit 1 (HA1) and subunit 2 (HA2) and both the N- and C- terminal parts of HA1 together with HA2 comprise the stalk of the molecule (Wilson *et al.,* 1981). Vaccines designed to elicit antibodies against the stalk of HA are reported to confer protection against IV infection in mice (Steel *et al.,* 2010). HA1, although highly variable, encodes specific and highly conserved domains which may be involved in determining the recognition and targeting (RTD) of influenza viruses to their receptor as revealed by the Informational Spectrum Method (ISM) (Veljkovic *et al.,* 2009a). This includes the VIN1 domain,

located within the site E in the N-terminus of HA1 (Veljkovic *et al.,* 2009b). In contrast with the high variability suffered by the globular part of the HA1 molecule, which is directly responsible for the receptor tropism, the site E remains relatively highly conserved (Matrosovich *et al.,* 2000). Thus, representing potential targets to develop broad array of protective therapies and vaccines against IV infection.

Due to the already mentioned recent cases related to H5N1 and H1N1 IV subtypes, and because their potential to cause future outbreaks among the population, we focused our efforts on designing a vaccine capable of conferring protection against both viral subtypes. As previously reported, RTD of HA1 from different H1N1 strains and HA1 from the recently emerged in Egypt H5N1 IV encode the same information. However, HA1 from H3N2 and all other H5N1 viruses encode different RT information (Veljkovic *et al.,* 2009a; Veljkovic *et al.,* 2009b). Thus, aiming to increase the vaccine coverage, one HA1- peptide from the VIN1 domain of H1N1 and three HA1-peptides from two different H5N1 IV strains were designed and selected based on ISM.

In order to test the immunogenicity of our experimental vaccine, we decided to immunize conventional pigs with the combination of the synthesized peptides. Pigs allow the evaluation of the protective efficacy of experimental vaccines against several viral strains, including the recently pandemic H1N1 virus, pH1N1 (Busquets *et al.,* 2010). Confirming the rationale behind their use as a preclinical animal model, immunization of conventional pigs with the VIN1-peptide cocktail allow us to demonstrate the induction of peptide-specific antibody and T-cell responses in every single animal, independently of their swine leukocyte antigen (SLA)-haplotype. Specific B and T-cell responses were induced against each one of the H1 and H5-peptides used, confirming their immunogenicity *in vivo*. Interestingly, the elicited antibodies also recognized several heterologous viruses *in vitro*, including the pH1N1, two swine influenza field isolates (SwH1N1 and SwH3N2) and a H5N1 highly pathogenic avian virus. This, together with the fact that the specific T-cell responses induced were also able to recognize the inactivated pH1N1, encouraged us to

challenge all pigs with the pH1N1 influenza virus. Albeit preliminary, our results demonstrate that VIN1-vaccination was able to confer a partial protection against intranasal challenge with pH1N1, as demonstrated with the partial and total viral clearance from the lung lavages in two out of four immunized pigs. We believe that our results could contribute to the obtainment of a broader array of protective vaccines against future influenza outbreaks or even pandemics.

6.3. Materials and Methods

6.3.1. Ethics statement

All experiments with the pH1N1 IV were performed at the Biosafety Level 3 facilities of the *Centre de Recerca en Sanitat Animal* (CReSA-Barcelona). Sample from the patient infected by pH1N1 IV was coded prior to isolating the virus to ensure anonymity. For this reason, the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona* (UAB) exempted this study from the requirement to have the consent of the patient, who was infected with pH1N1 IV. The present study was performed in accordance with the Guidelines of the Good Experimental Practices and under the supervision and approvement of the Ethical and Animal Welfare Committee of the UAB (*Permit Number:* DMAH-5796).

6.3.2. Animal experimental design

A total of eight 8-week-old conventional crossbreed pigs from a three-way cross (Duroc x Landrace hybrids paired with Pietrain boars) seronegative against influenza A virus were immunized three times two weeks apart. We immunized the pigs with either 15 μg of the VIN1-peptide cocktail (3.5-4 μg of each peptide) or saline solution in complete Freund's adjuvant (first immunization), incomplete Freund's adjuvant (second dose) and without adjuvant (last dose), by intramuscular (i.m.) administration. Four weeks after the second boost, the pigs were intranasally inoculated with 10^6 tissue culture infectious doses 50% ($TCID_{50}$) of the pH1N1 virus. Animals were

monitored daily for flu-like clinical signs. Sera and peripheral blood mononuclear cells (PBMC) obtained before each immunization, before the challenge and at 6 days post-infection (dpi), were used to detect specific humoral and cellular responses, respectively. Animals were euthanized at 6 dpi and a complete necropsy was carried out for each animal. Bronchoalveolar lavages (BAL) from the right lung of each pig were performed in 200 ml of PBS 1x immediately after post-mortem examination. BAL were frozen at -80ºC until their use for viral RNA extraction and quantification. For histopathological analysis, samples from lung (apical, middle and diaphragmatic lobes), nasal turbinate and trachea were collected and fixed by immersion in 10% neutral buffered formalin. In the lung, broncho-interstitial pneumonia (BIP) intensity was assessed by means a semi-quantitative scoring (0 to 3 indicating lack of, mild, moderate or severe pneumonia lesions, respectively) as previously described (Busquets *et al.,* 2010).

6.3.3. Virus and purified hemagglutinins

Viruses used were pH1N1 virus (the pandemic swine origin A/Catalonia/63/2009 H1N1 IV) [GenBank GQ464405-GQ464411 and GQ168897], SwH1N1 virus (A/Swine/Spain/003/2010 H1N1 IV) [GenBank JQ319725 and JQ319727], SwH3N2 virus (A/Swine/Spain/001/2010 H3N2 IV) [GenBank JQ319724 and JQ319726] and H5N1 HPAI virus (A/great crested grebe/Basque Country/06.03249/2006 H5N1 HPAIV) [GenBank EU636810 and EU636811].

After propagation at 37°C in the allantoic fluid of 11-day-old embryonated chicken eggs from a specific-pathogen-free flock, the infectious virus titer was determined in Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) cells and measured as $TCID_{50}$ by following the Reed and Muench method (Reed and Muench, 1938). Purified hemagglutinin for A/VietNam/1203/04 (H5) and A/New Caledonia/20/99 (H1) were purchased from Abcam.

6.3.4. Peptide synthesis

Four peptides were designed based on ISM predictions (Veljkovic *et al.,* 2009a; Veljkovic *et al.,* 2009b) and were mixed and used to immunize conventional pigs. The peptides were produced by GL Biochem (Shanghai) Ltd. Sequences from the synthetic peptides (thereafter referred as VIN1-peptides) are shown in *Table 6-1*.

Table 6-1. Amino acid sequences from the peptides used for immunization compared to the homologue sequence of the HA receptor recognition domain of the challenging strain (pH1N1) and the HA purified proteins used for the serologic tests.

Strain	Short name	Residues	Sequence
Challenge			
A/Catalonia/63/2009 (H1N1)	pH1N1	59-92	SSDNGTCYPGDFIDYEELREQLSSVSSFERFEIF
Immunization			
A/South Carolina/1/18 (H1N1)	NF-34	87-120	NSENGTCYPGDFIDYEELREQLSSVSSFEKFEIF
A/Egypt/0636-NAMRU3/2007 (H5N1)	ES-34	99-132	EELKHLLSRINHFEKIQIIPKNSWSDHEASGVSS
A/Hong Kong/213/03 (H5N1)	LE-35.1	41-75	LCDLDGVHPLILRDCSVAGWLLGNPMCDEFINVPE
A/Hong Kong/213/03 (H5N1)	LE-35.2	41-75	LCNLDGVKPLILRDCSVAGWLLGNPMCDEFINVPE
HA purified proteins			
A/VietNam/1203/04 (H5)	VN04	115-149 57-91	EELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSS LCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPE
A/New Caledonia/20/99 (H1)	NCD99	101-134	NPENGTCYPGYFADYEELREQLSSVSSFERFEIF

Grey shaded amino acids represent differences between the pH1N1 virus and the H1-peptide (NF-34) in homologous positions within the HA receptor recognition domain. Black shaded amino acids represent differences in the two H5-HK derived peptides (LE-35.1/2). Amino acid differences between the ES-34 and the H5-protein are marked in dark blue. Light blue represent the differences between the H5-HK peptides and the H5-protein. In red, the differences between the NF-34 and the H1-protein are marked.

The selected peptides were highly conserved and mapped to the flanking region of the HA1 within the VIN1 domain. Two peptides (LE-35.1 and LE-35.2) were derived from A/Hong Kong/213/03 (H5N1) [GenBank AB212056] and one (ES-34) from A/Egypt/0636-NAMRU3/2007 (H5N1) [GenBank EF382359]. The fourth peptide (NF-34) was derived from the HA1 of the human A/South Carolina/1/18 (H1N1) strain [GenBank AF117241].

6.3.5. Quantitative real time RT-PCR (RT-qPCR)

Viral RNA quantification using TaqMan RT-qPCR was performed in BAL. Viral RNA was extracted with QIAamp Viral Mini kit (Qiagen, Inc.). Amplification of a matrix (*M*) gene fragment was carried out using primers, probe, one-Step RT-PCR Master Mix Reagents (Applied Biosystems) and amplification conditions as described previously by Busquets *et al.* 2010 (Busquets *et al.,* 2010) in Fast7500 equipment (Applied Biosystems).

6.3.6. Influenza nucleoprotein (NP)-specific ELISA

Sera from animals before starting the experiment were examined for the presence of specific antibodies against influenza NP using the ID Screen® Influenza A Antibody Competition ELISA (ID VET, France), following manufacturer's instructions. Pig serum samples were used at 1:100 dilution. Known positive and negative sera were used as test controls.

