Universitat Autònoma de Barcelona

Conserved Hemagglutinin Peptides of Influenza Virus as Potential Multivalent Vaccine Candidate: Characterization of Immune Response in different Animal Models

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Tesis doctoral presentada por **Juliana González Zabala** para acceder al grado de Doctora dentro del programa de Doctorado en Medicina y Sanidad Animal de la Facultat de Veterinaria de la Universitat Autònoma de Barcelona, bajo la dirección del Dr. **Ayub Ismail Darji.**

Bellaterra, 2015

El Dr. **Ayub Ismail Darji**, investigador del Centre de Recerca en Sanitat Animal y del Instituto de Investigación Agroalimentaria (CReSA-IRTA) y el Dr. **Mariano Domingo Alvarez**, profesor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona e investigador adscrita al CReSA-IRTA

Informan:

Que la memoria titulada, **"Conserved Hemagglutinin Peptides of Influenza Virus as Potential Multivalent Vaccine Candidate: Characterization of Immune Response in different Animal Models"** presentada por Juliana González Zabala para la obtención del grado de Doctor en Veterinaria, se ha realizado bajo su supervisión y tutoría en la *Universitat Autònoma de Barcelona* y el *Centre de Recerca en Sanitat Animal*.

Para que conste a los efectos oportunos, firma el presente certificado en Bellaterra (Barcelona), a 1 de Diciembre de 2015.

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Director	**Tutor**	**Doctoranda**

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A mis Padres &

El Chino

Solo nosotros sabemos estar distantemente juntos

ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
CRNA	anti-genomic copies of the genome
AIV	avian influenza viruses
BSA	bovine serum albumin
BAL	bronchoalveolar lavages
RBC	chicken red blood cells
CS	cloacal swabs
C-Elisa	competitive enzyme-linked immunosorbent assay
CFA	complete freund adjuvant
CIS	consensus informational spectrum
CLRS	c-type lectin receptors
CTL	cytotoxic t cell
Dpi	days post-infection
Dc	dendritic cells
DSRNA	double-stranded rna
DMEM	dulbecco's modified eagle's medium
EID	egg infective dose
ELD	egg lethal dose
EIIP	electron-ion interaction potential
ER	endoplasmic reticulum
FP	feather pulp
F	frequency
Gal2	galactose
HI	hemagglutination inhibition test
HA	hemagglutinin
HA1	hemagglutinin subunit 1
HA2	hemagglutinin subunit 2
H/E	hematoxylin/eosin
Hpaiv	highly pathogenic avian influenza viruses
IFA	incomplete freund adjuvant
IF	indirect immunofluorescence
IS	informational spectrum
ISM	informational spectrum method
KLH	keyhole limpet hemocyanin
LPAIV	low pathogenic avian influenza virus
LRT	lower respiratory tract
M2e	M2 extracellular
MDCK	madin-darby canine kidney
MHC	major histocompatibility complex
M2	matrix protein 2
M1	matrixprotein 1
MDT	mean death time

MPLA	monophosphoryl lipid a
MDP	muramyl dipeptide
Glcnac3	n-acetylglucosamine
Nk	natural killer cells
NA	neuraminidase
Nabs	neutralizing antibodies
NDV	newcastle disease virus
NOS2	nitric oxide synthase 2
NLRP3	nod-like receptor family pyrin domain containing 3
NLRS	nod-like receptors
NS1	nonstructuralprotein1
NS2	nonstructuralprotein2
NEP	nuclear export protein
NP	nucleoprotein
OD	optical density
OS	Oropharyngeal swabs
PAMP	pathogen-associated molecular pattern
PAIV	pathogenic avian influenza
PAMPS	pathogens associated molecular patterns
PRR	pattern recognition receptor
PRRS	pattern-recognition receptors
PBS-TW	pbs-tween20
PBS	phosphate buffer saline
PA	polymerase acid protein
PB1	polymerase b1 protein
PB2	polymerase b2 protein
IFNS	proinflammatory cytokines and type i interferons
QIV	quadrivalent influenza vaccine
RBD	receptor-binding domain
RTD	recognition and targeting domain
RBC	red blood cells
RIG-I	retinoic acid inducible gene-i
RNP	Ribonucleoprotein complex
RNPS	ribonucleoproteins
RLRS	rig-i-like receptors
RT	room temperature
SN	seroneutralization assay
SA	sialic acid
S/N	signal-to-noise ratio
SPF	specific pathogen free
SIV	swine influenza viruses
TH	T- helper
TMB	tetramethylbenzidine

IAVS	**influenza a viruses**
PB1	**polymerase basic protein 1**
TCID50	**tissue culture infectious dose 50%**
TLRS	**toll like receptors**
TIV	**trivalent influenza vaccine**
TNF-A	**tumor necrosis factor alpha**
URT	**upper respiratory tract**
VAERD	**vaccine-associated enhanced respiratory disease**
VN	**viral neutralization**
VRNPS	**viral ribonucleoproteins**
VLPS	**virus-like particles**
WHO	**world health organization**

RESUMEN / SUMMARY

Influenza A viruses (IAVs) are responsible for pandemic outbreaks of influenza, and for most of the well-known annual flu epidemics, in humans, poultry and pigs. IAVs are divided into subtypes, based on the nature of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The HA is a homotrimeric surface glycoprotein that mediates influenza viral entry via cellular attachment and membrane fusion events. The receptor-binding pocket of HA is surrounded by antigenically variable antibody binding sites. Therefore, antibodies bounded to these sites should, in principle, block the binding to receptor proteins, inhibiting viral entry, demonstrating hemagglutinin inhibition activity and viral neutralization activity. However, the subunit 1 of HA (HA1) is highly variable across viruses and tends to change under immune pressure and, hence, easily evades the neutralizing antibodies induced by previous vaccinations or infections. The negative implications of influenza virus infections push the world to promote the development of multivalent flu vaccines that protect against all human influenza strains. Therefore, the field of bioinformatics has become a major part of the identification and early validation of new therapeutic targets and could be an essential first step in the development of an effective vaccine for influenza virus that represents the high variability of its antigenic determinants.

Therefore, in this thesis it was postulated that the HA1 could represent a potential target for a multivalent vaccine of influenza infection. Consequently, the general objective of this thesis was to select conserved peptides from the HA1 of influenza viruses and to evaluate the efficacy of the selected candidates to induce immunity that can protect animal against infection. To achieve this objective, three studies were undertaken in mice (Chapter 1), pigs (chapter 2) and chickens (Chapter 3).

In the **first study**, we evaluated the protective effect of improved HA1-peptides against the pandemic H1N1 2009 virus and a H7N1 highly pathogenic influenza virus (HPAIV) in a mouse model (**Chapter 1**). In this

study, mice were intraperitoneally vaccinated with the peptide mix (NG34+DC89), and next challenged with either the pH1N1 or the H7N1 strain of Influenza virus. Conversely to the 85% mortality observed in control mice, independently of the virus used for challenge, 80% and 66% of the peptide-vaccinated mice survived the challenge with pH1N1 and H7N1, respectively, without detection of influenza viruses (IV). Vaccinated mice surviving correlated with the presence of cross-reactive neutralizing antibodies in sera prior to challenge. The immunization with NG34+DC89 also induced mucosal immune responses demonstrated with the presence of IgA in bronchoalveolar lavage in 50% of the animals. Our results also show that NG34+DC89 is capable to induce cross-neutralizing antibodies and protection against two heterologous IV, pH1N1 and H7N1. Thus, NG34+DC89 represent an attractive immunogen, which could be further optimized for future multivalent vaccine formulations against influenza virus.

In the **second study**, we tested the immunogenicity of a HA1-peptide cocktail in a pig model to assess whether this new formulation can confer immunity to a wide range of IAVs *in vitro* (**Chapter 2**). Four peptides (NG34, DC55, RA22 and SS35) within the HA1 from H1 viruses were selected, and evaluated their immunogenicity in conventional farm pigs against homologous and heterologous viruses of influenza. Peptides immunizations induced HA neutralizing and inhibiting antibodies against homologous viruses. Those also cross-reacted against heterologous viruses like H7N1 and H5N2 and, most importantly, the circulating H3N2. Moreover, secretory IgA-specific HA antibodies in nasal swabs were detected. Altogether, the results show that the peptides tested were immunogenic in pigs. The humoral response with hemagglutinin-inhibiting and cross-neutralizing activity generated after vaccination could be used in further studies of protective heterosubtypic immunity.

In the **third study** we evaluated the protective effect of improved HA1-peptides against H7N1 highly pathogenic influenza virus (HPAIV) in chickens, a natural host model (**Chapter 3**). In this study, based on ISM, we selected two highly conserved peptides (NG34 and SS35) of a H1

influenza virus strain and used them to vaccinate free-range chickens. The vaccination with both NG34 and SS35 peptides induced specific antibodiesthat recognized heterologous viruses, as H7N1 HPAIV and H5N2 Low pathogenic avian virus (LPAIV) *in vitro*. Vaccination with NG34 peptide elicited a protective antibody response that conferred partial protection against a lethal challenge with H7N1 HPAIV. Furthermore, NG34 peptide induced a mucosal immune response, which correlated with reduced viral shedding in oropharyngeal/cloacal swabs and feather pulp. On the contrary, SS35 peptide vaccinated animals failed to produce an efficient protective immune response as no survival against lethal H7N1 challenge was achieved.

Finally, it remains to point out that all HA1-peptides from H1 subtype of influenza virus were selected by the method of informative spectra (ISM).

Four main general conclusions can be drawn from these studies:
(**i**) HA1-peptides are immunogenic in all the animals models tested (mice, pigs and chickens) and induce humoral and mucosal immune response. (**ii**) Novel conserved immunogenic peptides from the hemagglutinin subunit 1 protein of influenza viruses confer partial protection against different viral subtypes in mice; (**iii**) Pigs vaccinated with HA1 peptides elicit neutralizing and hemagglutination-inhibiting antibody responses against different subtypes of Influenza A virus and (**iv**) Synthetic peptides from the hemagglutinin of influenza viruses confer partial protection against highly pathogenic A/H7N1 virus in a free-range chicken model. Overall, these data provide insights on new approaches for vaccination in influenza and understanding of the immune response against influenza viruses in mice, pigs and chickens.

Los virus de influenza A (IAVs) son los responsables de brotes pandémicos y de la mayoría de las epidemias anuales en los seres humanos, aves y cerdos. Los virus de influenza A se dividen en subtipos, basado en la naturaleza de sus glicoproteínas de superficie, hemaglutinina (HA) y neuraminidasa (NA). La HA es una glicoproteína de superficie homotrimérica que media la entrada del virus de influenza a través de la unión celular y

eventos de fusión de membranas. El bolsillo de unión al receptor de HA está rodeado por los sitios de unión de anticuerpos antigénicamente variables. Por lo tanto, los anticuerpos limitados a estos sitios debería, en principio, bloquear la unión a las proteínas del receptor, inhibiendo la entrada del virus, lo que demuestra actividad de inhibición de la hemaglutinina y la actividad de neutralización viral. Sin embargo, la subunidad 1 de HA (HA1) es altamente variable a través de los virus de influenza y tiende a cambiar bajo presión inmune; por lo tanto, fácilmente evade los anticuerpos neutralizantes inducidos por la vacunación o infecciones anteriores. Las consecuencias negativas de las infecciones por virus de influenza incentivan al mundo de la ciencia para promover el desarrollo de vacunas multivalentes contra la gripe, que tengan eficaz protección frente a todas las cepas de influenza. Por consiguiente, el campo de la bioinformática se ha convertido en una parte importante de la identificación y validación temprana de nuevas dianas terapéuticas que podrían ser un primer paso esencial en el desarrollo de una vacuna eficaz para el virus de influenza, que representa la alta variabilidad de sus determinantes antigénicos.

En esta tesis se postuló que la HA1 podría representar una diana potencial para una vacuna multivalente frente a la infección por influenza virus. Por ende; el objetivo general de esta tesis fue seleccionar péptidos conservados de la subunidad 1 de la proteína hemaglutinina del virus de la gripe, para evaluar la eficacia de los candidatos seleccionados, para inducir la inmunidad que pueda proteger a los animales contra la infección. Para desarrollar este objetivo, se llevaron a cabo tres estudios experimentales: en ratones (Capítulo 1), cerdos (Capítulo 2) y pollos (Capítulo 3).

En el primer estudio, se evaluó el efecto protector de la mejora de péptidos HA1 contra la pandemia del virus H1N1 2009 (pH1N1) y el virus de influenza de alta patogenicidad H7N1 en un modelo de ratón (Capítulo 1). En este estudio, los ratones fueron vacunados por vía intraperitoneal con la mezcla de péptido sintético (NG34 + DC89) y desafiados ya sea con el pH1N1 ó H7N1 del virus de influenza. Una mortalidad del 85% fue observada en los ratones de control, independientemente del virus utilizado para el desafío, 80% y 66% de los ratones vacunados con péptido

sobrevivieron al desafío con pH1N1 y H7N1, respectivamente, sin la detección de virus de la influenza (IV). Los ratones vacunados supervivientes se correlacionaron con la presencia de anticuerpos neutralizantes de reactividad cruzada en sueros antes de la exposición. La inmunización con NG34+DC89 también indujo respuesta inmune en mucosas; demostrándose con la presencia de IgA en lavado broncoalveolar en el 50% de los animales. Nuestros resultados también muestran que la vacunación de NG34+DC89 es capaz de inducir anticuerpos y la protección cruzada neutralizante frente a dos cepas heterólogas, pH1N1 y H7N1. Por lo tanto, péptidos NG34+DC89 representan un inmunógeno atractivo, lo que podría ser aún más optimizado para futuras formulaciones de vacunas multivalentes frente el virus de influenza.

En el segundo estudio, hemos probado la inmunogenicidad de un cóctel HA1-péptido en un modelo porcino para evaluar si esta nueva formulación puede conferir inmunidad a una amplia gama de virus de influenza *in vitro* (Capítulo 2). En este estudio, hemos utilizado cuatro péptidos de la HA1 del subtipo H1 del virus de influenza (NG34, DC55, RA22 y SS35), evaluamos su inmunogenicidad en cerdos convencionales de granja frente a virus de influenza homólogos y heterólogos. Las vacunas de péptidos sintéticos indujeron anticuerpos con capacidad neutralizante y de inhibición frente a virus homólogos. Aquellos también cros-reaccionaron frente a los virus heterólogos de origen aviar como el virus H7N1 y virus de influenza de baja patogenicidad H5N2 y además el virus circulante H3N2 de origen porcino. Por otra parte, se detectaron anticuerpos específicos de IgA-secretora en hisopos nasales. Los resultados muestran que los péptidos evaluados fueron inmunogénicos en cerdos. La respuesta humoral con la actividad neutralizante y de inhibición de hemaglutinina generada después de la vacunación podría ser utilizada en otros estudios de inmunidad protectora heterosubtípica.

En el tercer estudio se evaluó el efecto protector de la mejora de péptidos de HA1 contra el virus altamente patógeno virus de la influenza H7N1 en pollos, un modelo huésped natural (capítulo 3). En este estudio, se utilizaron dos péptidos sintéticos NG34 y SS35 para vacunar pollos de

granja. La vacunación tanto con péptidos NG34 ó SS35 indujeron anticuerpos específicos que reconocen virus heterólogos, como H7N1 y H5N2 *in vitro*. La vacunación con péptido NG34 provocó una respuesta de anticuerpos protectores que confirió una protección parcial frente un desafío letal con H7N1. Además, el péptido NG34 indujo una respuesta inmune de la mucosa, que correlacionaba con la diseminación viral reducida en hisopos orofaríngeos/ cloacales y la pulpa de la pluma. Por el contrario, animales vacunados con el péptido SS35 no pudieron producir una eficiente respuesta inmune protectora frente el desafío letal H7N1.