6.3.7. Peptide-specific ELISA

A peptide-based ELISA method was developed for the evaluation of the presence of specific antibodies in serum samples. Briefly, 96 well plates (Costar, Corning Incorporated) were coated with 1 µg/ml of each peptide individually, the VIN1-peptides cocktail or H5-/H1- purified hemagglutinin in coating buffer (sodium bicarbonate 0.1 M) overnight at 4ºC. After blocking with 1% casein/PBS 1x for 1-h at 37ºC, serum from individuals were added to the coated plate diluted at 1:100 or titrated with 10-fold dilutions (starting from 1:10), followed by 2-h incubation at 37ºC. Plates were washed four times with PBS 1x/0.1% Tween20 and anti-pig IgG (whole molecule)-Peroxidase (Sigma) diluted 1:20,000 was added to wells followed by 45 min incubation at 37ºC. After washing the plates four times (PBS 1x/0.1%Tween20), fifty µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution were added to the wells

and allowed to develop for 8-10 min at room temperature (RT) protected from light. Optical density (OD) was measured at 450 nm.

6.3.8. Hemagglutination inhibition (HI) assay

An HI assay was performed following the standard procedures (World Organization for Animal Health, 2008) using chicken red blood cells (RBC) and 4 hemagglutination units of either, pH1N1 IV, SwH1N1 IV, SwH3N2 IV or H5N1 HPAIV. To avoid unspecific inhibitions, sera from individuals were treated prior to use. Briefly, one volume of serum samples was treated overnight at 37ºC with four volumes of Receptor Destroying Enzyme (Sigma) solution (100 U/ml). Next day, serum samples were incubated for 30 min at 56ºC after the addition of five volumes 1.5% sodium citrate. Finally, one volume of a 50% suspension of RBC was added and incubated for 1-h at 4ºC. Known positive and negative sera were used as controls. HI titers of ≥20 were considered positive.

6.3.9. Seroneutralization (SNT) assay

A SNT assay was done following the protocol described by Sirskyi and collaborators (2010) (Sirskyi *et al.,* 2010), with some modifications. Serum samples were diluted serially and incubated with 100 $TCID_{50}$ of pH1N1 virus for 2-h at 37ºC. The mixture was then added to 10^5 MDCK cells/well and incubated overnight. After two washes with PBS 1x, the cells were fixed with cold 80% acetone for 10 min. Cells were air-dried, washed five times with PBS 1x/0.05% Tween-20 and incubated at RT for 1-h and a half with biotinylated influenza A anti-NP primary antibody (CAT # MAB8252B, Millipore, CA) diluted 1/2,000 in 5%FBS/PBS 1x. Plates were then washed five times with PBS 1x/0.05% Tween-20 and incubated 30 min in the dark with HRP-conjugate streptavidin (Millipore, CA) diluted 1/10,000 in 5%FBS/PBS 1x. Finally, after five washes with PBS 1x/0.05% Tween-20, TMB substrate (Sigma) was

added to develop the reaction and stopped with Stop-solution H_2SO_4 (1N). Plates were then read at 450 nm.

6.3.10. IFN-γ ELISPOT Assay

An IFN-γ ELISPOT was performed as previously described (Argilaguet *et al.,* 2011), with some modifications. Briefly, PBMC were isolated from whole blood by Histopaque-1077 gradient (Sigma). Ninety-six-well plates (Costar, Corning Incorporated) were coated overnight with IFNγ-capture antibody (P2G10 clon, BD Pharmingen) diluted 1:100. After blocking the plates 1-h at 37ºC, 500,000 PBMC/well were seeded and stimulated with either 2 μg/ml of VIN1-peptide cocktail or individually, or with 10^5 $TCID_{50}$ of inactivated pH1N1 IV per well for 20-h. Cells were removed and a biotin mouse anti-pig IFN-γ detection antibody (BD Pharmingen), diluted 1:1,000, was used followed by streptavidin-peroxidase (0.5 μg/ml). Insoluble TMB blue (Calbiochem) was added as final substrate. Positive spots were counted using a microscope.

6.3.11. Immunofluorescence microscopy

MDCK cells (300,000 cells/well) were either mock infected or infected with pH1N1 IV, SwH1N1 IV, SwH3N2 IV or H5N1 HPAIV for 16-h at a MOI of 0.01. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then blocked with 3%BSA/PBS 1x for 1-h and incubated with the sera from the pigs (1:100) for 1-h in the blocking solution at RT. After three washes with PBS 1x, the cells were incubated with anti-IgG pig:FITC antibody (1:300) (Jackson Immunoresearch Europe LTd) for 1-h in blocking solution at RT. Finally, nuclei were counterstained with DAPI (1 μg/ml) and coverslips were mounted with Vectaschield. Protocol was modified from the previously described by Ballester *et al.* 2011 (Ballester *et al.,* 2011).

Fluorescence images were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). The images were processed by using the Image J v1.45l software (http://rsb.info.nih.gov/ij).

6.4. Results

6.4.1. VIN1 as a synthetic peptide-vaccine

The highly conserved VIN1 domain, located within the E site in the N-terminus of the HA1 molecule, plays an important role in the recognition and targeting (RT) between virus and receptor, therefore representing an ideal target for an antibody-mediated therapy against influenza infection (Veljkovic *et al.,* 2009b). Informational spectral analysis revealed that the RT domains of HA1 from H1N1/1918, pH1N1/2009, seasonal H1N1 and H5N1 emerged in Egypt encode the same information despite differences in their primary structures. Thus, based on ISM and using information available on the properties of HA and its receptors, a single 34-mer peptide (NF-34) from the H1N1 subtype was selected from within the VIN1 region. NF-34 corresponds to positions 87-120 from the A/South Carolina/1/18 (H1N1) virus (*Table 6-1*). Additionally, a peptide (ES-34) from the VIN1 domain from the A/Egypt/0636-NAMRU3/2007 (E; H5N1) was also selected and included in the vaccine. In previous studies, we also showed that HA1 from H3N2 and all other H5N1 encode different RT information (Veljkovic *et al.,* 2009a; Veljkovic e*t al.,* 2009b). Aiming to increase the vaccine coverage, two additional peptides (LE-35.1 and LE-35.2) were selected from the A/Hong Kong/213/03 (HK; H5N1) IV and both peptides were added to NF-34 and ES-34. LE-35.1 and LE-35.2 differ only in positions 43 and 48 (*Table 6-1*.), representing "hot spots" of variability within this H5N1 sequence.

Amino acid sequences from pH1N1 virus and VIN1-peptides are given in *Table 6-1*. The identity between pH1N1 virus and NF-34 (H1-peptide) is 92%. The similarity between all the H5-peptides and the pH1N1 virus is less than 75% with

even lower identities, being less than 40% when comparing pH1N1 and ES-34. There was no homology between the HK-derived peptides (LE-35.1 and LE-35.2) and the E-derived peptide (ES-34). The two amino acid differences between LE-35.1 and LE-35.2 represent a 95% of identity between them.

6.4.2. Immunogenicity of VIN1 peptide in a pig model

The pig is a good model not only to study influenza pathogenesis and therapy (Barnard *et al.,* 2009), but also for developmental immunology (Rothkotter e*t al.,* 2002). Thus, to confirm the bioinformatic predictions related to the capacity of VIN1-peptides to elicit humoral responses, four of the influenza-seronegative pigs were immunized three times with the VIN1-peptide mixture with two week intervals between immunizations. Four extra-pigs (also negative for IV antibodies) were inoculated with saline solution and remained as negative controls during the assay. To evaluate the ability of the VIN1-peptide cocktail to elicit antibodies, sera obtained 15 days after each immunization were tested against the peptides. Results obtained by ELISA showed that the immunization was efficient since every single immunized pig produced specific antibodies against the VIN1-peptide pool, detectable after the second immunization (*Figure 6-1a*). VIN1-peptides also elicited high antibody titres against each one of the single peptides included in the vaccine (*Figure 6-1b*). In correlation with the high specificity shown in the peptide-ELISA, sera from peptide-immunized pigs specifically reacted against the purified hemagglutinin protein of H5- and H1- subtypes, with only one serum from the V1N1 group showing background OD values in the H1-ELISA (*Figure 6-1c*).

Finally, we were intrigued to find that VIN1-peptides also had the ability to induce T-cell responses. PBMC isolated from VIN1-immunized pigs specifically secreted IFN-γ in response to *in vitro* stimulation with VIN1-peptide cocktail (*Figure 6-2*). First, we noted that VIN1-PBMC specifically secreted IFN-γ two weeks after the first immunization. Second, a homogeneous T-cell activity against the V1N1-peptide

cocktail was detected between animals after the third immunization (*Figure 6-2a*). And third, that all peptides were recognized by the specific T-cell induced (*Figure 6-2b*). These results demonstrated that VIN1-peptides could act as productive immunogens in pigs.

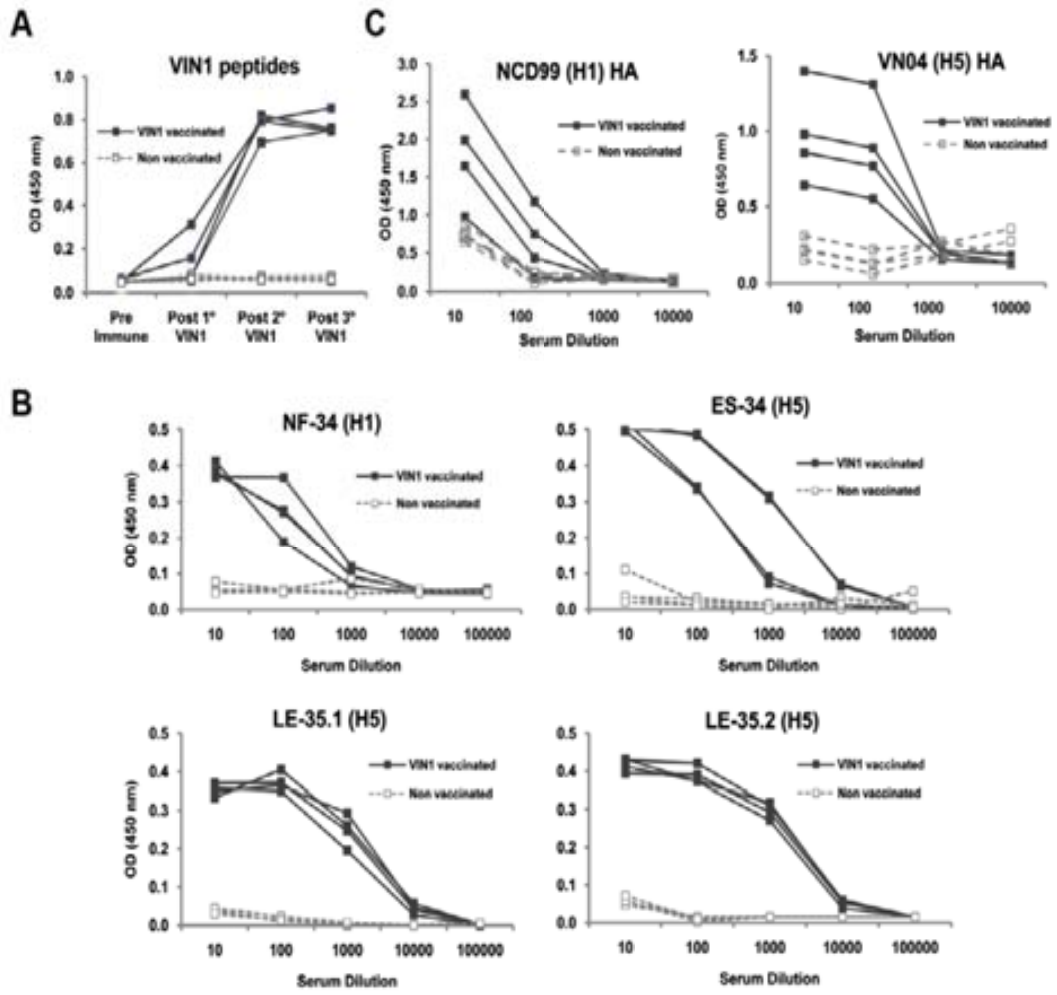

Figure 6-1. VIN1-peptide cocktail acts as a potent immunogen and the elicited sera react with different hemagglutinin subtypes and against VIN1-peptides. a| Sera from individuals were obtained 15 days after each immunization and were tested for binding to a mixture of the VIN1-peptides (serum dilution 1:100) by ELISA. **b|** Sera from individual pigs were obtained 15 days after the third immunization and were serially diluted and tested for binding to each single peptide by ELISA and **c|** Sera described in b| were tested for binding to H5- or H1- recombinant hemagglutinin by ELISA.

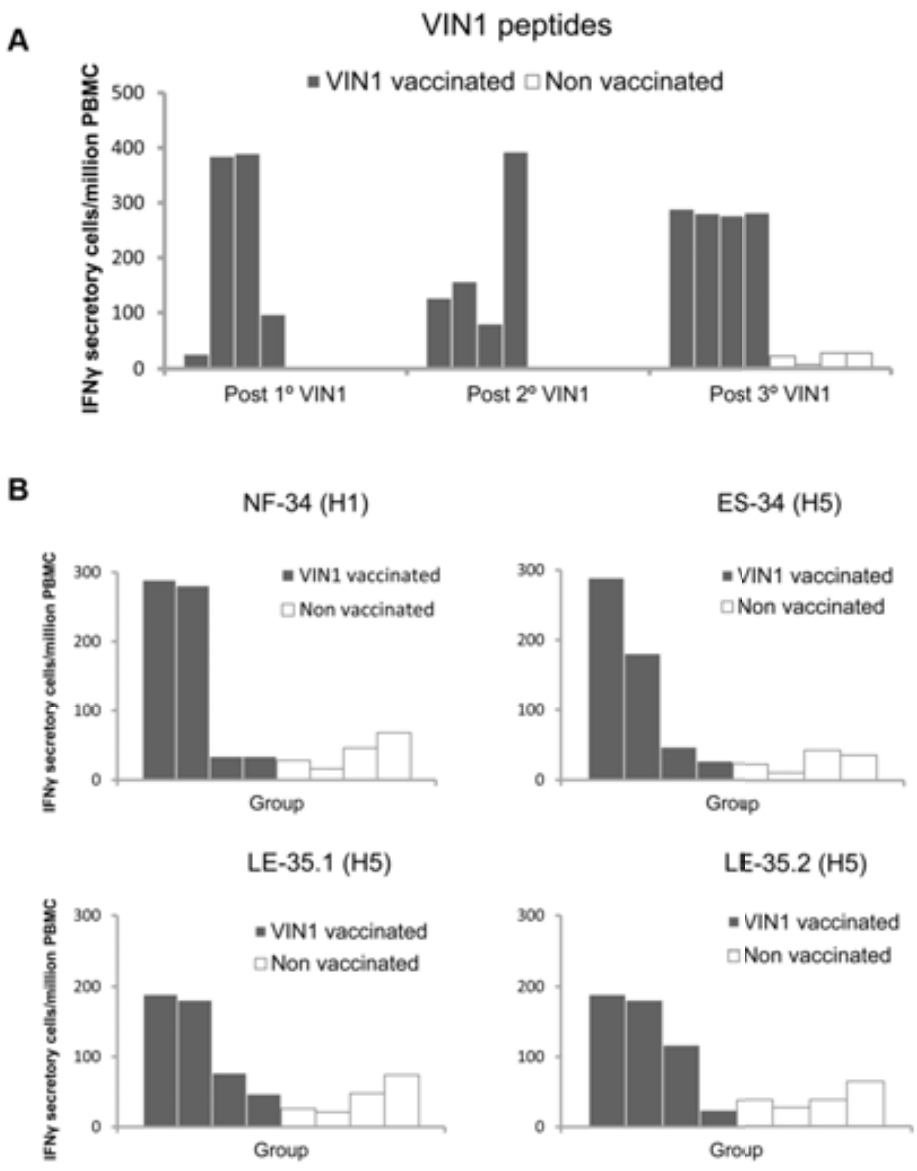

Figure 6-2. VIN1-HA1 derived peptides immunization induces strong T-cell responses in pigs. **a|** Kinetics of the VIN1 peptide-specific T-cell responses induced 15 days after all immunizations measured by IFNγ-ELISPOT. **b|** Specific T-cell responses induced 4 weeks after the third immunization were tested for each single peptide by IFNγ-ELISPOT.

6.4.3. VIN1 peptide immunization partially prevent pH1N1 virus replication in BAL

As previously reported, the pig can be used to evaluate the protection of experimental pH1N1 influenza vaccines since they are natural receptive hosts for this virus subtype (Busquets *et al.,* 2010). Aiming to evaluate the protective potential of our vaccine prototype, VIN1 peptide-vaccinated and control pigs were subjected to intranasal challenge with 10^6 $TCID_{50}$ of pH1N1 IV. The pH1N1 virus differs in three amino acids from the H1-peptide (NF-34) used in the vaccine (*Table 6-1.*).

Intranasal infection of control pigs caused a subclinical infection and minor hystopathological changes. Moreover, mild to moderate BIP was recorded at necropsy (6 dpi), albeit virus was recovered from BAL at this time-point (*Figure 6-3.*). These results are in concordance with previously reported data obtained using colostrums-deprived pigs (Busquets e*t al.,* 2010); therefore, validating the use of seronegative conventional animals for vaccine testing. We did not detect differences in the severity of the lesions in lungs of vaccinated and non vaccinated animals. However, in contrast with control pigs, 2 out of 4 VIN1-peptide vaccinated pigs showed no or less viral RNA in their BAL (*Figure 6-3.*), which demonstrates a partially protective effect of our experimental vaccine.

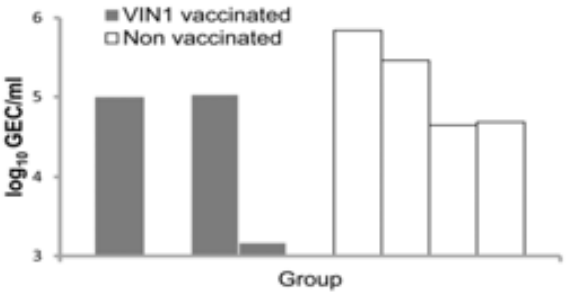

Figure 6-3. Immunization with VIN1-HA1 partially protects pigs *in vivo* against heterologous challenge with pH1N1. Influenza viral RNA quantification in BAL was performed by RT-qPCR at 6 dpi, corresponding to necropsy day. Bars indicated positive samples in genome equivalent copies (GEC) per ml of BAL. The detection limit in the assay was 3 log_{10} GEC/ml.

6.4.4. VIN1 peptides induce antibodies and T-cells that specifically recognize the pH1N1 virus

In an attempt to correlate the protection provided from the immunological outcome induced by our vaccine, sera from immunized and control pigs were used to evaluate their capability to *in vitro* recognize the pH1N1. Sera obtained before the challenge from pigs vaccinated with VIN1-peptides, specifically detected pH1N1 infected-MDCK cells, as shown by indirect IF (*Figure 6-4 panel A*). As expected, sera from control animals showed no reaction (*Figure 6-4 panel B*); thus, demonstrating the ability of the peptide-induced antibodies to specifically identify the virus. Importantly, every single cell infected by pH1N1 was also recognized by the specific NS1-monoclonal antibody (*Figure 6-4 panel C*) confirming the specificity of the reactions.

Furthermore, sera from 2 of the pigs immunized with VIN1-peptides showed detectable HI activity, albeit at low titre and only those obtained at 6 dpi (*Figure 6-5a*). As expected, sera from the control pigs did not show any specific response even at 6 dpi, which confirms the efficacy of our experimental vaccine to prime for viral-specific antibody responses. Regarding the presence of SNT antibodies, no significant differences were observed between the animal groups, at least at day 6pi (*Figure 6-5a*).

As occurred for the antibodies, the induced T-cell responses measured by IFN-γ ELISPOT, not only specifically recognized the synthetic peptides, but also the pH1N1 virus. Thus, before the challenge only one out of four of the VIN1-vaccinated pigs showed detectable T-cell responses in response to *in vitro* stimulation with the inactivated pH1N1, while all vaccinated pigs responded at 6 dpi (*Figure 6-5b*).

6.4.5. VIN1 peptides induce antibodies that recognize distinct viral subtypes

Current influenza vaccines protect mostly against homologous virus strains. The presented VIN1-peptide cocktail did not confer efficient neutralizing antibodies and only one pig did not show viral RNA in BAL 6 dpi. However, an IF was performed to

demonstrate that vaccination elicits antibodies that recognize different viruses. VIN1-sera obtained after three immunizations specifically detected SwH1N1 and H5N1 infected-MDCK cells, as shown by IF (*Figure 6-6*). Furthermore, antibodies elicited after VIN1-immunization specifically detected SwH3N2 (*Figure 6-6*).