Es necesario recalcar, que todos los péptidos HA1 del subtipo H1 del virus de influenza fueron seleccionados mediante el Método de espectros informativo (ISM).

Cuatro principales conclusiones generales pueden extraerse de estos estudios: (**i**) Péptidos HA1 son inmunogénicos en todos los modelos animales estudiados (ratones, cerdos y pollos) e inducen una respuesta immune humoral y de mucosa. (**ii**) Péptidos inmunogénicos de la subunidad 1 de la proteína hemaglutinina del virus de la influenza confieren una protección parcial contra diferentes subtipos virales en ratones; (**iii**) Los cerdos vacunados con péptidos HA1 provocan una respuesta de anticuerpos neutralizantes con actividad de inhibición de la hemaglutinina frente a diferentes subtipos de Influenza A virus y (**iv**) Péptidos HA1 confieren una protección parcial contra un virus altamente patógeno/ H7N1 en modelo de pollo.
En general, nuestros resultados proporcionan ideas sobre nuevos enfoques para la vacunación de la gripe y la comprensión de la respuesta inmune frente al virus de influenza en ratones, cerdos y pollos.

PART I
GENERAL INTRODUCTION AND OBJECTIVES

GENERAL INTRODUCTION

1.1. INFLUENZA A VIRUS

1.1.1. Classification and nomenclature

Influenza viruses belong to the family Orthomyxoviridae (from the Greek orthos meaning "straight" and myxa meaning ¨mucus¨)that comprise five genera: Influenza virus A,B, C, Thogotovirus and Isavirus (P. F. Wright, 2007; Palese P, 2007).The A, B and C types of virus also differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology (Fields et al., 2007). The influenza A viruses (IAVs) are responsible for pandemic outbreaks of influenza, and for most of the well-known annual flu epidemics. Few infectious diseases cause such a huge annual toll of morbidity, mortality, and economic loss as influenza.

IAVs are divided into subtypes, based on the nature of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To date, there are 18 different HA subtypes (H1 to H18) and 11 different NA (N1 to N11) subtypes for IAV (Tong et al., 2013b; Wu et al., 2014). All influenza A virus subtypes are circulating in wildbirds, which are therefore considered the natural influenza A virus reservoir, except H17, H18, N10 and N11. Viruses of subtypes H17N10 and H18N11 were recently identified in bat samples, raising the possibility that birds are not the exclusive influenza A virus reservoir (Munster et al., 2006; Tong et al., 2012; Tong et al., 2013)However; some isolates of influenza A virus cause severe disease both in domestic poultry and, rarely, in humans (WHO, 2014a). Occasionally, IAVs are transmitted from wild aquatic birds to domestic poultry, and this may cause an outbreak or give rise to human influenza pandemics as demonstrated in the three major pandemics of the last century (H1N1, H2N2 and H3N2 subtypes)(Kawaoka, 2006; Mettenleiter and Sobrino, 2008; Taubenberger and Morens, 2010). During recent outbreaks of highly pathogenic avian influenza (HPAI), there have been occasional transmissions of H5N1, H7N7 and H9N2 viruses to humans(Malik Peiris,

2009). IAVs are known to infect a remarkably diverse number of hosts. These hosts include dogs, horses, cats, non-human primates, cattle, seals, whales, pigs, guinea pigs, ferrets, mink, camels, and penguins, among others (Hinshaw et al., 1979; Manz et al., 2013; Webster et al., 1992).

The present nomenclature system to describe the type of influenza virus includes: the antigenic virus type or genera, the host of origin (except for humans), geographic site where it was first isolated, strain number, year of isolation, and virus subtype. The HA and NA subtype of influenza viruses is indicated in parenthesis, e.g. A/swine/Spain/SF32071/2007 (H3N2).

1.1.2. Genome description

The influenza virion is pleiomorphic, forming spherical virions with a diameter ranging from 80 to 120nm (Fujiyoshi et al., 1994). These particles consist of a host-derived lipid bilayer envelope in which the virus-encoded glycoproteins HA and NA, and M2 are embedded; an inner shell of matrix protein; and at the center, the nucleocapsids of the viral genome. The genome structure of IAV is represented in Figure 1.1A.

The genome of IAVs consists of eight unique segments of single-stranded RNA, which are of negative polarity (i.e., complementary to the mRNA sense). The RNA is loosely encapsidated by multiple NP molecules. To be infectious, a single virus particle must contain each of the eight unique RNA segments. Available evidence suggests that incorporation of RNAs into virions is at least partly random. The random incorporation of RNA segments allows the generation of progeny viruses containing novel combinations of genes (i.e., genetic reassortments) when cells are doubly infected with two different parent viruses (Webster et al., 1992).

Initially, each of the eight influenza A viral RNA segments was shown to encode a single virus polypeptide: segment 1 (PB2), segment 2 (PB1), segment 3 (PA), segment 4 (HA), segment 5 (NP), segment 6 (NA), segment 7 (M1 and M2) and segment 8 (NS1 and NS2/NEP) (Figure 1.1A)

(Palese, 1977)(Lamb and Lai, 1980; Lamb et al., 1981). At least two of these genome segments also encode alternate translation products including PB1-F2, PB1-N40, PA-X, PA-N155 and PA-N182 (Chen et al., 2001; Jagger et al., 2012; Muramoto et al., 2013; Wise et al., 2009) (Figure 1.1B). These proteins play significant roles in influenza virus replication, including their assistance in cell membrane recognition and endosomal fusion and acidification, inducing ribonucleoprotein (RNP) delivery into the nucleus, catalyzing polymerase holoenzyme of transcription and replication, and promoting protein and RNA binding and sialidase activity (Zhang et al., 2014). Specific functions of these proteins and their implications in viral pathogenicity are summarized below.

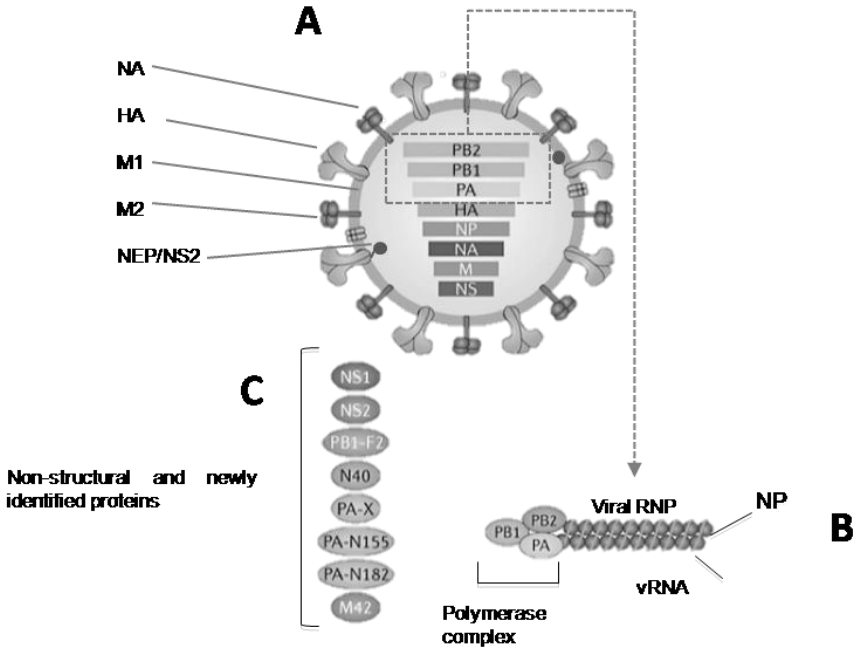

Figure 1.1. Representation of Influenza A Virus. A) Structure of IAV particle: HA, NA and M2 are inserted into the host-lipid membrane, the M1 underlies this lipid envelope and NEP/S2 is also associated with the virus. B) The two newly identified proteins N40 (Wise *et al.*, 2009) and PA-X (Jagger *et al.*, 2012), which represses cellular gene expression, are encoded by the PB1 and PA segments, respectively. Another two forms of PA, named PA-N155 and PA-N182, which have important functions in the replication cycle of influenza A viruses, were recently identified. C) The NP and the components of the RNA-dependent RNA polymerase complex (PB1, PB2 and PA) are expressed from their respective genome segments Modified from (Shi *et al.*, 2014).

The polymerase complex formed by polymerase acid protein (PA) and the polymerase basic protein 1 (PB1) and 2 (PB2)(Area et al., 2004; Detjen et al., 1987; Torreira et al., 2007)are situated at the ends of the nucleocapsids (Figure 1.1C). The PB1 subunit has been shown to have

polymerase activity, and is the responsible for transcribing the anti-genomic copies of the genome (cRNA) and then, transcribing these back into multiple copies of the genome (Biswas and Nayak, 1994). PB1-F2 is a virulence factor, causing apoptosis of infected host cells, and is not found in all strains of influenza viruses. It is encoded by a second ORF in the PB1 segment. (Chen et al., 2001; Zell et al., 2007). The PB2 subunit is a cap-binding protein that plays a role in initiation of viral mRNA synthesis by recruiting capped RNA primers (Blaas et al., 1982). The PB2 subunit has also been shown to contain nuclear localization signals that play a role in restricting the host range of influenza virus (Manzoor et al., 2009; Mukaigawa and Nayak, 1991; Subbarao et al., 1993). The PA plays a role in both transcription and replication (Fodor et al., 2002; Perales et al., 2000).

The nucleoprotein (NP) is a core antigen that plays an essential role in viral replication and transcription. Together with the polymerase proteins, it forms the ribonucleoprotein complex (RNP) and one RNP is associated with each segment of the viral genome (Compans et al., 1972; Rees and Dimmock, 1981).

M1 protein is important in RNP coating during viral assembly and it is the most abundant viral protein (Nayak et al., 2004; Smirnov Yu et al., 1991).

M2 protein is a trans-membrane protein that forms an ion channel tetramer, exhibiting pH inducible proton transport activity. During initial virus infection, M2 regulates the pH of viral core after virus uptake into the host cells endosomal compartment, and at the late stage of infection, it transports viral transmembrane proteins to the cell surface(Gerhard et al., 2006).

The NS1 protein has multiple functions in infected cells. It has been demonstrated to control both viral RNA replication and viral protein synthesis (Falcon et al., 2004; Hatada et al., 1990). It is also a major virulence factor of the virus, as it is known to inhibit host interferon responses (García-Sastre et al., 1998).

NS2 protein may help catalyze the nuclear export of newly synthesized viral RNPs from nucleus to cytoplasm, where assembly of the progeny virions occurs(O'Neill et al., 1998).

NA protein is the second most abundant glycoprotein, behind HA (Laver and Valentine, 1969). NA forms knob-like structures on the viral surface to catalyze progeny virus from infected cells, thereby allowing the virus to spread with resulting infection of new host cells (World Health Organization Global Influenza Program Surveillance, 2005). Inhibition of this protein leads to virus clumping on the surface of host cells and is the target of the NA inhibitor drugs (Palese et al., 1974).

HA protein is a homotrimeric surface glycoprotein that mediates influenza viral entry via cellular attachment and membrane fusion events (Bullough et al., 1994; Skehel et al., 1982). Up-to-date, 18 HA subtypes of the influenza A viruses (H1-H18) have been described (Tong et al., 2013), which are classified into two phylogenetic groups according to their amino acid sequence homologies. (Figure 1.2)

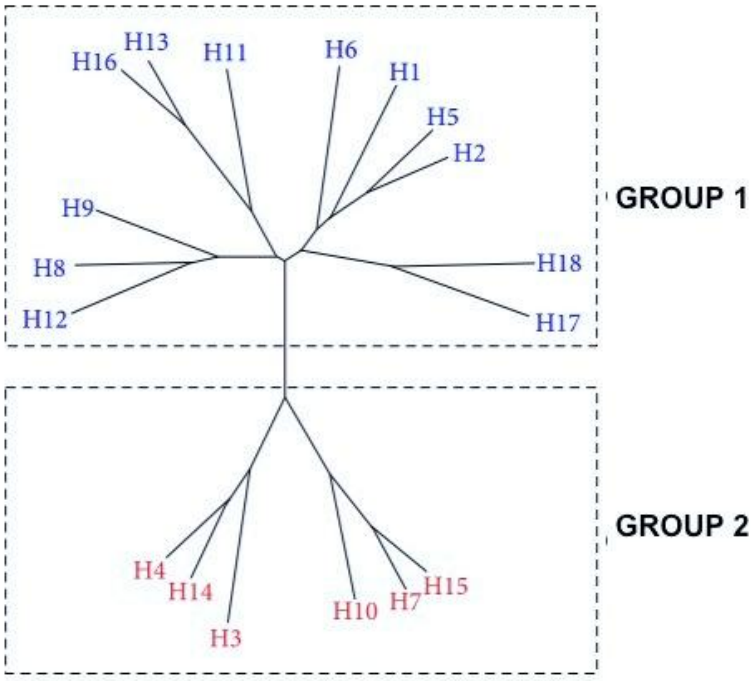

Figure 1.2. Phylogenetic tree of all subtypes of (HA) of influenza A. Representative HA protein sequences were selected for each subtype from viruses. Figure from (Jang and Seong, 2014)

The major envelope glycoprotein HA is synthesized in the infected cell as a single polypeptide chain (HA0) with a length of approximately 560 amino acid residues, which is subsequently cleaved into two subunits, HA1 and HA2. These subunits are linked by two intramonomer disulfide bridges that are presumably formed during the folding of HA0 in the endoplasmic reticulum (ER). The HA glycoprotein has a cylindrical shape with approximate dimensions of 135 Å (length) X 35-70 Å (radius) forming spikes that project externally (Isin et al., 2002). Each subunits of the HA molecule consist of a globular head domain and stem domain shown in Figure 1.3.

The globular heads of HA are formed by the HA1 residues 116 –261 folded into a jelly-roll motif of eight antiparallel β-strands. The distal tips of the globular heads contain the highly conserved shallow pockets that are the receptor binding sites (Weis *et al.*, 1988). The virus exploits the sialic acid residues in carbohydrate side chains of cellular receptor proteins. The receptor binding pocket of HA is surrounded by antigenically variable antibody binding sites. Antibodies bound to these sites should, in principle, block the binding to receptor proteins. Thereby, inhibit viral entry, demonstrating hemagglutinin inhibition (HI) activity and viral neutralization (VN) activity. However, the globular head domain is highly variable across viruses and tends to change under immune pressure and, hence, easily evades the neutralizing antibodies induced by previous vaccinations or infections (Bizebard *et al.*, 1995; Gerhard *et al.*, 1981; Isin *et al.*, 2002; Jang and Seong, 2014; Skehel *et al.*, 1982; Webster and Laver, 1980; Wiley and Skehel, 1987).

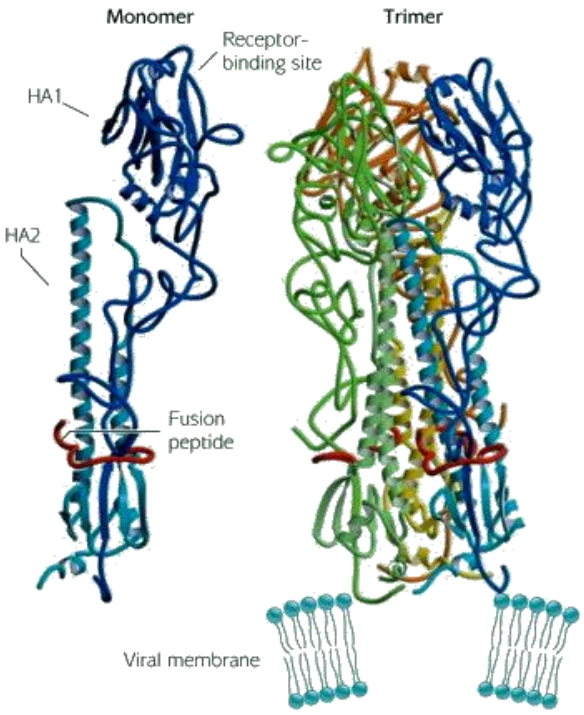

Figure 1.3. The three-dimensional structure of the influenza hemagglutinin (HA). The HA monomer (left) and trimer (right) are shown. In the monomer, the globular HA1 subunit is shown in dark blue, the HA2 subunit in light blue, with the "fusion peptide" in red. The receptor-binding site of HA1 is located at the tip of the molecule. This figure was produced by André van Eerde (University of Groningen); using MOLSCRIPT, on the basis of the co-ordinate file from the Protein Data Bank, code 3HMG.