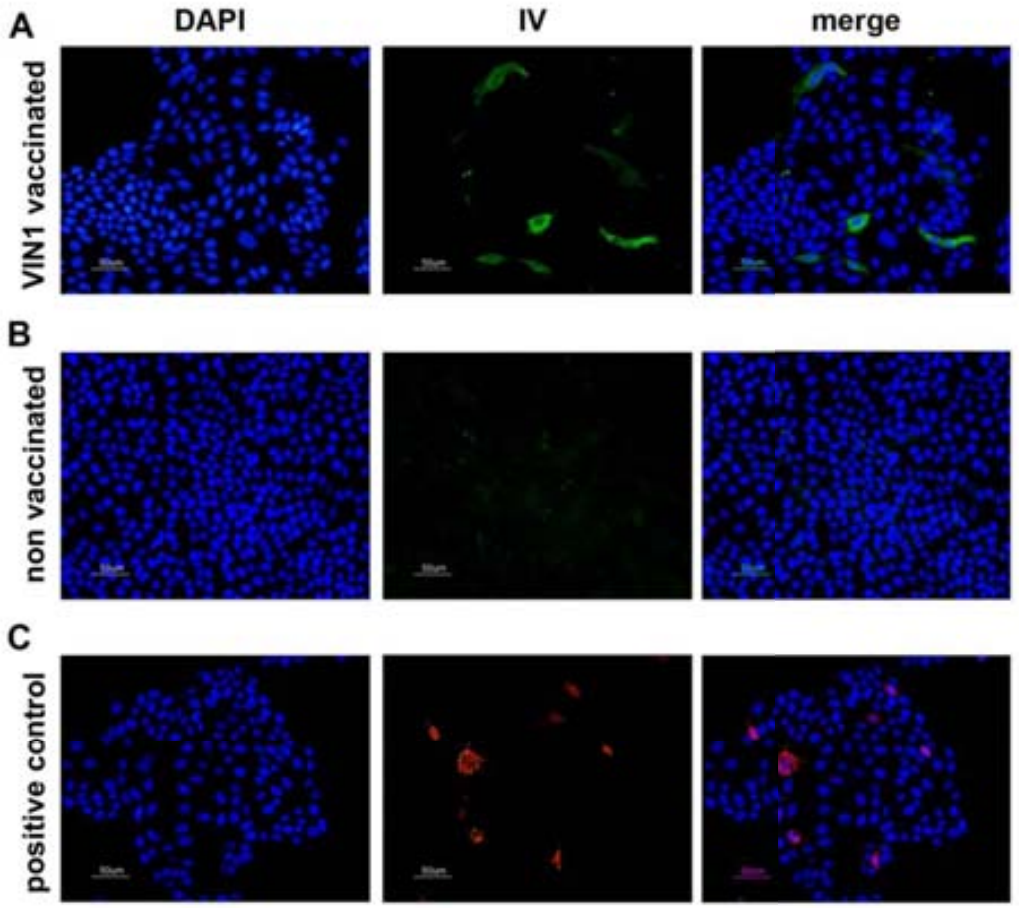

Figure 6-4. VIN1-sera recognize pH1N1 virus *in vitro.* Indirect immunofluorescence of pH1N1-infected MDCK cells at 16 hpi using as primary antibody: **a|** the serum from one pig (representative of the group), immunized three times with VIN1-peptides; **b|** the serum from one negative control pig (representative of the group), immunized three times with PBS; and **c|** A monoclonal antibody against the NS1 protein was used as control for the infection (right panel).

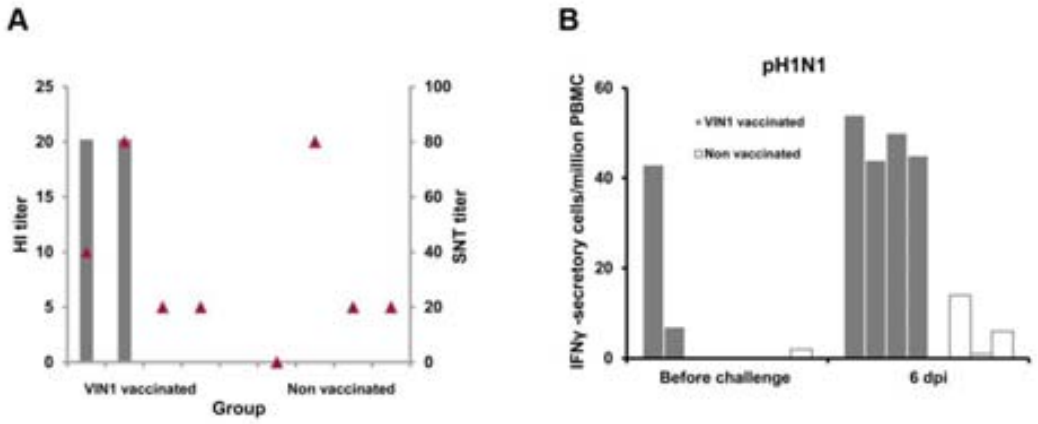

Figure 6-5. Immunization with the VIN1-HA1 peptide induces specific antibodies and T-cells against the heterologous pH1N1 virus. a| HI and SNT titers obtained with sera from pigs immunized either with the VIN1-peptides or with saline solution (control), at 6 dpi with the pH1N1 virus. Grey bars represent HI titers and red triangles show SNT. **b|** IFNγ-ELISPOT using pH1N1 virus as stimulus and PBMCs from pigs immunized either with the VIN1-peptides or with saline solution (control). The assay was done using PBMCs isolated either before the infection with the pandemic H1N1 virus or at 6 dpi.

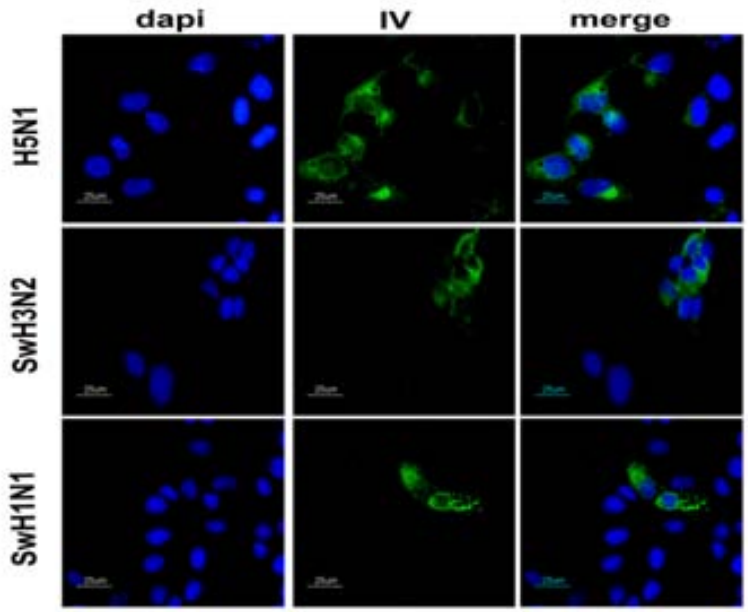

Figure 6-6. VIN1-sera recognize distinct viral subtypes. Indirect immunofluorescence of either H5N1, SwH3N2 or SwH1N1-infected MDCKs cells at 16 hpi using as primary antibody the serum from one pig (representative of the group), immunized three times with VIN1 peptides.

To further investigate the antibody response, an HI assay was performed against the same virus subtypes: H5N1 HPAIV, SwH1N1 IV and SwH3N2 IV. No inhibition activity was recorded against any of the mentioned virus for any sera.

6.5. Discussion

The search for universal vaccines against influenza viruses is a must. Most efforts have been focussed on driving the immune response against well conserved epitopes or proteins of IV, such as the influenza ion channel M2 protein, and conserved epitopes from the influenza NP and matrix 1 (M1) (Tompkins *et al.,* 2007; Kitikoon *et al.,* 2009; El Bakkouri e*t al.,* 2011). More recently, the potential use of highly conserved synthetic peptides from HA2 as an efficient vaccine in mice has also been demonstrated (Wang e*t al.,* 2010). In this report, we show evidence of the potential use of conserved HA1 peptides in future vaccine formulations using conventional pigs.

Peptides derived from the HA1-VIN1 domain were selected by ISM (Veljkovic e*t al.,* 2009a; Veljkovic e*t al.,* 2009b) and were used for the immunization carried out in the present study. As predicted, immunization of pigs with VIN1-peptides induced specific anti-VIN1 peptides antibodies that recognized the VIN1-peptides (*Figure 6-1a and b*), the H1 and H5 recombinant proteins (*Figure 6-1c*) and also the heterologous pH1N1 IV (*Figure 6-4*). Even though it was not predicted, VIN1-peptide immunization was also able to induce T-cell responses in every single conventional pig that, again, not only recognized the specific peptides but also the heterologous pH1N1 IV. Interestingly enough, not all peptides seemed to be equally recognized, with both the NF-34 and ES-34 from the H1 and H5 hemagglutinin, respectively, being optimally recognized. The fact that these two epitopes are located in equivalent regions within the primary structure of the HA1 subunit, validate even more the ISM predictions.

An ideal vaccine should elicit both humoral and cellular responses in the context of highly variable Major Histocompatibility Complex (MHC), which is what we found with our vaccine. The fact that swine and human MHC complexes are remarkably similar (Molder e*t al.,* 2009) opens avenues for the extrapolation of these and future results for human medicine. We observed an increase in virus clearance after the challenge with pH1N1 virus, which differs in 3 amino acids from NF-34 (the H1-peptide used in the VIN1-vaccine) (*Table 6-1*.), in 2 out of 4 of the immunized pigs. This also opens new expectations for the use of VIN1-modified peptides in future vaccine formulations. Apart from sequence diversity, there is a clear lack of correlation between the protection observed and the immune responses detected at the individual level. Intriguingly, as soon as at day six post infection, only 2 pigs (pigs 1 and 2 from the V1N1 peptide-immunized group) showed concomitant detection of neutralizing and HI activity that did not totally correlate with protection. While pig 2 showed a clear reduction in viral load, pig 1 showed virus titers indistinguishable from those found in the control group. Although disappointing, our results seem to point towards the very important role of T-cells in the protection afforded which could be an important tool for developing more efficient vaccines for the future. Thus, the partial protection observed might correspond with the induction of non-detectable specific cytotoxic T-cell activity (CTL), as has been reported before for influenza (Riberdy e*t al.,* 1999; Christensen *et al.,* 2000) or with any other kind of T-cell activity independent from the induction of IFN-γ that might be involved in cross-protection (Hillaire *et al.,* 2011). We are currently addressing these issues, including the identification of shorter specific CTL-peptides.

The length of the peptides used, as well as the fact that the T-cells specifically secreted IFN-γ in response to *in vitro* stimulation with both the NF-34 peptide and the pH1N1 IV, point towards the induction of specific CD4+-T cells in every single vaccinated farm pig and independent of its SLA II haplotype. This, together with the fact that the specific antibodies induced are also able to recognize the pH1N1 virus, seem to validate the use ISM to optimize the prediction of highly conserved epitopes

with better protective ability and to design future vaccine formulations, capable of inducing concomitantly, universal B and T-cell responses against H1N1 influenza viruses (Stanekova *et al.,* 2010).

Unexpectedly, the reduction in the viral loads shown by pigs 2 and 4 did not correlate with less severity in the lung lesions. All pigs from either control or immunized groups show indistinguishable minor hystopathological changes. Despite the fact that these results could reflect a limitation of our T cell-centric vaccines to reduce disease, pigs might not be ideal models to test so, mainly because of the mild disease found after pH1N1 infection. For that reason, our hypothesis are also being tested in mice and chickens, which are ideal models for the characterization of the protective capability of experimental vaccines against an infection with highly pathogenic H5N1 IV; most probably, the responsible of future pandemic episodes (Watanabe *et al.,* 2011).

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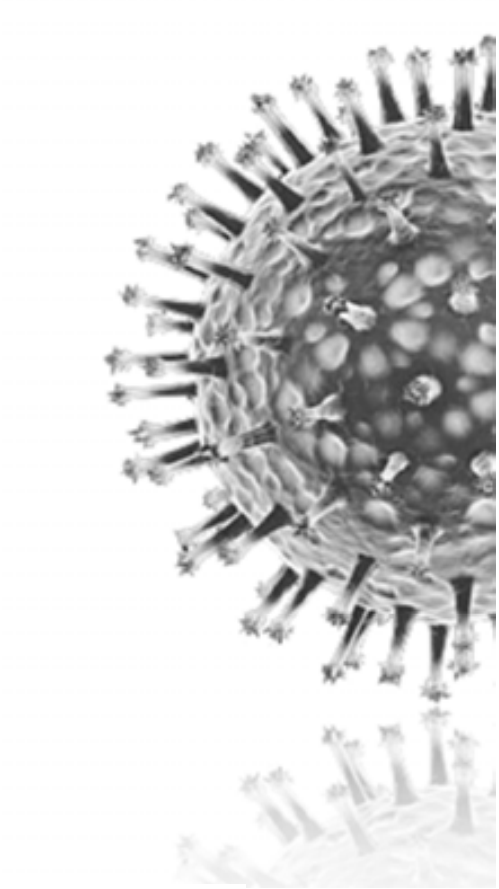
The manuscript was edited by both Dr. Maureen V. Foster and Dr. Kevin Dalton.

PART III:

Summarizing Discussion and Conclusions



"Science is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth"

[Jules Verne]

Chapter 7

Summarizing Discussion

Influenza A viruses have demonstrated to cross the species barrier increasing the host range and consequently, the probability of pandemic in humans (Claas *et al.,* 1998; Subbarao *et al.,* 1998; Peiris *et al.,* 1999; Lin *et al.,* 2000; Fouchier *et al.,* 2004). To adapt and to spread in humans, influenza viruses need to be efficient in: (i) animal-to-human transmission; (ii) virus-cell interaction barrier; and (iii) human-to-human transmission (Reperant *et al.,* 2012). Although many questions concerning IAV have been answered, there are still gaps in understanding the immunity in different hosts.

With the intention to further characterize some of the steps required by influenza virus to become pathogenic, and with the last objective to find an efficacy vaccine to combat multiple strains of IAV, the present thesis was formulated. Four studies were undertaken, each one with a specific objective, trying to better understand different aspects of the immune response elicited either to influenza infection or to vaccination. In this section, a general discussion of the main findings is reviewed; however, specific aspects of each study are discussed in the discussion section of the correspondent chapter.

Several studies have pointed towards the importance of different genes in the determination of influenza virulence and host range. However, most of the works are focused in mammalian species (mainly in mice and pigs) and little is known in birds. In the first study (chapter 3), using the HPIAV strain A/FPV/Rostock/34 (H7N1; FPV) and single NS-gene reassortant viruses created from it (FPV NS GD and FPV NS VN), the role of NS1 associated to virulence was tested in chickens, the natural host.

Our data evidence that, in chickens, FPV does not lose its virulence when NS-genes from both H5N1 are introduced in its genome, as reported in mammalian cell culture and in mice (Ma *et al.,* 2010; Wang *et al.,* 2010), where FPV is non pathogenic but with the introduction of NS1 from an H5N1, it gained pathogenicity. However, it was shown that a reassortant carrying the NS segment of A/Viet Nam/1203/2004 (H5N1; FPV NS VN) was more virulent than the original FPV, but also than FPV NS

GD. Thus, showing clinical signs earlier than the other groups and with a mortality rate of 100%. This observation is also in concordance with previous data which describe the enhanced virus replication in mammalian cells of those H5N1 viruses isolated after 1998 (Twu *et al.,* 2007), compared to the ones isolated previously.

Although little is known related to the link between the IAV virulence and the immune responses elicited in chickens, there are some reports which assess the roles of NS1 protein of AIV in their natural hosts (Kochs *et al.,* 2007; Long *et al.,* 2008; Zhu *et al.,* 2008; Zielecki *et al.,* 2010; Penski *et al.,* 2011). With the first study presented in this thesis, we were also intrigued to investigate some of these responses. Our experiments with FPV and NS-reassortants demonstrate an increase of mRNA IFN activity in infected chickens, mainly in those infected with NS-reassortant viruses. This is in concordance with previous reports which also demonstrate an increase in IFN production (Penski *et al.,* 2011). However, we could not detect an increase of production of IFN-β protein in the blood of infected animals at early time points.
Interestingly, we did detect an overexpression in both transcriptional and post-transcriptional levels of the proinflammatory protein IL-1β in all the infected-chickens. At 48 hpi the percentage of cells producing IL-1β suffer a dramatically increase in both NS-reassortant infected animals. By separating the blood cell populations by size and complexity we observed a reduction in lymphocytes cells but an increase of macrophage/monocyte-like cells. These lasts cells were suggested to be the ones secreting the mentioned proinflammatory cytokine.

Despite these clear observations concerning both, the pathological manifestation and the immunological outcome, it is important to notice that FPV is, by itself, a HPAIV. This is well known since the virus was first isolated, and we could confirm it in our study. Thus, our results support the notion that the NS1 protein has a significant effect on the viral pathogenicity, as demonstrated by the exchange of NS segment. The NS-reassortant viruses not only keep the virulence of FPV which, by itself, is a HPAIV in chickens, but also showed higher severity. We strongly believe

that these findings can help in finding better vaccine strategies trying to improve the immunogenic response of the existing ones (Ferko *et al.,* 2004).

One of the most important obstacles that a zoonotic IAV need to overcome is the cross-species transmissions, from animals to humans. Although wild birds play an important role in the persistence of IAV, human exposure to IAV of wild water-bird reservoirs is relatively rare. The human exposure to IAV of *bridge species* is more frequent. Thus, it is important to control both the poultry and other avian species and know the ecological implications of possible occurring infections.

Since it was demonstrated in several animal models (Kreijtz *et al.,* 2009; Bodewes *et al.,* 2010; Jourdain *et al.,* 2010; Bodewes *et al.,* 2011; Costa *et al.,* 2011) that experimental infection with IAV could provide certain protection against challenge with IAV of different subtype, an experimental infection with three AIV was carried out in chickens (chapter 4).

Results obtained in the second study evidence that a pre-existing immunity can have an important role in chickens to determine whether they will succumb to a lethal IAV infection or not. Although HA-heterosubtypic protection demonstrated in other studies (Jourdain *et al.,* 2010; Costa *et al.,* 2011) these were done in other species rather than chickens. In our work we observed a cross-protection among different viral subtypes, but sharing the same HA. Despite no protection against an H5-HPAIV was afforded even after subsequent infections with two H7-viruses, a slight delay in the clinical manifestations was detected.

The results obtained in this study, together with all the previous reports done in other species and already mentioned, highlight the wide variability of responses obtained among the different species. This is an important fact to take into account when designing vaccines. The protection afforded after either vaccination or infection can depend on several factors including: host, viral strain and time between infections or immunizations (Kida *et al.,* 1890).

In relation with the last conclusions resulting from the second study, results obtained in the third work (chapter 5) supports the evidence of the differences among species concerning immunological evaluations.

A comprehensive study to evaluate the immune responses in a wide number of wild avian species after vaccination was carried out in Spain. It is important to be prepared in case another outbreak similar (or worst) than the one cause by H5N1 HPAIV occur. This virus was shown to affect thousands of birds, including the natural reservoir with an obvious economic and ecological implication (Ellis *et al.,* 2004). Between 2006 and 2007 Spanish zoos and wildlife center carried out a vaccination program with two different H5-commercial inactivated vaccines. We assess the responses afforded by both vaccinations as well as the durability of HI antibodies and interesting results were obtained. The main conclusion we can obtain from this study is that to cover a huge number of species, the better option is to combine two vaccines. Each vaccine used per separate is not able to elicit antibodies in some of the studied species; however, when using both vaccines, the results concerning humoral responses improve a lot. Previous studies (Philippa *et al.,* 2005; Bertelsen *et al.,* 2007; Philippa *et al.,* 2007) reported interesting data obtained in zoos and wildlife centers from other European Countries, but this work show the importance of combining different vaccines to increase the efficacy of the vaccination programs. Therefore, our suggestion is that in case of an outbreak, the use of a combination of biosecurity measures as well as a good vaccination program which consider protecting a wide number of species is the best option to protect animals.