HA1 also contains the major antigenic epitopes of the molecule. Five antigenic sites have been described in the head domain of the H1 subtype: Sa (residues 128-129, 156-160, 162-167), Sb (residues 187-198) ,which are located near the spike tip; Ca1 (residues 169-173, 206-208. 238-240), Ca2 (residues 140-145, 224-225), which are between adjacent HA monomers (subunits interface) about halfway down the globular head; and Cb (residues 74-79), that is within the vestigial esterase domain near the base of globular head, surrounding the receptor binding pocket as shown in Figure 3.and 4 These sites are designated as A, B, C, D, and E in the H3 subtype (Wiley and Skehel, 1987; Wiley et al., 1981; Wilson and Cox, 1990) shown in Figure 1.4. Substitutions of amino acids at these antigenic sites during infection are associated with antigenic change (Sriwilaijaroen and Suzuki, 2012). However, the mutations within the site E are rare. This indicates that variable antigenic sites A and B located in the globular head of HA1 could represent an immune decoy which protects the important functional site E, determining the conserved long-range properties of the molecule. A similar structural organization was previously reported for HIV-

1 gp120 (Veljkovic and Metlas, 1990; Veljkovic et al., 2004) and it was pointed out as an important obstacle in development of AIDS vaccine (Veljkovic et al., 2001). Therefore, HA1 has been the major target of the present work.

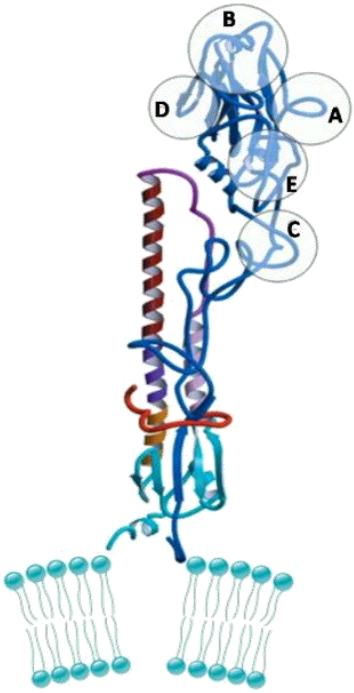

Figure 1.4 The location of the five major antigenic epitopes, A–E, on the HA1 subunit of the influenza virus hemagglutinin. In the intact HA trimer, epitope D is not exposed and thus may not be involved in antibody induction. This figure was produced by André van Eerde (University of Groningen); using MOLSCRIPT, on the basis of the co-ordinate file from the Protein Data Bank, code 3HMG.

On the other hand, HA2, which is a major part of the stem domain, is folded mainly into a helical coiled-coil structure by which the 80-Å-long helix forms the stem backbone. HA2 contains the hydrophobic peptide required for membrane fusion that is in a trimer interface far (about 35 Å) from the bottom of the molecule (Ekiert et al., 2009; Wiley and Skehel, 1987; Xu et al., 2010). This domain has been shown to remain relatively well conserved across viruses, but is far less immunogenic than the bulky globular head domain. Thus, antibodies directed to this domain occur only at a low frequency (Corti et al., 2010; Sui et al., 2011).

1.1.2.1. Virus replication

In the initial stages of IAV replication, the viral HA binds to sialic acid residues on glycoproteins or glycolipids on the cell surface. The fine specificity of HA's receptor binding depends on the nature of the glycosidic linkage between the terminal sialic acid and the penultimate galactose residue on the receptor. Human influenza viruses preferentially bind to sialic acids attached to galactose in an α2,6 configuration, whereas avian viruses have a preference for sialic acids attached to galactose in an α2,3 linkage(Connor *et al.*, 1994; Rogers *et al.*, 1983). This difference is thought to be the basis for the less efficient transmission of avian influenza viruses to humans. Pigs, on the other hand, have receptors with either type of linkage between sialic acid and galactose, and thus, are readily susceptible to infection with both human and avian viruses(Ito *et al.*, 1998; Shinya *et al.*, 2006).

The virus enters the cell by receptor-mediated endocytosis (Wiley and Skehel, 1987), the viral and endosomal membranes fuse under low pH conditions(Huang et al., 1981; Maeda and Ohnishi, 1980; White et al., 1981)(Figure 1.5). The viral genome is subsequently released into the cytoplasm and migrates to the nucleus, where it is transcribed and replicated. Viral segments then associate with nucleoprotein (NP) to form viral ribonucleoproteins (vRNPs), which are exported to the cytoplasm for packaging with the assistance of matrix protein 1 (M1) and non-structural protein 2 (NS2; also known as NEP). NS1 is not included in the virion but is abundantly expressed in infected cells(Shaw *et al.*, 2008). The vRNPs translocate to the cell membrane along with the envelope proteins, HA, NA, M1 and the ion channel protein M2, and virus particles are formed(Leser and Lamb, 2005). Finally, release of the virus particle from the host cell is mediated by NA, and the infection spreads to other host cells.

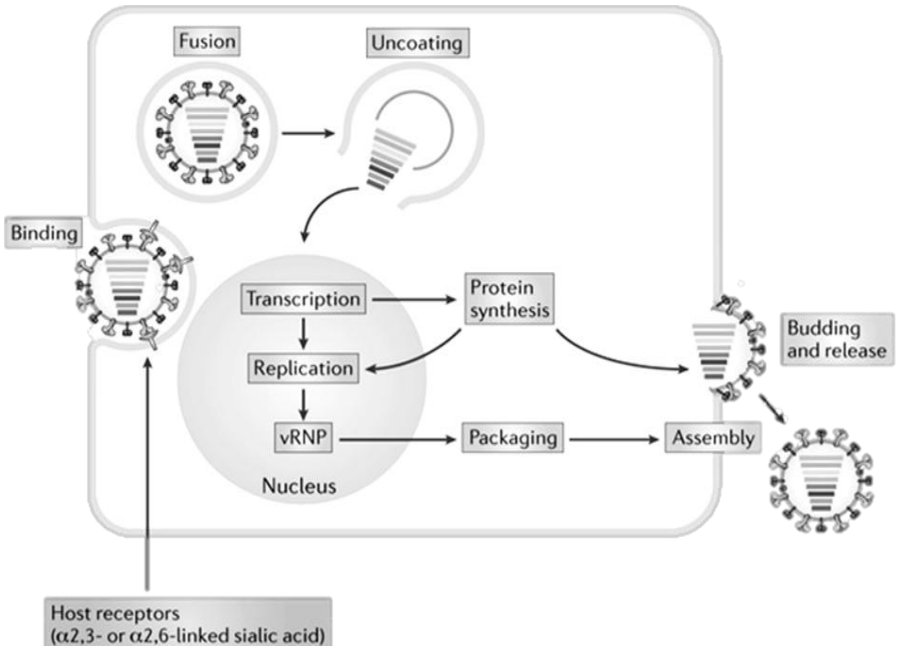

Figure 1.5 Representation of the replication cycle of Influenza A virus.(Shi *et al.*, 2014).

1.2. IMMUNE RESPONSE TO INFLUENZA INFECTION

1.2.1. Innate immune response

The innate immune system forms the first line of defense against influenza virus infection. It consists of components (e.g. mucus and collectins) that aim to prevent infection of respiratory epithelial cells. In addition, rapid innate cellular immune responses are induced that aim at controlling virus replication.

Influenza A virus infection is sensed by infected cells via pattern-recognition receptors (PRRs) that recognize viral RNA, the main pathogen-associated marker pattern (PAMP) of influenza A viruses. The PRRs are toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) and the NOD-like receptor family pyrin domain containing 3 (NLRP3) protein (Pang and Iwasaki, 2011). TLR7 binds single-stranded viral RNA (especially in plasmacytoid dendritic cells) and TLR3 and RIG-I bind double-stranded viral

RNA (in most other infected cells). Signalling of these receptors leads to production of proinflammatory cytokines and type I interferons (IFNs) (Alexopoulou et al., 2001; Heil et al., 2004; Lund et al., 2004).

Alveolar macrophages, dendritic cells (DC) and natural killer cells (NK) play an important role in innate response to limit viral spread after of the alveoli infection. Alveolar macrophages become activated and phagocytose (apoptotic) influenza virus-infected cells (Kim et al., 2008; Tumpey et al., 2005). Alveolar macrophages in the lungs produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF-α), and this way contribute to influenza virus induced pathology (Jayasekera et al., 2006; Lin et al., 2008; Peper and Van Campen, 1995). However, alveolar macrophages also help to regulate immune responses, especially the development of antigen-specific T cell immunity (Wijburg et al., 1997). DCs present the influenza virus-derived antigens to T cells and activate them. DCs degrades the viral proteins and the immuno-peptides (epitopes) are presented by major histocompatibility complex (MHC) class I or class II molecules to activate a T cells response(GeurtsvanKessel et al., 2009). MHC class I/peptides are recognized by $CD8^+$ cytotoxic T cell (CTL). Resulting MHC class II/peptides are recognized by $CD4^+$ T helper (Th) cells. Finally, NK cells can recognize antibody-bound influenza virus infected cells and lyse these cells, a process called antibody dependent cell cytotoxicity (ADCC). These cells can recognize influenza virus-infected cells with their cytotoxicity receptors (NCR) NKp44 and NKp46 (Arnon et al., 2001; Mandelboim et al., 2001).

1.2.2. Adaptive immune response

The adaptive immune system forms the second line of defense against influenza virus infection. It consists of humoral and cellular immunity mediated by virus-specific antibodies and T cells respectively (Figure 1.6).

1.2.2.1. Humoral response

Influenza virus infection induces virus-specific antibody responses (Mancini et al., 2011; Potter and Oxford, 1979). Especially antibodies specific for the two surface glycoproteins HA and NA are of importance since the presence of antibodies recognizing these proteins correlates with protective immunity (Gerhard, 2001).

The HA-specific antibodies bind to the trimeric globular head of the HA predominantly and inhibit virus attachment and entry in the host cell. Thus, HA-specific antibodies can neutralize and confer protection against influenza virus(de Jong et al., 2000).Also antibodies to the NA do not directly neutralize the virus but by inhibiting the release of newly produced virus particles and shorten the severity and duration of illness(Chen et al., 2000; Johansson et al., 1993; Johansson et al., 1998). Furthermore, NA-specific antibodies also facilitate ADCC and also may contribute to clearance of virus-infected cells (Mozdzanowska et al., 1999).The M2 play an important role in the virus replication cycle. Compared to HA and NA, it is a minor antigen on mature virions; however, its expression in virus-infected cells can be readily detected (Lamb et al., 1985; Pinto et al., 1992). NP is an important target for protective T cells, and NP-specific antibodies may contribute to protection against influenza virus infection (Carragher et al., 2008; Lamere et al., 2011). The leading antibody isotypes in the influenza specific humoral immune response are IgA, IgM and IgG. Mucosal or secretory IgA antibodies are produced locally and transported along the mucus of the respiratory tract. These antibodies are also able to neutralize influenza virus intracellularly(Mazanec et al., 1995).

1.2.2.2. Cellular response

The CD4+ T helper cell response after influenza virus infection and vaccination is multi-specific and also HA- and NA-specific CD4+ T cells are induced (Swain et al., 2006). CD4+ T cells are crucial for the optimal

activation and early expansion of B cells, for the initiation and maintenance of germinal center reaction and the generation of long-lived plasma and memory B-cells (Bernasconi et al., 2002; Lanzavecchia, 1985; McHeyzer-Williams and McHeyzer-Williams, 2005). They also play a role in the control of virus infection by promoting CD8+ cytotoxic T cell responses. After influenza virus infection the virus-specific CD4+ T cell responses are directed against a variety of proteins, including NP, M1, PB1, PB2, PA and NS1. Also the CD8+ T cell responses are largely directed against these proteins, and a large number of epitopes have been identified which are recognized by virus-specific CD8+ T CTL (Bodewes *et al.*, 2010). The main function of CD8+ T cells is the elimination of virus-infected cells through the release of perforin and granzyme. Perforin permeabilizes the membrane of the infected cells and subsequently granzymes enter the cell and induce apoptosis. In addition, activated CD8+ T cells produce cytokines like IFN-γ and TNF-α which can modulate the immune response. The majority of CTL epitopes is fully conserved between intra-subtypic variants of influenza A viruses. However, it has been demonstrated that some immunodominant CTL epitopes display variability that is associated with escape from recognition by specific CTL (Berkhoff *et al.*, 2007; Rimmelzwaan *et al.*, 2004; Voeten *et al.*, 2000).

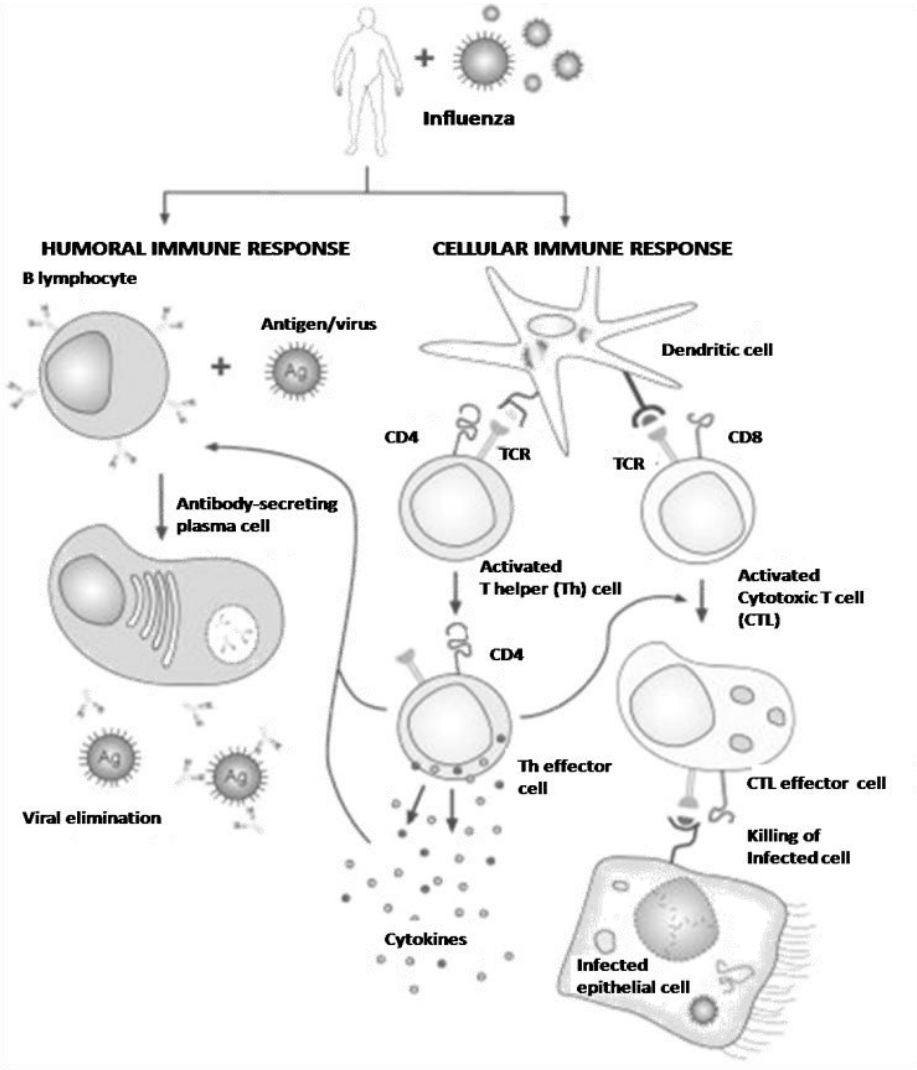

Figure 1.6. Induction of humoral and cellular immunity. The humoral branch of the immune system comprises B-lymphocytes *(left)*, which after interaction with influenza differentiate into antibody-secreting plasma cells. The cellular response *(right)* starts with antigen presentation via MHC I and II molecules by dendritic cells, which then leads to activation, proliferation and differentiation of antigen-specific T cells (CD4 or CD8). These cells gain effector cell function to either help directly, release cytokines, or mediate cytotoxicity following recognition of antigen. The formation of a cellular memory immune response and the various forms of innate immunity induced by influenza is not represented(Behrens G, 2006).