There are evidences indicating that the reactivity of antibodies and the cell-mediated immunity, particularly CD8+ cytotoxic lymphocytes (CTL), correlates with reduced virus shedding (McMichael *et al.,* 1983; Kreijtz *et al.,* 2007) and contribute to elicit cross-protective immunity to multiple viral subtypes (Rimmelzwaan *et al.,* 2007; Grebe *et al.,* 2008; Epstein and Price, 2010). Thus, they should be considered as vaccine

candidates. In the last study (chapter 6) it was shown that conserved peptides, from the HA1-VIN1 domain (or VIN1-vaccine), are able to elicit both humoral and cellular responses, contributing to *in vitro* recognition of different viral subtypes. However, these findings do no correlate with the protective immunity afforded. VIN1-vaccine only confers a partial protection in pigs after challenge with the pandemic H1N1 virus, as demonstrated with the increase in virus clearance.

The pH1N1 virus is not able to cause clinical signs in pigs, as observed in this study and in previous work (Busquets *et al.,* 2010). Although results concerning protection are not convincing and we could only evaluate the amount of viral RNA in BAL, we strongly believe that this vaccine formulation could be a good path which can lead us to find a better formulation. Our efforts will be now focused on changing the formulation trying to improve the protection after IAV-infection in other animal models.

Taken together, the data resulted from this thesis demonstrate the importance of studying the immunity to IAV infection, not only in mammalian species, but also in birds. Therefore, to know the role of the NS1 protein (a viral determinant) during IAV infection in chickens and to assess the effect of pre-existing immunity in subsequent infection can help in understanding the mechanisms by which the immunity can block IAV (or fail to do it). Moreover, to determine the vaccination efficacy of conventional vaccines used in a wide range of wild birds-species can lead to improve future vaccination programs. Finally, findings presented in this thesis also show promising results concerning the searching of a vaccine able to elicit cross-protective immunity.

The information given might contribute to the production of better vaccines against influenza virus, a potential tool to control and combat future pandemics.

Chapter 8

Conclusions

1. NS1 protein is demonstrated to be an important viral virulence factor in chickens, showing more severe clinical signs at earlier time points, higher mortality rates and an increase in viral shedding.

2. No evident up-regulation of IFN-β expression was observed in any group. However, a severe increase of IL-1β production was detected in both reassortant-infected groups, mainly in FPV NS GD-infected chickens at 48 hours after infection.

3. A pre-exposure to H7N2 LPAIV prevent chickens from lethal H7N1 HPAIV-infection and from viral shedding. This protection coincided with the presence of specific H7-hemagglutining inhibitory antibodies before challenge.

4. The immunity conferred by subsequent challenges do not protect against a final infection against H5N1 HPAIV. The lack of protection correlated with the absence of anti-H5 antibodies prior to challenge.

5. The inactivated water-in-oil adjuvanted H5N9 and H5N3 vaccines are demonstrated to be equally effective in eliciting high titers of HI H5-antibodies among most of the taxonomic orders. However, receiving a single vaccine subtype is not enough to elicit detectable antibody response in some of the taxonomic orders and/or species.

6. Successive vaccination programs with hetersubtypic vaccines are suggested to be the key to obtain a wide protection in wildlife birds, especially from those belonging to taxonomic orders and/or species which did not develop HI antibody to a unique vaccine. To maintain HI titers among different wild species a revaccination between 6 and 12 months after vaccination is required.

7. VIN1-vaccination, which consists of an HA1-based peptide vaccine, elicits specific humoral and cellular responses against both, VIN1-peptides and pH1N1 virus. These findings validate the use of ISM to predict highly conserved epitopes with optimal immunogenic ability.

8. Vaccination with VIN1-peptides confers a partial reduction of viral load in BAL. However, there is no correlation between the increase of viral clearance in BAL and the absence of lung lesions after VIN1-immunization.

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Apprendix

Figure S1. (Figure legend, next page)

CHAPTER 4: Figure S4-1. Histopathology of different tissues from chickens experimentally infected with LPAIV H7N2 and/or HPAIV H7N1 Chicken from group 1 (G1; pre-exposed to LPAIV H7N2 and subsequently challenged with HPAIV H7N1) at 10 days after challenge, and from group 2 (G2; inoculated with HPAIV H7N1) at 4 days post inoculation. (A.1) Brain; no microscopic lesions, hematoxylin-eosin staining (HE). (A.2) Brain; moderate diffuse vacuolation of neuropil and gliosis, HE. (B.1) Heart; no microscopic lesions, HE. (B.2) Heart; moderate diffuse degeneration of myocytes, HE. (C.1) Liver; no microscopic lesions, HE. (C.2) Liver; mild multifocal necrosis of hepatocytes, associated with infiltration of lymphocytes and plasma cells, HE. (D.1) Cloacal Bursa; no microscopic lesions, HE. (D.2) Cloacal Bursa; severe diffuse follicular degeneration.

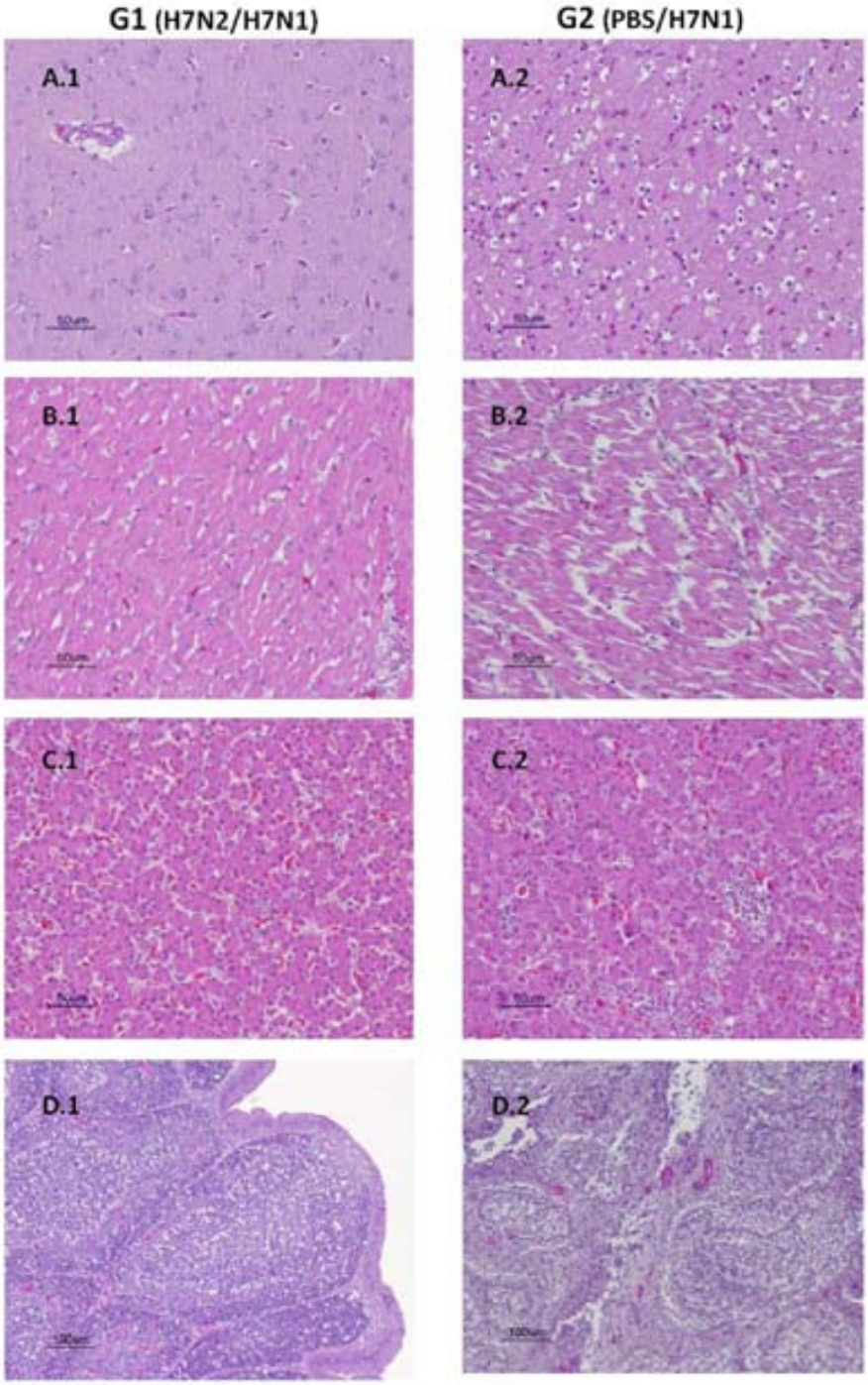
G1 (H7N2/H7N1)
G2 (PBS/H7N1)
A.1
A.2
B.1
B.2
C.1
C.2
D.1
D.2

TABLE 1. Humoral immune response of avian species in zoos, vaccinated twice (within a 3-week interval) with an inactivated H5N9 vaccine (VP1)[a]