1.3. INFLUENZA EPIDEMICS AND PANDEMICS

1.3.1. Antigenic variation

Influenza antigenic properties are determined by both HA and NA(Oxford, 2000). HA acts to attach the virus into host cells and subsequently, fuse it to cell membranes, which is essential for the virus life cycle(Skehel and Wiley, 2000). HA is synthesized as a single peptide but cleaved into HA1 and HA2 by specific host protease. The amino acids at the

cleavage site are important in determining the virulence of the virus, that is the virus becomes highly virulent if these amino acids are lipophilic(Skehel and Wiley, 2000). Immunity induced by HA has been shown to increase host resistance to influenza and reduce the likelihood of infection and severity(Clements et al., 1986). However, such protection is not effective against newly emerging influenza viruses that contain antigenic variations known as antigenic drift and shift (Ping et al., 2008).

1.3.1.1. Antigenic drift

Antigenic drift refers to represents mutations leading to slight antigenic changes incurred by the circulating HA or NA subtypes within a population over a period of time(Subbarao, 2006). Antigenic drift does not cause the HA subtype to change, but is sufficient to necessitate a newly designed seasonal influenza vaccine each year because antibodies can no longer neutralize, or can only weakly neutralize, the mutated form of the virus(Subbarao, 2006). In contrast, antigenic shift represents a significant change in which a circulating HA or NA subtype is exchanged either for a different one, or for the same subtype from a different virus. This creates a mosaic virus, which then may be introduced into an immunologically naive population.

1.3.1.2. Antigenic shift

This occurs when there is a genetic reassortment of different influenza viruses within the same cell in a common host, resulting in a virus with new antigenic proteins(Bouvier and Palese, 2008). The most recent example of this type of shift is that which resulted in the 2009 H1N1 pandemic, which originated and reassorted in swine (Fraser *et al.*, 2009; Medina and Garcia-Sastre, 2011; Neumann *et al.*, 2009; Smith*et al.*, 2009; Vijaykrishna *et al.*, 2010). Also, the transfer of a whole virus from one host

species to another and the re-emergence of previously non circulating virus strain are considered an antigenic shift (Suarez, 2008).

1.3.2. Avian Influenza virus

The avian influenza virus (AIV) cause a wide range of clinical manifestations from asymptomatic to a severe acute disease with mortality rates reaching 100% in avian species(Swayne, 2008).AIV can generally be classified according to their virulence in poultry into low and high pathogenic avian influenza (LPAIV and HPAIV, respectively)(Capua and Alexander, 2004; Perkins and Swayne, 2001; Suarez, 2010; Swayne and Suarez, 2000; Webster *et al.*, 1992).HPAIV produce severe disease in poultry, mainly chickens and turkeys, causing devastating economic losses. Meanwhile, LPAIV causes little morbidity and mortality (Capua and Alexander, 2006). HPAIV affecting poultry have been limited to theH5 and H7 subtypes. Moreover, there is a great risk for subtypes H5 and H7 low pathogenic virus can become highly pathogenic as results of mutations or as a consequence of reassortments between LPAIV subtypes that co-infect birds (Alexander and Brown, 2009; Capua and Alexander, 2004; Dugan et al., 2008; Kalthoff et al., 2010; Li et al., 1990; Sharp et al., 1997; Suarez, 2010). More than 28 outbreaks have been recorded worldwide, of which half of them have occurred in the past 10 years. The potential impact of HPAI was demonstrated when the disease became widespread round the world affecting a huge number of avian species, causing enormous economic losses and zoonotic risk (Lin *et al.*, 2000; Xu *et al.*, 1999).

1.3.3. Swine influenza viruses

Pigs are suggested to act as mixing vessel host to generate new assortments of influenza viruses potentially pathogenic (Ma et al., 2008;Van Reeth, 2007). Although, pigs can be experimentally infected with subtypes of AIVs (Kida et al., 1994), only SIVs of the subtypes H1N1, H1N2 and

H3N2 are enzootic and widespread in swine producing regions of Western Europe (Simon-Grife et al., 2011; Van Reeth et al., 2008). The outbreaks in swine are common and cause significant economic losses in industry, primarily by causing stunting and extended time to market (Kay et al., 1994). In Central Europe, SIV activity is low and the circulation of H3N2 and H1N2 remains to be confirmed (Van Reeth et al., 2008), however, IAV infections in pigs can be potential risk.

1.3.4. Human influenza viruses

IAV causes annual epidemics and occasional pandemics that have claimed the lives of millions. Currently, in humans, IAVs of the H1N1 and H3N2 subtypes and influenza B viruses are responsible for the seasonal outbreaks of influenza. In 1918 IAVs of the H1N1 subtype were introduced in the human population causing the 'Spanish Flu' outbreak. These viruses circulated in humans until 1957 when influenza A(H2N2) viruses caused the 'Asian Flu' outbreak and completely replaced viruses of the H1N1 subtype. Within a decade, viruses of the H2N2 subtype were replaced by viruses of the influenza A(H3N2) subtype which caused the 1968 'Hong Kong' pandemic. In 1977 influenza A viruses of the H1N1 subtype were reintroduced without causing a major pandemic.

In 2009, a new influenza A(H1N1) virus of swine-origin caused the first influenza pandemic of the 21st century (WHO, 2009). In 2015 the instances of Swine Flu continue to rise substantially to five-year highs with over 10,000 cases reported and 774 deaths in India (Suresh Rewar, 2015). In addition to these worldwide introductions, IAVs of various subtypes (e.g. H5N1, H7N7 and H9N2) are transmitted from animals to humans sporadically(de Wit et al., 2008). Especially, HPAI viruses of the H5N1 subtype have caused severe disease and death in an ever increasing number of humans. Thus far, more than 600 human cases have been reported between 2003-2015 of which 60% proved to be fatal (WHO, 2015). Thus, the pathogenesis of infections with these avian H5N1 viruses in humans differs from that of seasonal IAVs of the H1N1 and H3N2

subtypes(Uiprasertkul et al., 2007; Uiprasertkul et al., 2005; van den Brand et al., 2010; van Riel et al., 2006; van Riel et al., 2007).

Recently, at the end of March 2013, a novel LPAI H7N9 virus emerged in China, resulting in 137 confirmed human infections including 45 fatalities (WHO, 2014b). This particular A(H7N9) virus had not previously been seen in either animals or people. However, since then, infections in both humans and birds have been observed. Most of the cases of human infection with this avian H7N9 virus have reported recent exposure to live poultry or potentially contaminated environments, especially markets where live birds have been sold. This virus does not appear to be easily transmitted from person to person (WHO, 2014b).

1.3.5. Economical and social impact of influenza

The societal burden of recurring annual influenza epidemics and occasional pandemics are often underestimated. It is important to understand the extent to which influenza impacts on society, its financial burden and strategies to minimize the impact. This section provides an overview of the social and economic implications of influenza.

Each outbreak of influenza takes a significant toll on societies in terms of morbidity, mortality, productivity and financial resources. The WHO estimates that there are 3–5 million cases of severe influenza illness, resulting in 250,000–500,000 deaths annually in the industrialized world. Clinics and hospitals can be overwhelmed when large numbers of sick people appear for treatment during peak periods of illness. In industrialized countries, influenza is associated with a considerable economic burden in terms of healthcare costs, lost days of work or education, general social disruption and workforce productivity losses (WHO, 2014).

In addition to its social impact, influenza also has a significant economic impact. Influenza A is a major cause of concern to the swine and

poultry industry. However, the current global influenza situation is characterized by avian flu outbreaks that continue to affect mainly in Southeast Asia, and also in developing countries as Egypt, Romania, Nigeria, China, Iran, Turkey, Russia, India and Pakistan. A lower incidence in Ukraine, Saudi Arabia and some African countries is observed. Japan and Republic of Korea are in this group; however, these are likely to be affected by the proximity to countries where the incidence high. In most economically developed countries the effect is very low although cases have been reported for example Germany (8), United Kingdom (3), Sweden (1) and France (1) (See Figure 1.7). This means that avian flu outbreaks in Asia and other countries as they progressively moves westward are prompting the imposition of import bans on poultry products from disease-affected countries (OIE, 2015).

The global market impact of these bans over the past year include a decline by 1% to 12.6 million tonnes. Although the rate of trade growth has been waning since 2012. For 2015, outbreaks of highly pathogenic avian influenza (HPAI) in some areas of the United States from January onwards caused numerous countries to suspend imports as reported by Food and Agriculture Organization of the United Nations (FAO, 2015).

In summary, negative implications of influenza has revived efforts to develop "universal" flu vaccines that protect against all human influenza strains. However, OIE reported that data on vaccination coverage show that even in industrialized countries, large proportions of the population at risk do not receive the influenza vaccine. The target of the WHO of reaching 75% vaccination coverage of high-risk people is critical to establish measures to control of influenza virus infection in poultry and swine.

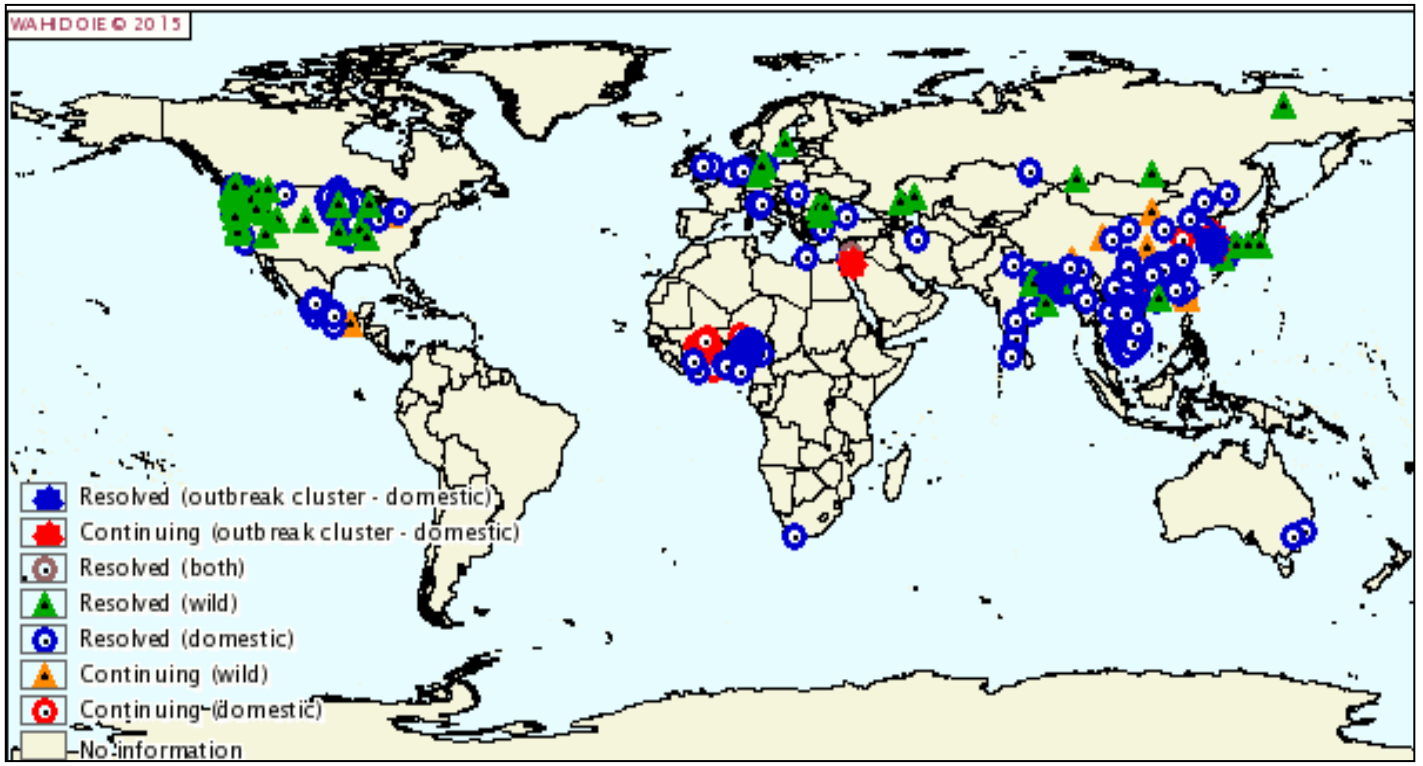

Figure 1.7 Outbreaks of Highly Pathogenic Avian influenza (subtype H5N1) in poultry. Notified to the OIE end of 2003 to 11 August 2015 (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/20

1.4. INFLUENZA VACCINES

1.4.1. Current influenza vaccines and their limitations

Vaccines are the most effective methods of preventing influenza virus infection and further transmission in humans. Current influenza vaccines are ineffective when it comes to protecting against newly introduced influenza strains and currently circulating strains that have undergone genetic drift. Vaccines that are able to elicit heterologous immunity to many influenza subtypes are desirable.

Currently developed influenza vaccines include seasonal trivalent influenza vaccine (TIV) formulations which contain either inactivated influenza antigens or live attenuated influenza viruses, derived from two influenza A strains and B strain. Quadrivalent influenza vaccine (QIV) formulations, which add an additional influenza B strain, have entered the market recently.. In addition, several pandemic vaccines have been developed to be prepared for a possible future outbreaks of highly pathogenic influenza strains (Soema et al., 2015a).

Novel alternative types of antigens such as virus-like particle (VLP)-based, subunit vaccines, recombinant proteins, viral vectors (Kyriakis et al., 2009), synthetic peptides (Vergara-Alert et al., 2012; Wang et al., 2010) and DNA-based vaccines(Chen et al., 2008) have been reported. The advantages and disadvantages of these vaccine types, as well as the specific adjuvants used for each formulation, are summarized in Table 1.