Order	Species		No. of birds	GMT	% of birds with HI titers of ≥32
	Common name	Scientific name			
Anseriformes	Total		179	61	67.2
	Mandarin duck	*Aix galericulata*	1	4	0
	Egyptian goose	*Alopochen aegyptiacus*	6	13	33.3
	Northern pintail	*Anas acuta*	1	256	100
	Northern shoveler	*Anas clypeata*	1	256	100
	Baikal teal	*Anas formosa*	6	226	100
	Eurasian wigeon	*Anas penelope*	2	181	100
	Mallard	*Anas platyrhynchos*	8	19	37.5
	Chiloe wigeon	*Anas sibilatrix*	9	4	0
	Greylag goose	*Anser anser*	14	24	57.1
	Swan goose	*Anser cygnoides*	4	19	50
	Bar-headed goose	*Anser indicus*	17	234	94.1
	Magpie goose	*Anseranas semipalmata*	1	32	100
	Canada goose	*Branta canadensis*	1	2,048	100
	Barnacle goose	*Branta leucopsis*	2	256	100
	Red-breasted goose	*Branta ruficollis*	9	299	100
	Hawaiian goose	*Branta sandvicensis*	1	512	100
	Muscovy duck	*Cairina moschata*	16	18	50
	Ringed teal	*Callonetta leucophrys*	3	81	100
	Cape Barren goose	*Cereopsis novaehollandiae*	7	32	57.1
	Southern screamer	*Chauna torquata*	5	111	80
	Australian wood duck	*Chenonetta jubata*	3	8	33.4
	Andean goose	*Chloephaga picta*	3	323	100
	Black swan	*Cygnus atratus*	16	146	87.5
	Black-necked swan	*Cygnus melancoryphus*	1	256	100
	Mute swan	*Cygnus olor*	11	53	54.5
	Fulvous whistling-duck	*Dendrocygna bicolor*	1	1,024	100
	Marbled duck	*Marmaronetta angustirostris*	1	32	100
	Rosybill	*Netta peposaca*	9	299	88.9
	Red-crested pochard	*Netta rufina*	8	91	62.5
	Knob-billed duck	*Sarkidiornis melanotos*	3	51	66.7
	Ruddy shelduck	*Tadorna ferruginea*	6	7	16.7
	Raja shelduck	*Tadorna radjah*	1	1,024	100
	Common shelduck	*Tadorna tadorna*	2	362	100
Charadriiformes	Total		17	20	47.1
	Eurasian oystercatcher	*Haematopus ostralegus*	4	23	50
	Audouin's gull	*Larus audouinii*	1	4	0
	Caspian gull	*Larus cachinnans*	5	7	20
	Pied Avocet	*Recurvirostra avosetta*	5	42	60
	Masked lapwing	*Vanellus miles*	2	64	100
Ciconiiformes	Total		82	14	33.7
	Abdim's stork	*Ciconia abdimii*	1	256	100
	White stork	*Ciconia ciconia*	20	13	30
	Ibis stork	*Ciconia ibis*	3	51	100
	Scarlet ibis	*Eudocimus ruber*	18	5	5.6
	Northern bald ibis	*Geronticus eremita*	4	64	100
	Marabou stork	*Leptoptilos crumeniferus*	9	13	22.2
	Yellow-billed stork	*Mycteria ibis*	1	4	0
	Roseate spoonbill	*Platalea ajaja*	3	32	66.6
	African spoonbill	*Platalea alba*	3	128	66.7
	African sacred ibis	*Threskiornis aethiopicus*	19	15	37
	Straw-necked ibis	*Threskiornis spinicollis*	1	8	0
Columbiformes	Total		79	6	12.5
	Nicobar pigeon	*Caloenas nicobarica*	6	20	66.7
	Speckled pigeon	*Columba guinea*	7	4	0
	Rock pigeon	*Columba livia*	56	5	7.1
	Common wood pigeon	*Columba palumbus*	1	4	0
	Victoria crowned pigeon	*Goura victoria*	2	4	0
	Barbary dove	*Streptopelia risoria*	7	10	28.6
Coraciiformes	Total		27	5	7.4
	Knobbed hornbill	*Aceros cassidix*	2	4	0

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TABLE 1—*Continued*

Order	Species		No. of birds	GMT	% of birds with HI titers of ≥32
	Common name	Scientific name			
	Mindanao wrinkled hornbill	*Aceros leucocephalus*	2	4	0
	Black hornbill	*Anthracoceros malayanus*	2	4	0
	White-crowned hornbill	*Berenicornis comatus*	2	4	0
	Great hornbill	*Buceros bicornis*	1	4	0
	Rhinoceros hornbill	*Buceros rhinoceros*	1	4	0
	Abyssinian ground hornbill	*Bucorvus abyssinicus*	1	4	0
	Southern ground hornbill	*Bucorvus leadbeateri*	8	6	12.5
	Silvery-cheeked hornbill	*Bycanistes brevis*	1	4	0
	Trumpeter hornbill	*Bycanistes bucinator*	1	4	0
	Gray-cheeked hornbill	*Bycanistes subcylindricus*	2	4	0
	Laughing kookaburra	*Dacelo novaeguineae*	4	7	25
Falconiformes	Total		75	42	64
	Cinereous vulture	*Aegypius monachus*	3	8	33.3
	Steppe eagle	*Aquila nipalensis*	3	51	100
	Verreaux's eagle	*Aquila verreauxii*	1	128	100
	Red-tailed hawk	*Buteo jamaicensis*	1	16	0
	Variable hawk	*Buteo poecilochrous*	1	128	100
	Royal hawk	*Buteo regalis*	4	32	50
	Turkey vulture	*Cathartes aura*	4	11	25
	Short-toed eagle	*Circaetus gallicus*	3	20	66.7
	Black vulture	*Coragyps atratus*	1	4	0
	Lanner falcon	*Falco biarmicus*	1	512	100
	Lesser kestrel	*Falco naumanni*	3	203	100
	Black-chested buzzard eagle	*Geranoaetus melanoleucus*	2	4	0
	Palm-nut vulture	*Gypohierax angolensis*	1	8	0
	White-backed vulture	*Gyps africanus*	1	4	0
	Griffon vulture	*Gyps fulvus*	3	102	100
	Himalayan vulture	*Gyps himalayensis*	1	256	100
	White-tailed eagle	*Haliaeetus albicilla*	2	32	100
	Bald eagle	*Haliaeetus leucocephalus*	4	54	50
	African fish eagle	*Haliaeetus vocifer*	4	38	50
	Black kite	*Milvus migrans*	3	64	66.7
	Red kite	*Milvus milvus*	5	194	100
	Hooded vulture	*Necrosyrtes monachus*	6	81	83.3
	Egyptian vulture	*Neophron percnopterus*	2	362	100
	Osprey	*Pandion haliaetus*	3	40	66.7
	Harris's hawk	*Parabuteo unicinctus*	2	256	100
	Honey buzzard	*Pernis apivorus*	1	4	0
	Southern caracara	*Polyborus plancus*	4	54	75
	King vulture	*Sarcoramphus papa*	2	64	100
	White-headed vulture	*Trigonoceps occipitalis*	1	4	0
	Andean condor	*Vultur gryphus*	3	4	0
Galliformes	Total		69	30	59.4
	Vulturine guineafowl	*Acryllium vulturinum*	3	25	66.7
	Lady Amherst's pheasant	*Chrysolophus amherstiae*	3	8	33.3
	Golden pheasant	*Chrysolophus pictus*	1	4	0
	Great curassow	*Crax rubra*	1	32	100
	Red junglefowl	*Gallus gallus*	26	55	69.2
	Silver pheasant	*Lophura nycthemera*	2	4	0
	Indian peafowl	*Pavo cristatus*	31	29	61.3
	Common pheasant	*Phasianus colchicus*	2	4	0
Gruiformes	Total		31	10	25.8
	Blue crane	*Anthropoides paradisea*	3	4	0
	Demoiselle crane	*Anthropoides virgo*	10	8	20
	Black crowned crane	*Balearica pavonina*	1	4	0
	Gray crowned crane	*Balearica regulorum*	11	16	45.5
	Seriema	*Cariama cristata*	3	6	0
	Common crane	*Grus grus*	3	13	33.3
Passeriformes	Total		9	8	11.1
	Pied crow	*Corvus albus*	3	4	0
	Carrion crow	*Corvus corone*	1	4	0
	Corn bunting	*Emberiza calandra*	1	16	0

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TABLE 1—*Continued*

Order	Species		No. of birds	GMT	% of birds with HI titers of ≥32
	Common name	Scientific name			
	Rosy starling	*Pastor roseus*	1	128	100
	Red-billed chough	*Pyrrhocorax pyrrhocorax*	1	16	0
	Common blackbird	*Turdus merula*	2	4	0
Pelecaniformes	Total		31	15	38.7
	Great white pelican	*Pelecanus onocrotalus*	20	32	60
	Pink-backed pelican	*Pelecanus rufescens*	8	4	0
	Great cormorant	*Phalacrocorax carbo*	3	4	0
Phoenicopteriformes	Total		93	122	86
	Lesser flamingo	*Phoeniconaias minor*	19	143	89
	Chilean flamingo	*Phoenicopterus chilensis*	5	256	80
	American flamingo	*Phoenicopterus ruber*	69	111	85.5
Psoformes	Total		3	13	33.3
	Toco toucan	*Ramphastos toco*	1	4	0
	Keel-billed toucan	*Ramphastos sulfuratus*	1	128	100
	Black-mandibled toucan	*Ramphastos ambiguus*	1	4	0
Psittaciformes	Total		177	15	42.9
	Blue-fronted amazon	*Amazona aestiva*	3	8	33.3
	Orange-winged amazon	*Amazona amazonica*	2	16	50
	Yellow-shouldered amazon	*Amazona barbadensis*	9	30	56
	Festive amazon	*Amazona festiva*	5	21	40
	Yellow-crowned amazon	*Amazona ochrocephala*	3	32	66.7
	Red-spectacled amazon	*Amazona pretrei*	3	10	0
	Vinaceous amazon	*Amazona vinacea*	3	10	0
	Hyacinth macaw	*Anodorhynchus hyacinthinus*	1	128	100
	Great green macaw	*Ara ambigua*	3	51	100
	Blue-and-yellow macaw	*Ara ararauna*	27	16	66.7
	Red-and-green macaw	*Ara chloroptera*	17	9	23.5
	Scarlet macaw	*Ara macao*	15	37	86.7
	Military macaw	*Ara militaris*	13	19	38.5
	Red-fronted macaw	*Ara rubrogenys*	13	16	53.8
	Chestnut-fronted macaw	*Ara severa*	4	8	25
	Blue-crowned parakeet	*Aratinga acuticaudata*	1	4	0
	Finsch's parakeet	*Aratinga finschi*	1	4	0
	White cockatoo	*Cacatua alba*	8	9	25
	Sulfur-crested cockatoo	*Cacatua galerita*	5	11	40
	Goffins cockatoo	*Cacatua goffini*	1	8	0
	Salmon-crested cockatoo	*Cacatua moluccensis*	1	32	100
	Western corella	*Cacatua pastinator*	8	4	0
	Yellow-crested cockatoo	*Cacatua sulphurea*	1	64	100
	Eclectus parrot	*Eclectus roratus*	7	16	57.1
	Golden parakeet	*Guaruba guarouba*	6	11	16.7
	Scaly-headed parrot	*Pionus maximiliani*	1	4	0
	Pesquet's parrot	*Psittrichas fulgidus*	1	4	0
	African gray parrot	*Psittacus erithacus*	15	13	40
Sphenisciformes	Total		16	9	18.8
	Humboldt penguin	*Spheniscus humboldti*	5	21	60
	African penguin	*Spheniscus demersus*	11	6	0
Strigiformes	Total		12	7	16.7
	Little owl	*Athene noctua*	2	11	50
	Eurasian eagle owl	*Bubo bubo*	7	7	14.3
	Snowy owl	*Bubo scandiacus*	2	4	0
	Barn owl	*Tyto alba*	1	4	0
Struthioniformes	Total		88	11	30.8
	Emu	*Dromaius novaehollandiae*	9	7	22.2
	Greater rhea	*Rhea americana*	19	9	21.1
	Ostrich	*Struthio camelus*	5	37	80
All			933	103	48.2