VACCINE TYPES	ADVANTAGES	DISADVANTAGES	ADJUVANTS USED	MODEL	COMMERCIAL	REF
Inactivated virus and live attenuated virus- based	Induces robust humoral and cellular immune responses and protection	Safety problems; potential allergic reactions to eggs	Rintatolimod, MF59, AS03B	Human, Chickens, Pigs	MedImmune®,Sanofi Pasteur®, Novartis® bioCSL®, GlaxoSmithKline®	(Overton *et al.*, 2014; Richt and Garcia-Sastre, 2009)
DNA-based	Induces humoral and cellular immune responses and protection; safe and easy to scale up	Low immunogenicity; potential host autoimmunity; risk of malignancy.	NA	Mice, pigs, ferrets,	Preclinical models	(Bragstad et al., 2013; Prather et al., 2003; Shamlou, 2003; Tompkins et al., 2007
Viral vector-based	Induces high humoral and cellular responses and cross-protection	Safety problems, pre-existing immunity	NA	Mice, macaques, ferrets, humans	Paxvax®(Clinicaltrial phase I)	(Fan *et al.*, 2015; Gurwith *et al.*, 2013; Nayak *et al.*, 2009; Vemula *et al.*, 2013
VLP-based	Genome-free particles containing only viral envelope proteins; can be prepared in a variety of expression systems; induces immune responses and protection	Relatively lower immunogenicity	NA	Mice, pigs, chickens, ferrets, humans	Novavax® (Clinical trial phase II)	(Ali *et al.*, 2000; Choi *et al.*, 2013; Green *et al.*, 1993; Prabakaran *et al.*, 2010; Roldao *et al.*, 2010; Zheng *et al.*, 2004)
Subunit vaccines (HA, M2e, NP, NA)	High safety profile without involving infectious viruses; rapid, stable, consistent and scalable production; induces humoral and cellular immune responses, neutralizing antibodies and cross-protection	Require appropriate adjuvants, suitable administration route, fusion with several proteins, or combination with different vaccination strategies to improve immunogenicity	Adjuvants can be fused with influenza protein vaccines or mixed together with these vaccines	Mice, chickens, pigs, ferrets	Animal trial, Clinical trial phase I	(Bodewes *et al.*, 2011; Mallajosyula *et al.*, 2014; Rajão *et al.*, 2014)

Table adapted from (Soema *et al.*, 2015a; Zhang *et al.*, 2015)

1.4.2. Formulation strategies: adjuvants and antigens

The limitations of current influenza vaccines may be resolved through the implementation of new technologies in the field of influenza production and vaccine formulation. These antigens demand novel production methods, which carry their own advantages and disadvantages. In addition, it often needs to be formulated with excipients and adjuvants to be sufficiently immunogenic (Soema *et al.*, 2015a). These formulation strategies for influenza vaccines will be discussed below.

1.4.2.1 Adjuvants

A common limitation of some of the novel strategies, but also of existing influenza vaccines, is their limited immunogenicity (Wiersma. L et al., 2015) . Many novel antigens such as peptides and DNA antigens require the addition of adjuvants to steer the immune response toward a specific cellular immune response.

The most commonly used vaccine adjuvants are Alum and emulsions which have been used to boost the immunogenicity of human vaccines for decades (Mohan et al., 2013).

Alum adsorbs the vaccine antigen and makes the antigen stay longer in the injection site, so that immunogenicity of the antigen is increased. In an immunological mechanism study, alum was shown to activate NALP3 inflammasome and induce IL-1β production to stimulate the innate immune system. Alum is likely to induce T helper type 2 immune responses to co-administered antigens in humans (Eisenbarth et al., 2008).

Emulsions oil-in-water or water-in-oil, such as Freund's Incomplete Adjuvant (IFA), MF59® and Montanide™, can trigger depot generation and induction of MHC responses. IFA induces a predominantly Th2-based

response with some Th1 cellular response, and is widely used in inactivated poultry vaccines. However, the precise mode of action of emulsion-based adjuvants is still unclear(Mohan et al., 2013).

MF59 and AS03 have been shown to be more effective and are used in seasonal and pre-pandemic vaccine preparations (Smith et al., 2010).MF59is composed of squalene, polysorbate 80, sorbitan trioleate, trisodium citrate dehydrate, citric acid monohydrate, and water for injection. The use of MF59 adjuvant resulted in stronger antibody responses and vaccine antigen dose sparing effects (Durando et al., 2010). MF59 stimulates cells in the sites of injection to express chemokines and cytokines. These chemokines and cytokines recruit innate immune cells and APCs. Some APCs subsequently uptake antigen-MF59 complex and migrate to draining lymph nodes for the induction of adaptive immune responses(O'Hagan et al., 2012).AS03is a modified form of MF59 and is an oil-in-water emulsion with a tocopherol. This form of adjuvant is used in influenza vaccines, and it is also able to confer seroprotection in immunocompromised patients infect with HIV-1 (Soema et al., 2015b).

Montanide™ is able to induce a strong long-term humoral immune response and is widely used in inactivated poultry vaccines. W/O emulsions allow the reduction of the vaccine dose or the antigen concentration, which is important as vaccines must be cost effective. It can also enhance cellular immune responses by inducing higher IgG2a antibody levels than other type of emulsions(Aucouturier et al., 2001). In addition, Montanide™ has been used in trial vaccines against HIV, malaria and breast cancer (Jones et al., 1990). Liu et al., demonstrated the effects of mineral oil, Montanide™ provided 100% protection efficiency in SPF chickens after challenging with a HPAI virus (Liu et al., 2011). Chapter 5 focuses on HA1-peptide vaccine plus Montanide™ in a free-range chicken model against avian influenza.

Some saponin-based adjuvants, like the immunostimulating-complex or ISCOMS, can translocate antigen across the membrane of antigen presenting cells and thus, promote the endogenous route of antigen processing and presentation(Sun et al., 2009). Indeed the use of this type

of adjuvants result in increase of influenza-specific CD8+ T-cell responses as was demonstrates in animal models and clinical trials (Chung et al., 2015)

Other adjuvants, essentially ligands for pattern recognition receptors (PRR), act by inducing the innate immunity, predominantly targeting the APCs and consequently influencing the adaptative immune response. Members of nearly all of the PRR families are potential targets for adjuvants. These include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs).

PRR-ligands as classical adjuvants induce strong Th2 response with little or no Th1 response, the current challenge is to develop adjuvants which induce a strong Th1 bias important for vaccines against hepatitis, flu, malaria, and HIV. New adjuvants are being developed that are natural ligands or synthetic agonists for PRRs, either alone or with various formulations. PRR activation stimulates the production of pro-inflammatory cytokines/chemokines and type I IFNs that increase the host's ability to eliminate the pathogen. Thus, the incorporation of pathogens associated molecular patterns (PAMPs) in vaccine formulations can improve and accelerate the induction of vaccine-specific responses. A number of these agonists are now in clinical or late preclinical stages of development for hepatitis and human papillomavirus vaccines (Mbow et al., 2010; Steinhagen et al., 2011). When used in combination with alum or classical emulsion adjuvants, the immune response can be biased towards a Th1 response(Mbow et al., 2010).

TLR-based immune adjuvants are considered as potential adjuvants, so many different kinds of TLR agonists have been tried in experimental animal models and clinical trials. TLR3 and RLR-ligands, double-stranded RNA (dsRNA), which is produced during the replication of most viruses, is a potent inducer of innate immunity. Synthetic analogs of dsRNA, such as poly (I:C), have been tested as adjuvants. They act through TLR3 and RIG-I/MDA-5, inducing IL-12 and type I IFNs production, facilitating antigen

cross-presentation to MHC class II molecules, and improving generation of cytotoxic T cells(Edelman, 1980).

TLR4-ligands, bacterial lipopolysaccharides (LPS), which are ligands for TLR4, have long been recognized as potent adjuvants, but their pyrogenic-activity have prevented their clinical use. The development of less toxic derivatives led to the production of MPLA (monophosphoryl lipid A), which formulated with alum (AS04) triggers a polarized Th1 response and is approved for clinical use in Europe (Edelman, 1980; Steinhagen et al., 2011). TLR5-ligand, bacterial flagellin, is a potent T-cell antigen and has potential as a vaccine adjuvant. Unlike other TLR agonists, flagellin tends to produce mixed Th1 and Th2 responses rather than strongly Th1 responses. Flagellin can be used as an adjuvant mixed with the antigen but it is more frequently fused to a recombinant vaccine antigen (Huleatt et al., 2007; Mizel and Bates, 2010).

TLR7/8 pathway, specialized in the recognition of single stranded viral RNA, has demonstrated promising pre-clinical results as a target for potential vaccine adjuvants. Imidazoquinolines (i.e. imiquimod, gardiquimod and R848) are synthetic componds that activate TLR7/8 in multiple subsets of dendritic cells leading to the production of IFN-α and IL-12 thus promoting a Th1 response (Steinhagen et al., 2011).

TLR9-ligands, oligodeoxynucleotides containing specific CpG motifs (CpG ODNs such as ODN 1826 and ODN 2006) are recognized by TLR9. They enhance antibody production and strongly polarize Th cell responses to Th1 and away from Th2 responses (Kobayashi et al., 1999). NOD2-ligands, fragments of bacterial cell walls, such as muramyl dipeptide (MDP), have long been recognized as adjuvants. More recently, it was discovered that MDP triggers the activation of NOD2 and the NLRP3 inflammasome (Girardin et al., 2003).

Adjuvants may be combined to achieve a stronger effect or a more potent skewing of immune responses. Currently, much effort is devoted to combining alum with TLR9 agonists (Siegrist et al., 2004). In experimental

models, administration of other combinations such as CpG ODNs with MDP or MPLA has proven very effective (Kim et al., 2000). Adjuvants currently in clinical use enhance humoral responses but new adjuvants in clinical and preclinical trials are focused on generating multifaceted immune responses. The PRR pathways are an attractive source of novel adjuvants for vaccines due to their ability to induce strong cell-mediated immunity.

1.4.2.1. Antigens

1.4.2.1.1. Recombinant antigens

The use of recombinant technology enables the production of a wide array of influenza protein antigens that can induce different immune responses. These include not only conventional antibody responses against HA, but also immune responses against HA stalk regions(Steel *et al.*, 2010) and M2 ectodomain(Schotsaert *et al.*, 2009), which are potentially cross-reactive. Immunization with recombinant HA protein was shown to protect against homologous influenza strains in mice(Katz *et al.*, 2000), chickens (Crawford *et al.*, 1999). Immunization with recombinant M2 protein was shown to protect against both homologous and heterologous challenges in mice (Slepushkin *et al.*, 1995). Human trials with recombinant influenza proteins have found them to be safe and immunogenic, able to generate antibodies at titers that are considered to be protective in humans (Powers *et al.*, 1995; Treanor *et al.*, 1996).A similar approach to recombinant protein immunizations is vaccination with virus-like particles (VLPs). Recombinant headless HA2 protein was expressed on VLPs, which induced cross-reactive antibodies that showed immunogenicity against heterologous influenza strains in mice (Quan *et al.*, 2007)

1.4.2.1.2. Viral vectors

Vectored vaccines can be constructed to express full-length influenza virus proteins, as well as generate conformationally restricted epitopes,

features critical in generating appropriate humoral protection. Inclusion of internal influenza antigens (HA, M2e, M1 and NP) in a vectored vaccine can also induce high levels of protective cellular immunity (Tripp and Tompkins, 2014). One of the most studied viral vectors are vaccinia virus (Kreijtz et al., 2007), adenovirus, alphavirus, baculovirus; among others (Tripp and Tompkins, 2014).

1.4.2.1.3. DNA vaccines

DNA vaccines induce antigen production in the host itself. Previous studies have demonstrated that a DNA vaccine based on HAe, an HA1 region of the influenza A virus, induces neutralizing activity and protection against influenza viral challenge, suggesting that HAe and HA1 of the HA protein play significant roles in the immune response to viral infection and are attractive targets for vaccine development(Chen *et al.*, 2014). In addition, a DNA vaccine expressing full-length consensus-sequence M2 induced M2-specific antibody responses and protected against challenge with lethal influenza (Tompkins *et al.*, 2007). However, clinical trials have so far been only moderately successful (Drape *et al.*, 2006; Jones *et al.*, 2009), since plasmid DNA was poorly immunogenic in humans. Potentially, this could be improved by alternative delivery systems or the use of adjuvants. While influenza DNA vaccines are a promising concept, several concerns regarding safety have to be considered (Wiersma. L *et al.*, 2015). Antibodies against the DNA plasmid could render the vaccine ineffective. Also, the continued production of influenza antigens in the host might alter the immune system, or induce tolerance against influenza antigens. Arguably, the largest issue is the introduction of extraneous DNA into the vaccinated subject, which could lead to unwanted genetic changes such as tumor growth. Extensive safety and efficacy studies are therefore necessary to overcome these concerns(Wiersma. L *et al.*, 2015). Clinical trials have so far been only moderately successful since plasmid DNA was poorly immunogenic in humans. Newer techniques, such as self-amplifying mRNA-based vaccines, also appear to show promise clinical studies(Ulmer *et al.*, 2015).

1.4.2.1.4. Peptides

Compared with conventional vaccines, which are based on attenuated or inactivated microorganisms, synthetic peptides offer several advantages over other forms of vaccines, particularly with regards to safety and ease of production. The main benefit of vaccination with peptide epitopes is the ability to minimize the amount and complexity of a well-defined antigen. Peptides can be used for the induction of both influenza-specific immune B-cell and T-cell responses against conserved epitopes or proteins of the influenza M2, NP, M1 and HA.

The extracellular domain of the M2 protein (M2e), several studies have been reported that M2e appears to be an attractive target due to its surface localization, high level of conservation and the linear nature of its peptide. M2e-specific antibodies can limit spread of the virus by binding virus particles to the cell, thereby preventing release into the extracellular fluid (Subbarao and Joseph, 2007). Furthermore, these antibodies are thought to be mostly non-neutralizing, the proposed immunological mechanism that mediates the protection is antibody-dependent cellular cytotoxicity (ADCC), but also a role for alveolar macrophages has been demonstrated in a mouse model (El Bakkouri et al., 2011; Fiers et al., 2009). Even so, the M2e peptide can be either chemically conjugated to carrier proteins or recombinantly expressed on the surface of virions or VLPs. However, immune response induced by VLPs might be relatively lower such as demonstrated in mice (Song et al., 2011).

Other strategy, could improve immunogenicity of M2e. Zhao et al showed that mice vaccinated with M2e-multiple antigenic peptide (MAP)-based vaccine induced strong M2e-specific IgG antibodies, protecting

immunized mice against with PR8 A/H1N1, or divergent H5N1 influenza viruses (Ma et al., 2013; Zhao et al., 2010a; Zhao et al., 2010b).Some studies have been demonstrated that influenza A virus infection of humans induces a weak anti-M2 antibody response that is of short duration (Feng et al., 2006). A possible explanation for this low reactivity is the small size of M2e and its low abundance of M2 in virions compared to the large glycoproteins, HA, and NA (Hutchinson et al., 2014).

Internal protein NP is also an ideal antigen for induce Immunity cellular. Some studies showed that peptides of two conserved epitopes ($NP_{265-274}$ and $NP_{174-184}$) elicited CD4+ T cell response (Roti et al., 2008b), suggesting that these two epitopes may be candidates able to provide partial immunity to pandemic H5N1 virus. NP peptides presented on MHC class I molecules may also induce specific CD8+ T cell response which correlates with protection (Macleod et al., 2013; Thomas et al., 2006; Yewdell et al., 1985). However, immunity induced by NP might be low, and with the absence of neutralizing activity, it would be unable to induce highly potent protection against virus infection. Therefore, some studies are considering the inner proteins not for a stand-alone vaccine but as adjuvants that could broaden the immune response to HA-based vaccines (Kaiser, 2006).

Apart from the conserved antigens described above; other protein such as M1, also has potential for application as targets to develop influenza vaccines. Highly conserved epitopes and consensus sequences have been employed in various vaccine platforms. An example of one such epitope of interest is the immunodominant HLA-A*0201 restricted $M1_{58-66}$ epitope (Gotch et al., 1987), which was recently shown to be presented not only by HLA-A but also by HLA-C molecules (Choo et al., 2014). Of interest, this highly conserved epitope is under functional constraints, since mutations were not tolerated without loss of viral fitness (Choo et al., 2014).

Among one of the most important surface proteins, HA has been a promising antigen target, which is central to the virus to bind to cell membrane and infect the cells. As mentioned above, functionally, influenza

virus HA is composed of a receptor binding globular head domain of HA1 and a membrane fusion inducing stalk domain of HA2 for virus entry (see section 1.1.2), (Wiley et al., 1981). However, the host immune system usually recognizes the bulky and highly variable-immunodominant globular head domains in the HA which shield the more conserved regions such as those in the stem part (Nabel and Fauci, 2010; Steel et al., 2010). In addition, memory immune response is usually elicited against these immunodominant epitopes from previously encountered strain. Therefore, development of broadly neutralizing anti-HA antibodies against diverse viral strains could be challenging as influenza A HA varies among not only the different 18 subtypes (H1–H18) which fall into two distinct phylogenetic groups as shown in Figure 1, but also among the different strains within each subtype. Nonetheless, several research groups have isolated Abs with broad inhibitory spectrum which bind to highly conserved epitopes in diverse influenza viruses from group 1, group 2, or both groups of the HA protein using naïve or immune phage display Ab libraries, Ab cloning from sorted plasmablasts and plasma cells, or hybridomas of memory B cells. Importantly, most of these studies have shown that cross-subtype broadly neutralizing anti-HA antibodies can be induced upon vaccination or infection in humans and animals which raises the prospects of HA-targeted universal influenza vaccine development.

1.5. NOVEL BIOINFORMATICS TOOLS TO SELECT POTENTIAL VACCINE CANDIDATES

1.5.1. Informational spectrum method (ISM)

The field of bioinformatics has become a major part of the identification and early validation of new therapeutic targets. It could be an essential first step in the development of an effective vaccine for the influenza virus that represents the high variability of its antigenic determinants. In order to solve this problem, herein was used the application of the Informational spectrum method (ISM), a virtual

spectroscopy for analysis of protein-protein interactions, protein-DNA interactions and structure/function relationship of proteins and DNA sequences (Veljkovic et al., 2008).

This method involves three basic steps:

i) Transformation of the alphabetic code of the primary structure into a sequence of numbers by assigning to each amino acid or nucleotide a corresponding numerical value representing the electron-ion interaction potential
ii) Conversion of the obtained numerical sequence by mathematical model into the informational spectrum.
iii) Cross-spectral analysis, which allows identification of frequency components in the informational spectrum of molecules, which are important for their biological function or interaction with other molecules.

The ISM platform has been successfully applied as the main bioinformatics tool for discovery of new anti-HIV drugs (Veljkovic et al., 2008). In an attempt, our group is using peptides selected by ISM analysis. HA1 domain was identified to be involved in virus/receptor interaction and represents candidate prophylactic and vaccine target. Moreover this domain is located within the E site, one of the five major antigenic domains of the HA molecule that is less prone to mutations as mentioned above.

Our preliminary results in a pig model against intranasal challenge with pH1N1 demonstrated that: i) HA-peptide elicited strong cellular response and ii) HA peptide-specific antibody is able to recognize distinct viral subtypes (Vergara-Alert et al., 2012). Based on findings, in the present thesis, our aim is to advance the field of vaccination and improve vaccine efficacy with better accessibility to memory B cells and better induction of cross-neutralizing antibodies. Chapters 3, 4 and 5 explore the ability of HA1-peptides to elicit immune responses against influenza virus in an experimental mouse infection as well as infections in its natural host pig and chicken.

HYPOTHESIS AND OBJECTIVES

Epidemics and sporadic pandemic outbreaks caused by influenza virus are a continuing threat to human health and to the world's economy. Currently, the hemagglutinin (HA) is the primary target of inactivated influenza virus vaccines. However, continual variation in the HA demands the annual reformulation of influenza vaccines. Despite this, site E is one of the five major antigenic domains of the HA, which is located in the HA subunit 1 (HA1), close to the receptor binding site. Since this region is involved in receptor-binding , it is less prone to mutations and highly conserved among influenza A viruses. Therefore, in this thesis it was postulated that the HA1 could represent a potential target for a multivalent vaccine of influenza infection.

Consequently, the general objective of this thesis is **to select conserved peptides from the hemagglutinin subunit 1 (HA1) of influenza viruses and to evaluate the efficacy of the selected candidates to induce immunity that can protect animals against infection.**

To accomplish the general purpose three specific objectives were established:

1. To evaluate the protective effect of improved HA1-peptides against the pandemic H1N1 2009 virus and a H7N1 highly pathogenic influenza virus (HPAIV) in a mouse model (*Chapter 1*)

2. To test the immunogenicity of an HA1-peptide cocktail in a pig model, and to asses whether this new formulation can confer immunity to a wide range of IAVs *in vitro* (*Chapter 2*)

3. To evaluate the protective effect of improved HA1-peptides against H7N1 highly pathogenic influenza virus (HPAIV) in chickens, a natural host model (*Chapter 3*)

PART II
STUDIES

CHAPTER 1

Study I: Novel conserved immunogenic peptides from the hemagglutinin subunit 1 protein of influenza virus protect mice against different viral subtypes

1.1. INTRODUCTION

During lasts years, an increase in the number of outbreaks involving highly pathogenic avian influenza viruses (HPAIV) has been reported in poultry and wild birds (Lupiani and Reddy, 2009). Some of these outbreaks were responsible for avian-to-mammals transmissions, affecting also humans; thus, representing a threat to public health (Morens and Fauci, 2012; WHO, 2014). The wide flow of influenza virus is of concern considering that the three human influenza pandemics reported in the twentieth century (1918 H1N1, 1957 H2N2 and 1968 H3N2) were the result of reassortments between human and avian or swine influenza viruses (IV) in non-human reservoirs (Cox and Subbarao, 2000; Webby and Webster, 2003). In 2009 a swine-origin IV A H1N1 (pH1N1) was isolated in humans and was the responsible of the first pandemic of the 21st century (Ghosh and Heffernan, 2010; Scalera and Mossad, 2009; Vincent *et al.*, 2008). More recently, an outbreak of influenza A/H7N9 virus in humans was identified in China. The A/H7N9 is a novel subtype for humans and still little is known about its zoonotic potential, although it is believed that the direct contact with poultry may be the main source of infection (WHO, 2014).

Among all IV proteins, the hemagglutinin (HA) plays an important role in the attachment of IV to host cells, determining the viral host range and pathogenicity (Gamblin and Skehel, 2010; Hatta *et al.*, 2001; Munster *et al.*, 2007). HA is a viral surface polypeptide formed by subunit 1 (HA1) and subunit 2 (HA2), with the stalk of the molecule comprising the N- and C-terminal portions of HA1 along with the HA2 molecule (Wilson *et al.*, 1981). Eighteen HA subtypes, based on antigenic differences, have been identified so far (Tong *et al.*, 2012; Wu *et al.*, 2014).

The HA proteins can be further divided into two major phylogenetic groups: group 1 contains subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; and group 2 contains H3, H4, H7, H10, H14, and H15 (Air, 1981; Nobusawa *et al.*, 1991; Tong *et al.*, 2012). Antibodies directed

to the stalk of the HA from an IV strain of the group 1 recognize a variety of strains and mediate neutralization of many virus strains within that group (Corti *et al.*, 2010). HA-specific antibodies can neutralize IV by interacting with the receptor-binding domain (RBD) or by blocking the conformational rearrangement associated with the membrane fusion (Rao *et al.*, 2010). Several studies have suggested the possibility of eliciting such antibodies through immunization; either against conserved regions of specific IV subtypes or through cross-reaction among subtypes (Du *et al.*, 2011; Roose *et al.*, 2009). Conserved synthetic peptides from the HA2 can provide protection against distinct IV subtypes in mice (Wang *et al.*, 2010a). Although HA1 is highly variable, it encodes specific and highly conserved information which may be involved in the recognition and targeting (RTD) of IV to their receptor as has been widely revealed by Informational Spectrum Method (ISM) (Veljkovic *et al.*, 2009b). Furthermore, HA1 is the main region for the induction of neutralizing antibodies (nAbs) against IV; therefore, becoming the ideal target to develop subunit influenza vaccines (Du *et al.*, 2011).

In fact, we have recently reported the potential use of conserved HA1 synthetic peptides in vaccine formulations that elicit specific-antibodies and cellular responses, as well as partial protection against the pH1N1 infection in pigs (Vergara-Alert *et al.*, 2012). In the latter case, synthetic peptides were designed using a bioinformatics approach (ISM) capable to identify functional conserved immunogenic domains, such as the VIN1 domain, with demonstrated vaccine potential (Veljkovic *et al.*, 2009a). Interestingly, this specific domain located within the site E in the N-terminus of HA1, below the globular head of HA1, is involved in the receptor binding (Rogers and D'Souza, 1989). In spite of its relative success, the VIN1-peptides (NF-34) showed concomitant detection of neutralizing antibody response and HI activity that did not totally correlate with protection in pigs (Vergara-Alert *et al.*, 2012). Thus, aiming to increase the vaccine efficacy, in the present study we describe the immunological responses and the protection afforded by a new vaccine formulation in a mouse model. We have *in silico* selected and modified NF34 and thus achieving the design of two new peptides, named NG34 and DC89.

Furthermore, we have analysed the immunological cross-reactivity between these peptides used for immunization and the HA1 protein of the virus strains used for challenge, represented by the characteristic peak frequencies in their consensus informational spectra. These findings suggested that informational similarity between our peptides is more important for the immunological cross-reactivity than their sequence similarity as reported (Krsmanovic *et al.*, 1998; Veljkovic *et al.*, 2003; Veljkovic *et al.*, 2004; Vergara-Alert *et al.*, 2012).

Our results demonstrate that the immunization with NG34+DC89 mix peptide elicits humoral and mucosal immunity that confers partial protection against pH1N1 and H7N1 IV in mice. We believe that our findings are a step forward to the search of a universal influenza vaccine.

1.3. MATERIALS AND METHODS

Bioinformatic analysis and peptides synthesis

The Informational spectrum method (ISM) is a virtual spectroscopy method for investigation of protein-protein interactions and structure-function analysis of proteins. Physical and mathematical basis of the ISM was described in details elsewhere (Cosic et al., 1986; Veljkovic et al., 2008).Briefly, protein sequences are transformed into signals by assignment of numerical values for each amino acid. These values correspond to electron-ion interaction potential (EIIP) (Cosic et al., 1986; Veljkovic et al., 2008; Cosic, 2012) corresponding with the electronic properties of amino acids which are essential parameters determining the properties of biological molecules. The result is a series of frequencies and their amplitudes (the informational spectrum, IS). The obtained frequencies

correspond to the distribution of structural motifs with defined physico-chemical characteristics determining the biological function of the sequence. When comparing proteins which share the same biological function, the ISM allows detection of code/frequency pairs in IS which are specific for their common biological properties (Veljkovic et al., 2008).This common characteristic of sequences is represented by peaks, a consensus informational spectrum (CIS) of proteins. Significance of information isdetermined by the signal-to-noise ratio (S/N), representing the ratio between the signal intensity at one particular IS frequency and main value of the whole spectrum. A higher S/N value at the characteristic frequency (F) in CS/CIS of two or more proteins suggests a higher specificity of cross-reactivity(Veljkovic et al., 2008; Veljkovic et al., 1985)(Veljkovic et al., 2009a; Veljkovic et al., 2009b). The ISM was successfully applied in structure-function analysis of HA1 protein sequences and de novo design of two biologically active peptides (NG34 and DC89).

On one hand, the modified VIN1-peptide (NF-34), referred as NG34, and derived from the IV strain A/Catalonia/63/2009 (H1N1) [GenBank: ACS36215]. NG34 was relatively conserved and mapped to the flanking region of the HA1 within the VIN1 domain (Vergara-Alert et al., 2012). On the other hand, DC89 peptide which contained the original NF-34 peptide fused to a highly conserved peptide sequence (DC55), derived from the HA1 of A/California/04/2009 (H1N1) IV strain [GenBank: ACS45035]. To analyse and align the amino acid sequences of HA proteins of pH1N1 and H7N1 IV available in GenBank we used Mega 6 Integrated software for Molecular Evolutionary Genetics. Sequences from the synthetic peptides are shown in Figure 1. The peptides were produced by GL Biochem (Shanghai) Ltd.

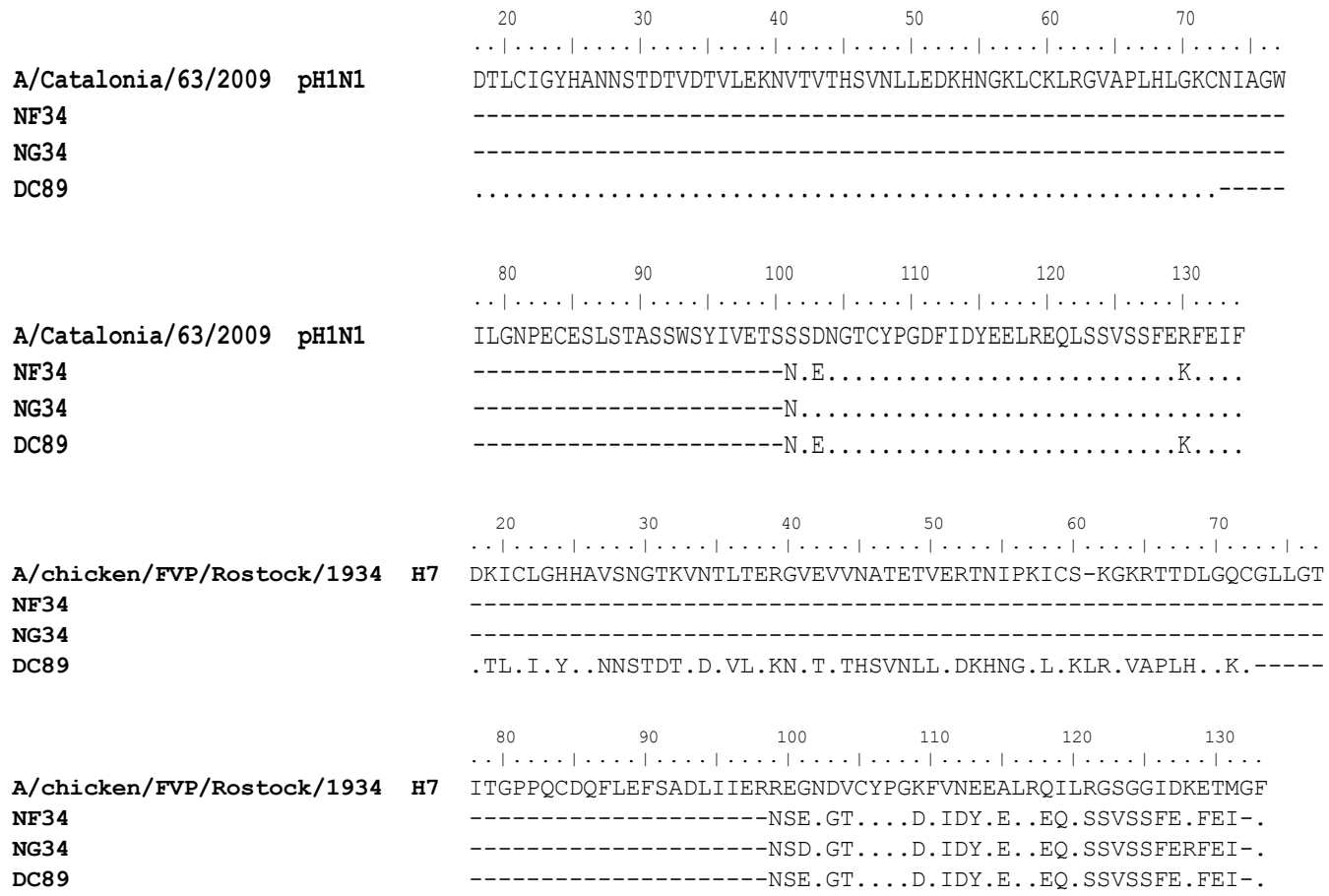

Figure 1.1. Amino acid sequences from the peptides used for immunization (NF-34, NG34 and DC89) compared to the homologous sequence of the HA1 of the IV (pH1N1 and H7N1 HPAIV) used for the challenge. The amino acid similarities between sequences from the HA1-peptides and the IV are represented in dots
.