[a] The geometric mean titers (GMT) and the percentages of birds with a postvaccination serum hemagglutination inhibition (HI) titer of ≥32 shown were measured 6 weeks after the second vaccination.

TABLE 2. Humoral immune *response of avian species in zoos vaccinated twice (within a 6-week interval) with an inactivated H5N3 vaccine (VP2)*[a]

Group	Order	Species		No. of birds	GMT	% of birds with HI titers of ≥32
		Common name	Scientific name			
Nonvaccinated in VP1	Anseriformes	Total		44	10	11
	Anseriformes	Egyptian goose	*Alopochen aegyptiaca*	4	4	0
	Anseriformes	Mallard	*Anas platyrhynchos*	12	10	9
	Anseriformes	Greylag goose	*Anser anser*	2	4	0
	Anseriformes	Bar headed goose	*Anser indicus*	2	32	100
	Anseriformes	Magpie goose	*Anseranas semipalmata*	1	4	0
	Anseriformes	Hawaiian goose	*Branta sandvicensis*	5	16	20
	Anseriformes	Cape Barren goose	*Cereopsis novaehollandiae*	1	4	0
	Anseriformes	Andean goose	*Chloephaga picta*	4	11	0
	Anseriformes	Black swan	*Cygnus atratus*	6	16	0
	Anseriformes	Mute swan	*Cygnus olor*	1	16	0
	Anseriformes	Fulvous whistling duck	*Dendrocygna bicolor*	1	64	100
	Anseriformes	Rosybill	*Netta peposaca*	5	7	20
	Columbiformes	Total		5	16	40
	Columbiformes	Common wood pigeon	*Columba palumbus*	1	4	0
	Columbiformes	Diamond dove	*Geopelia cuneata*	2	64	100
	Columbiformes	Barbary dove	*Streptopelia turtur*	2	8	0
	Coraciiformes	Total		2	4	0
	Coraciiformes	White-crowned hornbill	*Berenicornis comatus*	2	4	0
	Falconiformes	Total		4	49	75
	Falconiformes	Common buzzard	*Buteo buteo*	2	100	100
	Falconiformes	Griffon vulture	*Gyps fulvus*	1	4	0
	Falconiformes	Black kite	*Milvus migrans*	1	128	100
	Galliformes	Total		11	187	100
	Galliformes	Red jungle fowl	*Gallus gallus*	5	56	100
	Galliformes	Indian peafowl	*Pavo cristatus*	6	512	100
	Gruiformes	Total		1	4	0
	Gruiformes	Demoiselle crane	*Anthropoides virgo*	1	4	0
	Pelecaniformes	Total		4	152	75
	Pelecaniformes	Great white pelican	*Pelecanus onocrotalus*	3	512	100
	Pelecaniformes	Great cormorant	*Phalacrocorax carbo*	1	4	0
	Phoenicopteriformes	Total		4	8	0
	Phoenicopteriformes	American flamingo	*Phoenicopterus ruber*	4	8	0
	Strigiformes	Total		2	11	50
	Strigiformes	Barn owl	*Tyto alba*	1	4	0
	Strigiformes	Spectacled owl	*Pulsatrix perspicillata*	1	32	100
Vaccinated in VP1	Anseriformes	Total		91	20	42
	Anseriformes	White-cheeked pintail	*Anas bahamensis*	1	4	0
	Anseriformes	Chestnut teal	*Anas castanea*	2	4	0
	Anseriformes	Mallard	*Anas platyrhynchos*	11	4	0
	Anseriformes	Greylag goose	*Anser anser*	8	40	88.8
	Anseriformes	Emperor goose	*Anser canagicus*	5	4	0
	Anseriformes	Swan goose	*Anser cygnoides*	5	9	20
	Anseriformes	Barnacle goose	*Branta leucopsis*	1	4	0
	Anseriformes	Red-breasted goose	*Branta ruficollis*	3	16	33.3
	Anseriformes	Hawaiian goose	*Branta sandvicensis*	1	16	0
	Anseriformes	Cape Barren goose	*Cereopsis novaehollandiae*	1	11	0
	Anseriformes	Andean goose	*Chloephaga picta*	6	4	0
	Anseriformes	Ashy-headed goose	*Chloephaga poliocephala*	2	4	0
	Anseriformes	Ruddy-headed goose	*Chloephaga rubidiceps*	4	4	0
	Anseriformes	Black swan	*Cygnus atratus*	6	102	83.3
	Anseriformes	Black-necked swan	*Cygnus melancoryphus*	3	6	0
	Anseriformes	Rosybill	*Netta peposaca*	6	323	100
	Anseriformes	Red-crested pochard	*Netta rufina*	2	181	100
	Anseriformes	Ruddy shelduck	*Tadorna ferruginea*	14	61	78.6
	Anseriformes	Common shelduck	*Tadorna tadorna*	12	85	83.3
	Charadriiformes	Total		4	13	50.0
	Charadriiformes	Caspian gull	*Larus cachinnans*	4	13	50
	Ciconiiformes	Total		25	16	44
	Ciconiiformes	White stork	*Ciconia ciconia*	3	4	0
	Ciconiiformes	Glossy ibis	*Plegadis falcinellus*	17	31	64.7
	Ciconiiformes	African sacred ibis	*Threskiornis aethiopicus*	5	4	0
	Columbiformes	Total		9	4	0
	Columbiformes	Common wood pigeon	*Columba palumbus*	9	4	0
	Coraciiformes	Total		6	9	33.3
	Coraciiformes	Knobbed hornbill	*Aceros cassidix*	2	4	0
	Coraciiformes	Minanao wrinkled hornbill	*Aceros leucocephalus*	2	4	0
	Coraciiformes	Black hornbill	*Anthracoceros malayanus*	2	45	100
	Falconiformes	Total		7	9	28.6
	Falconiformes	Turkey vulture	*Cathartes aura*	2	64	100
	Falconiformes	Himalayan vulture	*Gyps himalayensis*	5	4	0
	Falconiformes	Bald eagle	*Haliaeetus leucocephalus*	1	4	0
	Falconiformes	Hooded vulture	*Necrosyrtes monachus*	1	4	0
	Falconiformes	Harris's hawk	*Parabuteo unicinctus*	2	4	0
	Galliformes	Total		22	437	95.5
	Galliformes	Indian peafowl	*Pavo cristatus*	22	437	95.5

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TABLE 2—*Continued*

Group	Order	Species		No. of birds	GMT	% of birds with HI titers of ≥32
		Common name	Scientific name			
	Gruiformes	Total		6	9	33.3
	Gruiformes	Blue crane	*Anthropoides paradisea*	3	4	4
	Gruiformes	Demoiselle crane	*Anthropoides virgo*	2	45	100
	Gruiformes	Gray crowned crane	*Balearica regulorum*	1	4	0
	Passeriformes	Total		1	4	0
	Passeriformes	European greenfinch	*Carduelis chloris*	1	4	0
	Pelecaniformes	Total		8	4	0
	Pelecaniformes	Pink-backed pelican	*Pelecanus rufescens*	8	4	0
	Phoenicopteriformes	Total		91	18	29.7
	Phoenicopteriformes	Lesser flamingo	*Phoeniconaias minor*	31	4	0
	Phoenicopteriformes	Chilean flamingo	*Phoenicopterus chilensis*	9	276	100
	Phoenicopteriformes	American flamingo	*Phoenicopterus ruber*	51	27	35.3
	Psittaciformes	Total		7	58	100
	Psittaciformes	Red-and-green macaw	*Ara chloroptera*	1	32	100
	Psittaciformes	Military macaw	*Ara militaris*	3	32	100
	Psittaciformes	Eclectus parrot	*Eclectus roratus*	3	128	100
	Sphenisciformes	Total		16	10	0
	Sphenisciformes	African penguin	*Spheniscus demersus*	4	4	0
	Sphenisciformes	Humboldt penguin	*Spheniscus humboldti*	12	14	0
	Strigiformes	Total		3	4	0
	Strigiformes	Eurasian eagle owl	*Bubo bubo*	2	4	0
	Strigiformes	Snowy owl	*Nyctea scandiaca*	1	4	0
	Struthioniformes	Total		3	128	33.3
	Struthioniformes	Emu	*Dromaius novaehollandiae*	1	16	0
	Struthioniformes	Greater rhea	*Rhea americana*	2	362	100
Total nonvaccinated in VPI				77	19	23.4
Total vaccinated in VPI				299	16	38.5
All				376	18	33.2

* The geometric mean titers (GMT) and the percentages of birds with a postvaccination serum hemagglutination inhibition (HI) titer of ≥32 shown were measured 6 weeks after the second vaccination. Animals are grouped into two groups: the nonvaccinated in VPI and the ones that were vaccinated in VPI.