Cell culture and viruses

Viruses used were the pandemic swine-origin A/Catalonia/63/2009 (H1N1) IV isolated in 2009 [GenBank accession number GQ464405-GQ464411 and GQ168897] (here after referred to as pH1N1) (Busquets *et al.*, 2010) and a reassortant virus carrying the NS-segment of A/Goose/Guangdong/1/96 (GD; H5N1) in the genetic background of the HPAIV A/FPV/Rostock/34 (here after referred to as H7N1) (Ma W, 2010; Wang *et al.*, 2010b). Viruses stocks were propagated in Madin-Darby Canine Kidney (MDCK; ATCC CCL-34) cells, cultured in Dulbecco's modified Eagle's medium (Life Technologies, S.A.) supplemented with 10% fetal calf serum (FCS) and antibiotics (100U/ml^{1}penicillin and 0.1mg/ml^{-1}streptomycin) at 37°C in a 5% CO_2 humidified atmosphere. Virus titers were determined in MDCK-cells and measured as tissue culture infectious doses 50% ($TCID_{50}$) following the Reed and Muench method (L. J. Reed and Muench, 1938; Villegas, 1998).

Animal experimental design

This study was conducted at the Biosafety Level 3 facilities of the *Centre de Recerca en Sanitat Animal* (CReSA-Barcelona). Animal procedures were approved by the Ethical and Animal Welfare Committee of *Universitat Autònoma de Barcelona* (Protocol #DMAiH-57567). Thirty-one mice were kept under BSL-3 containment conditions at CReSA. Animals were divided into two groups, and each group was further subdivided into three groups (Table 1.1). Animals were divided into two groups, and each group was further subdivided into three groups (Table *2*). Mice in group 1 (G1; n= 16) were either inoculated twice with NG34+DC89 (G1A; n=9) or with saline solution (G1B; n=4). Thirty days after last immunization both groups were challenged with 10^5 $TCID_{50}$ pH1N1 IV. Three animals were used as negative control. Mice in group 2 (G2; n= 15) were either immunized twice with NG34+DC89 (G2A; n=9) or with saline solution (G2B; n=3). Thirty days after last immunization both groups were challenged with 10^5 $TCID_{50}$ H7N1

IV. Three animals were used as negative control. All mice were monitored for clinical signs, and body weights and mortality rate were recorded. According to ethical procedures animals losing more than 25% of their initial weight were sacrificed with sodium pentobarbital and scored as dead. For immunological purposes, blood samples and bronchoalveolar lavages (BAL) were collected from each group. All samples were stored at -80ºC until tested.

Table 1.1. Experimental design

GROUP		Nº ANIMALS	IMMUNOGEN (CFA) DAY 0	IMMUNOGEN (IFA) DAY 15	INOCULUM DAY 45
G1	G1A	9	NG34+DC89	NG34+DC89	10^5 $TCID_{50}$ of pH1N1
	G1B	4	Saline solution	Saline solution	
	Negative control	3	-	-	-
G2	G2A	9	NG34+DC89	NG34+DC89	10^5 $TCID_{50}$ of H7N1
	G2B	3	Saline solution	Saline solution	
	Negative control	3		-	-

Abbreviations: CFA = complete Freund Adjuvant; IFA = incomplete Freund Adjuvant. Thirty-one mice were randomly distributed into two groups, and further subdivided into three groups each one. Animals from groups A (G1A and G2A) received the first NG34+DC89 immunization (day 0) and 2 weeks later (day 15) mice were boosted with a second dose. One month later, the animals were challenged with either pH1N1 (G1A) or H7N1 (G2A). Mice from groups B were used as positive controls for pH1N1 (G1B) and H7N1 (G2B) infections. Negative controls were used in both G1 and G2 groups.

IgG and IgA Antibody detection

An indirect ELISA was developed to measure the peptide-specific antibodies in serum and BAL samples from immunized mice following the protocol previously described by our group, with some modifications (Vergara-Alert *et al.*, 2012). Shortly, plates were coated with 5 µg/ml of each peptide, and incubated overnight at 4°C. After blocking with 3% bovine serum albumin (BSA)/PBS 1x for 1-h at 37ºC, mouse sera were added to the coated plate diluted at 1:100, followed by 2-h incubation at 37°C for the evaluation of IgG. Plates were then washed four times with PBS 1x/0.1% Tween20, and HRP-conjugated goat-anti-mouse IgG (Sigma-Aldrich) diluted 1:3,000 was added to the wells followed by 45 min

incubation at 37ºC. For IgA measurement, plates were incubated with BAL (direct sample), and then incubated with 1:1,000 HRP-conjugated goat-anti-mouse IgA (Sigma-Aldrich), followed by 45 min incubation at 37ºC. After washing the plates four times (PBS 1x/0.1% Tween20), 50 µl/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich) were added and allowed to develop for 8-10 min at RT. Optical density (OD) was measured at 450 nm.

Virus quantification by Real Time RT-PCR (RRT-PCR)

Viral RNA quantification using one step RRT-PCR was performed in lung tissue. 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), as previously reported (Busquets *et al.*, 2010; Spackman *et al.*, 2002) and amplification conditions as described by Busquets *et al.* 2010 (Busquets *et al.*, 2010) in Fast7500 equipment (Applied Biosystems) 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 2.68 log viral RNA copies/sample.

Seroneutralization (SN) assay

Serum samples were tested by SN using a rapid specific biotin-streptavidin conjugated system for detecting influenza antibodies following the protocol described by Sirskyj *et al.* 2010 with some modifications (Sirskyj *et al.*, 2010). Serum samples were diluted serially and incubated with 100 $TCID_{50}$/50 µL of the trypsin-treated pH1N1 virus (10 µg/ml; Sigma-Aldrich) or H7N1 IV for 2-h at 37ºC in a 5% CO_2 atmosphere. The

mixture was then added to the 96-well plates containing confluent MDCK-cells (5×10^4cells/well) and incubated overnight, followed by washings with PBS 1x and fixation with 80% acetone at 4ºC for 10 min. Cells were air-dried, washed 5 times with PBS 1x/0.05% Tween20 and incubated at RT for 1-h with 1:2,000 biotinylated influenza A anti-NP primary antibody (Millipore, CA). Plates were then washed five times with PBS 1x/0.05% Tween 20, and incubated at RT for 1-h with 1:10,000 HRP-conjugated streptavidin (Millipore, CA), followed by TMB substrate (Sigma-Aldrich). The reaction was stopped with 1N H_2SO_4, and plates were read at 450 nm.

Hemagglutination Inhibition (HI) assay

An HI assay was performed following the standard procedures (OIE) using chicken red blood cells (RBC) and 4 haemagglutination units of either pH1N1 IV or H7N1 IV. To avoid unspecific inhibitions, sera from individuals were treated prior to use. Briefly, one volume of serum samples was treated overnight at 37°C with four volumes of Receptor Destroying Enzyme (Sigma-Aldrich) solution (100 U/ml). Next day, serum samples were incubated for 30 min at 56°C 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 4°C. Known positive and negative sera were used as controls.

Histology examination

Lung tissue samples were collected and fixed by immersion in 10% neutral buffered formalin for 48 h, dehydrated through graded alcohols and embedded in paraffin. Tissue sections (4mm) were processed routinely for hematoxylin/eosin (H/E) staining. Each tissue was evaluated microscopically for the presence of inflammatory lesions in the lung.

Indirect Immunofluorescence (IF)

MDCK-cells (3×10^5cells/well) were infected with pH1N1 or H7N1 IV for 18h at a MOI of 0.1 following the protocol previously described by (Ballester *et al.*, 2011) with some modifications. The cells were blocked and incubated with the sera from the mice diluted 1:100 for 1h in the blocking solution at RT. After three washes, the pH1N1-infected cells were incubated at RT for 1h with mouse IgG: FITC-conjugated (1:300) (Jackson Immunoresearch Europe Ltd) and the H7N1-infected cells were incubated with goat-anti-mouse IgG: Cy3-conjugated (1:300) (Life Technologies) for 1-h at RT. Ananti-nucleoprotein (NP) monoclonal antibody (ATCC, HB-65H16L-10-4R5) (1:100) was used as control for the infection. Nuclei were counterstained with DAPI (1 µg/ml) and coverslips were mounted with Vectaschield. 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 Jv1.45l softwarehttp://rsb.info.nih.gov/ij).

Statistical analysis

The Mann–Whitney test was used to compare parameters among the different groups. This non-parametric analysis was chosen due to the number of animals used. For statistical analyses and graphics design, the GraphPad Software (San Diego-California, USA) was used. Differences were considered significant when $p< 0.05$.

1.3. RESULTS

In *silico* selection of immunogens

Based on ISM, a single 34-mer peptide (NF-34) from the H1N1 subtype was selected from within the conserved VIN1 region, and used in our previous study (Vergara-Alert et al., 2012). In order to elicit nAbs and to improve the protection conferred by NF-34, some modifications in the peptide synthesis were done. A new peptide (NG34) corresponding to positions 101–134 from the A/Catalonia/63/2009 (H1N1) virus was selected. ISM analyses revealed conserved information encoded between HA1-peptides and HA1 protein of the A/pH1N1 and A/H7N1, the consensus Informational spectra (CIS) of these sequences is characterized by one peak of the common frequency as shown in Figure 1.2

The modified NG-34 peptide according to ISM has higher S/N ratio at the characteristic F(0.257) (S/N=7.245compared to S/N of NF-34 (S/N=5.13), which suggest higher specificity of cross-reactivity between NG34 and HA1 of A/pH1N1 (Figure 1.2C) and A/H7N1 (Figure 2D)compared to NF-34 and HA1 of A/pH1N1 (Figure 2A)and A/H7N1 (Figure 1.2B). Aiming to increase the vaccine coverage, a new peptide (DC89) was designed and included in the vaccine formulation. DC89 comprises the original NF-34 sequence fused to DC55, a highly conserved peptide sequence corresponding to positions 18–72 from the A/California/04/2009 (H1N1) (Veljkovic et al., 2009a). The high specificity of cross-reactivity between NG34 and DC89, and the HA1 of the A/pH1N1 IV is represented by the characteristic frequencies F(0.257) and F(0.295) (Figure 1.2C and 1.2E), and might correlate with the induction of efficient nAbs. Further, we analysed the vaccine potential of the identical combination of peptides exposed to challenge with H7N1. The specificity of cross-reactivity between DC89 and H7N1 could be lower on the basis of inferior S/N as shown in Figure 2F. In contrast to expected high specificity of cross-reactivity

between NG34 and H7N1 (Figure 1.2D). With respect to the referred results we can anticipate that the antibodies elicited by this dual peptide immunogens could have lower specificity against H7N1, and therefore, could require a higher concentration to achieve neutralization.

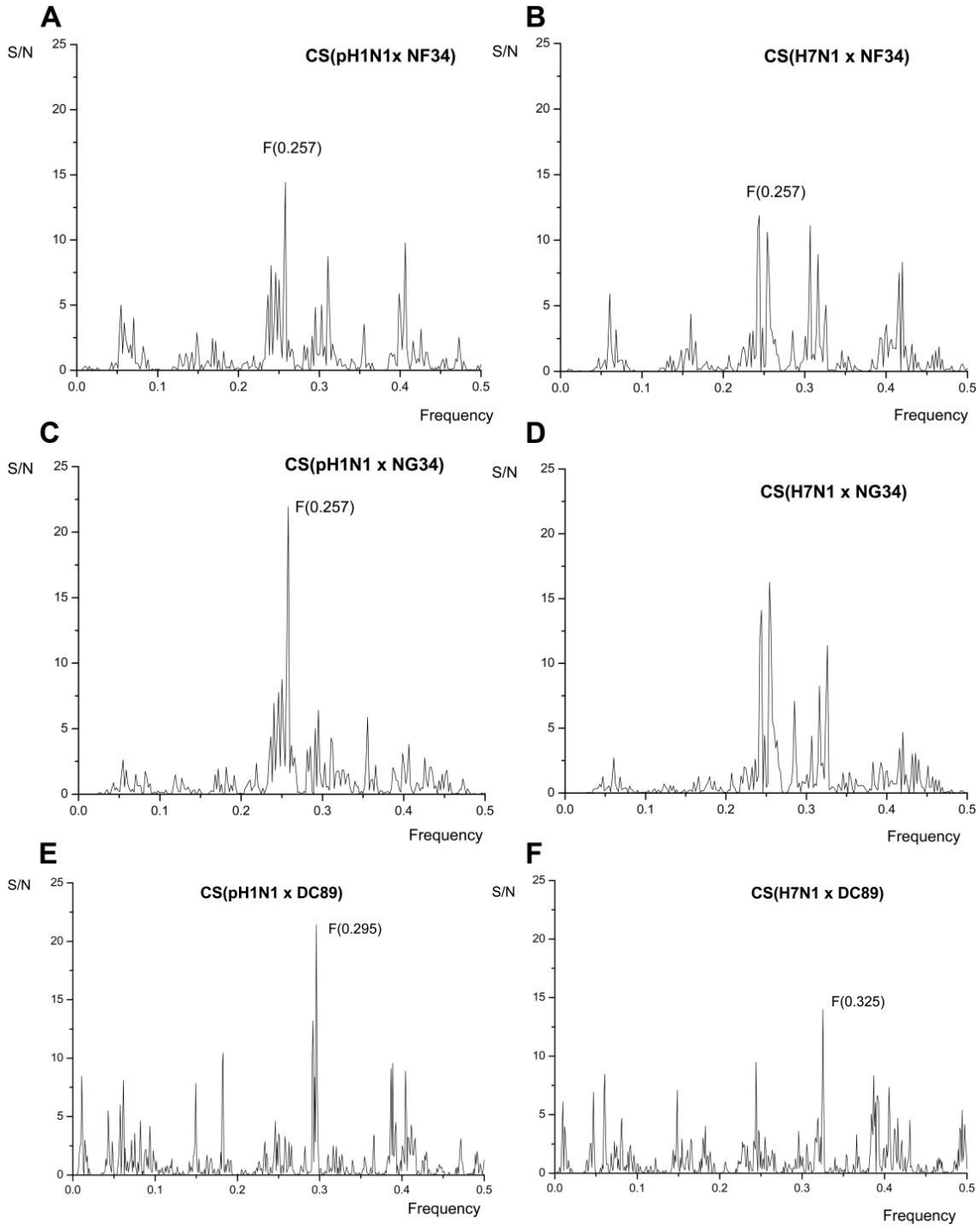

Figure 1.2 Consensus Informational spectra (CIS) for synthetic peptides and HA1 proteins of both virus strains used for challenge. (A) NF34 andHA1/pH1N1, (B) NF34 and HA1/H7N1, (C) NG34 and HA1/pH1N1, (D) NG34 and HA1/H7N1 (E) DC89 and HA1/pH1N1, (F) DC89 and HA1/H7N1. The dominant peak represents the long-range component of the protein-protein interaction between HA1 proteins of the virus strain and HA1-peptide.

Vaccination of mice with NG34+DC89 protects against homologous and heterologous IV challenges

With the aim to assess the protective potential of our newly designed peptide-vaccine, NG34+DC89-vaccinated and control mice were subjected to intranasal challenge with either 10^5 $TCID_{50}$ pH1N1 or $10^5 TCID_{50}$ H7N1 IV (Table 1). Immunized mice lost significantly less weight than control mice at 6 days post-infection (dpi). In addition, 7 out of 9 (77.77%) vaccinated-mice survived pH1N1 challenge, whereas 3 out of 4 from the control mice succumbed to infection by days 6, 7 and 9 (Figure 1.3A and 1.3C). Similarly, from the H7N1-control group all animals (3/3) developed progressive weight loss, mainly between days 6pi and 9pi (Figure 1.3D). The animals losing weight also showed other clinical signs, such as lethargy and ruffled hair. On the other hand, 6 out of 9 mice (66,66%) vaccinated with NG34+DC89 survived after H7N1 challenge while all mice from control group died (Figure 1.3B).

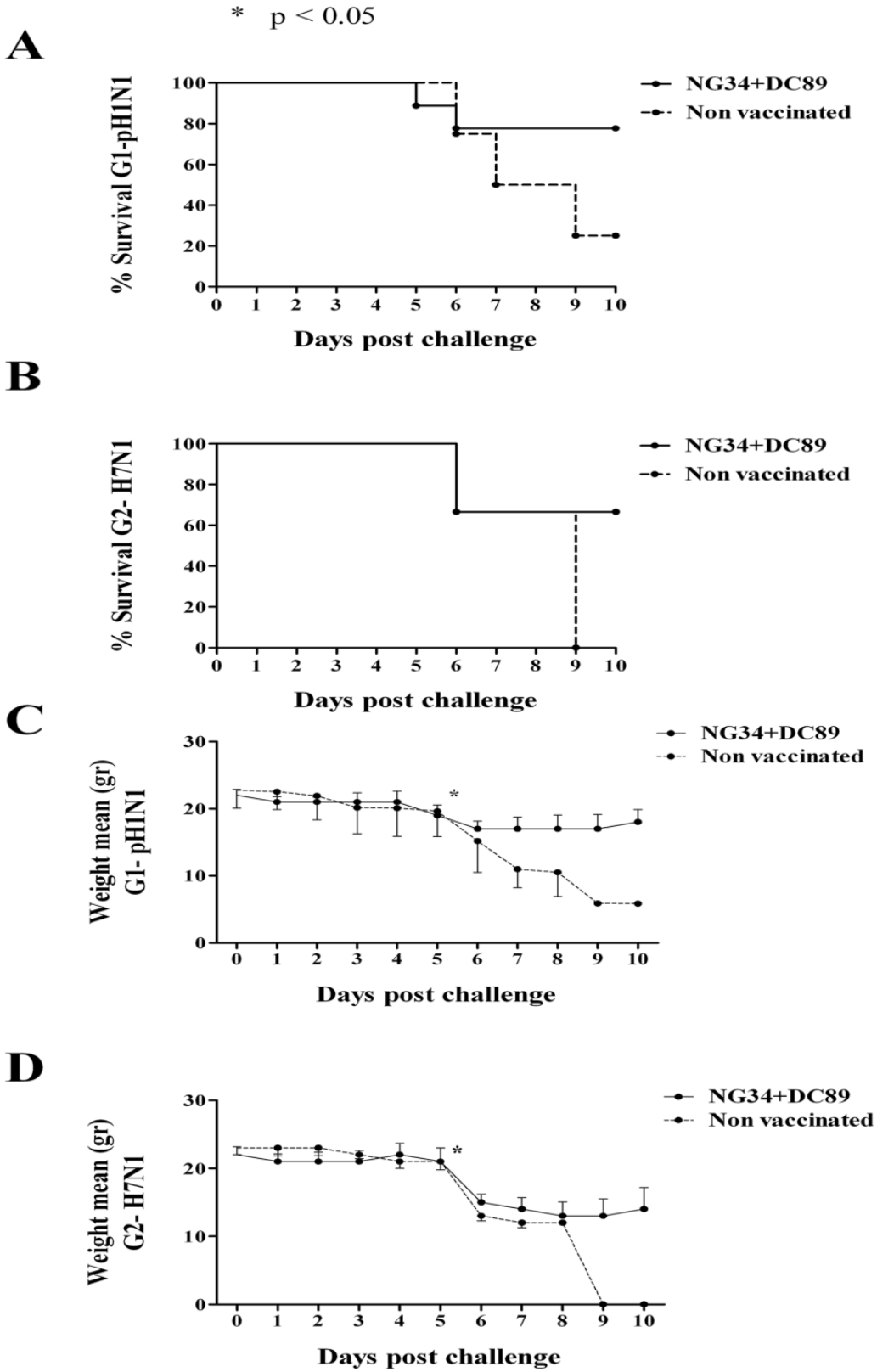

Figure 1.3. Vaccination of mice with NG34+DC89 confers protectionagainst homologous and heterologous IV challenges. Immunization with NG34+DC89 protects mice against challenge with pH1N1 IV (A and C) and H7N1 IV (B and D). Error bars represent standard deviations. (A) Asterisk indicates significant differences between groups ($p < 0.05$) Histopathological examination reveals that after NG34+DC89 vaccination, no influenza-like lesions were detected in lungs due to IV infection.

To examine whether NG34+DC89 could inhibit viral infection and reduce tissue damage in the lungs of challenged mice, viral load in the lungs was detected by RT-qPCR. Vaccinated-animals from G1A and G1B which survived after the infection did not show viral RNA in the lungs at 10 dpi. Only a survivor from the G1B resulted positive for pH1N1 RNA at 10 dpi (Figure 1.4A). Moreover, viral RNA copies in the lungs of vaccinated but non-protected mice from G1A and G1B were 2 logs lower than that in the non-vaccinated mice (G2A and G2B) (Figure 1.4A and 1.4B).

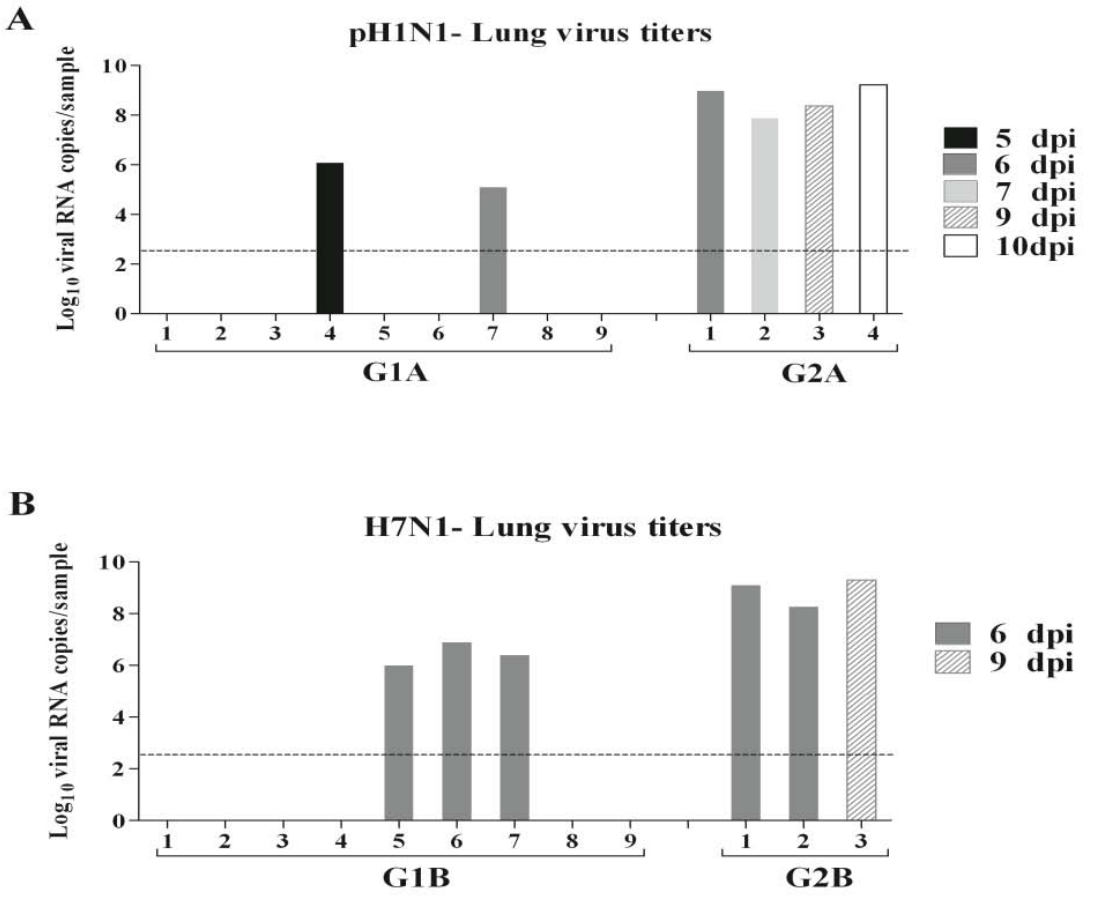

Figure 1.4. Viral RNA shedding detected by RRT-PCR in lung tissues from mice experimentally challenged with IV. Viral RNA detected by RT-qPCR expressed as log10 viral RNA copies per sample of the lung tissues. Viral RNA copies in the lungs of vaccinated non-protected mice from G1A and control group G1B after H1N1 challenge (A). Viral RNA copies in the lungs of vaccinated non-protected mice from G2A and control group G2B after H7N1 challenge (B). Bars represent only animals with detectable virus in lungs during the experiment. Limit of detection indicated by the dashed line (2.68 $\log_{10}$ viral RNA copies/sample).

Consistent with these results, H/E staining demonstrated that none of the survivor mice showed histopathological lesions after the infection with either pH1N1 or H7N1 IV (Figure 1.5A and 1.5B). Conversely, non-vaccinatedmice showed moderate lymphoplasmacytic interstitial inflammation at 7 days after pH1N1 challenge (Figure 1.5C). Histopathological examination of non-vaccinated mice revealed even more severe lesions at 6 days after H7N1 infection, showing bronchiolar epithelial cells hyperplasia, loss of cilia with exocitosis of lymphocytes, as well as lymphoplasmacytic peribronchial and interstitial inflammatory infiltrates. Hyperplasia of pneumocytes type II and an increased number of macrophages and sloughed epithelial cells in the alveolar lumens were also observed. Moreover, moderate multifocal peribronchiolar and perivascular lymphoid hyperplasia was also found (Figure 1.5D). As expected, non-vaccinated, non-infected animals did not show histopathologic lesions (Figure 1.5E and 1.5F).

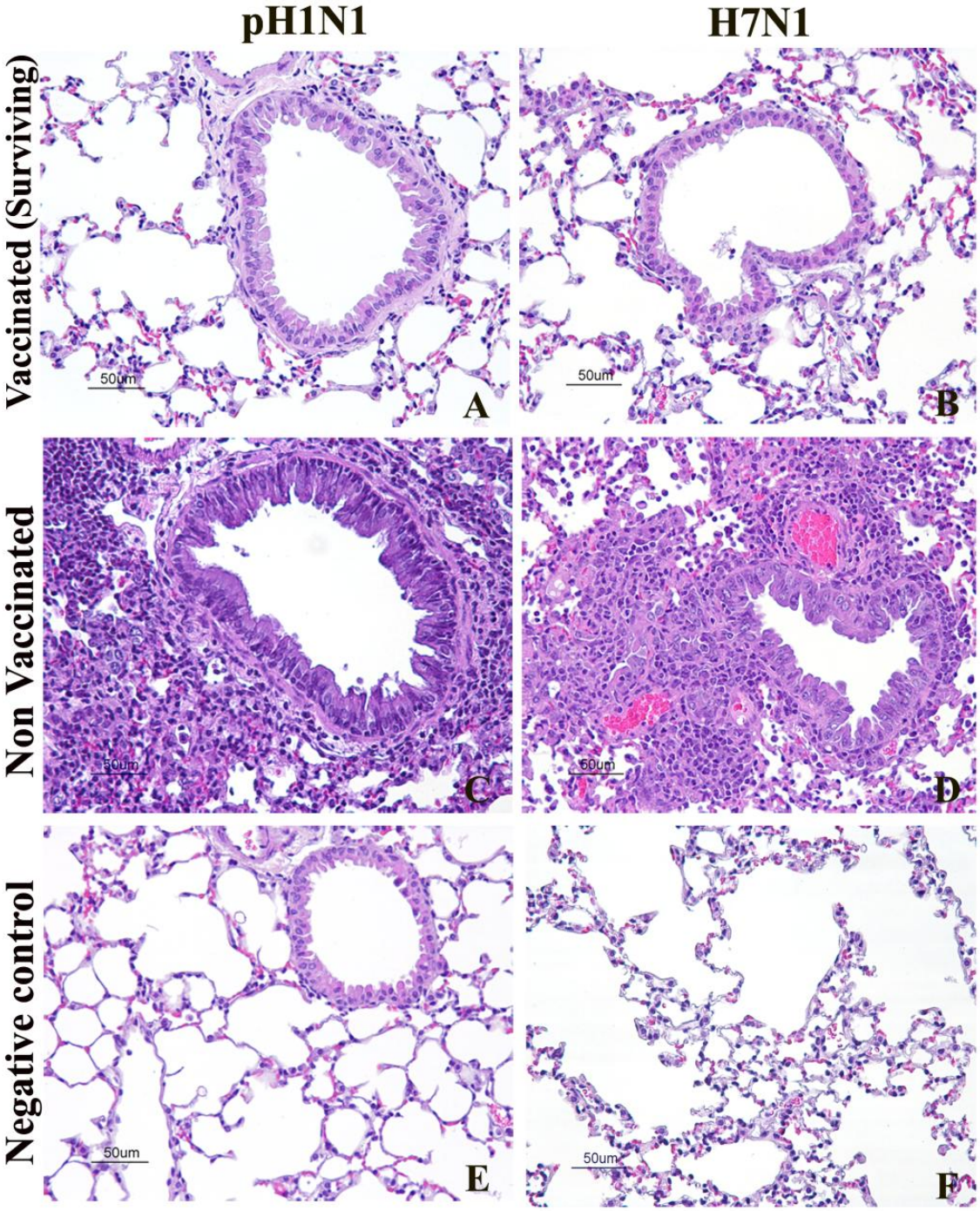

Figure 1.5. Histopathological examination reveals that after NG34+DC89 vaccination, no influenza-like lesions were detected in lungs due to IV infection. H/E staining of lungs at 10 dpi from surviving vaccinated mice: challenged with pH1N1 IV (A); and H7N1 IV (B); lungs at 6 dpi from control mice: challenged with pH1N1virus (C) and H7N1 IV (D); lung from two negative control mice before challenge (E and F)

NG34+DC89 induces peptide-specific neutralizing antibodies that recognize both pH1N1 and H7N1 IV

Aiming to correlate the protection afforded with the induction of specific immune responses in response to vaccination with NG34+DC89, an indirect ELISA was developed using the specific peptides as coating antigens. As expected, no detectable levels of peptide-specific antibodies were found in pre-challenge sera from control mice. In contrast, sera obtained from vaccinated mice from G1 and G2 groups before IV challenge showed NG34-specific IgGs (Figure 1.6A and 1.6B). Interestingly, at 10dpi all mice infected with pH1N1 (G1) showed lower level of specific-NG34 antibodies (Figure 1.6A). Similar results were found in two of the H7N1 challenged mice (G2) at 10dpi (Figure 1.6B). Immunization with NG34+DC89 also induced specific-antibodies against the DC55 peptide, which is included in the DC89 (Figure 1.6C and 1.6D), albeit always at lower levels when compared with NG34 peptide (Fig 1.6A and 1.6B)

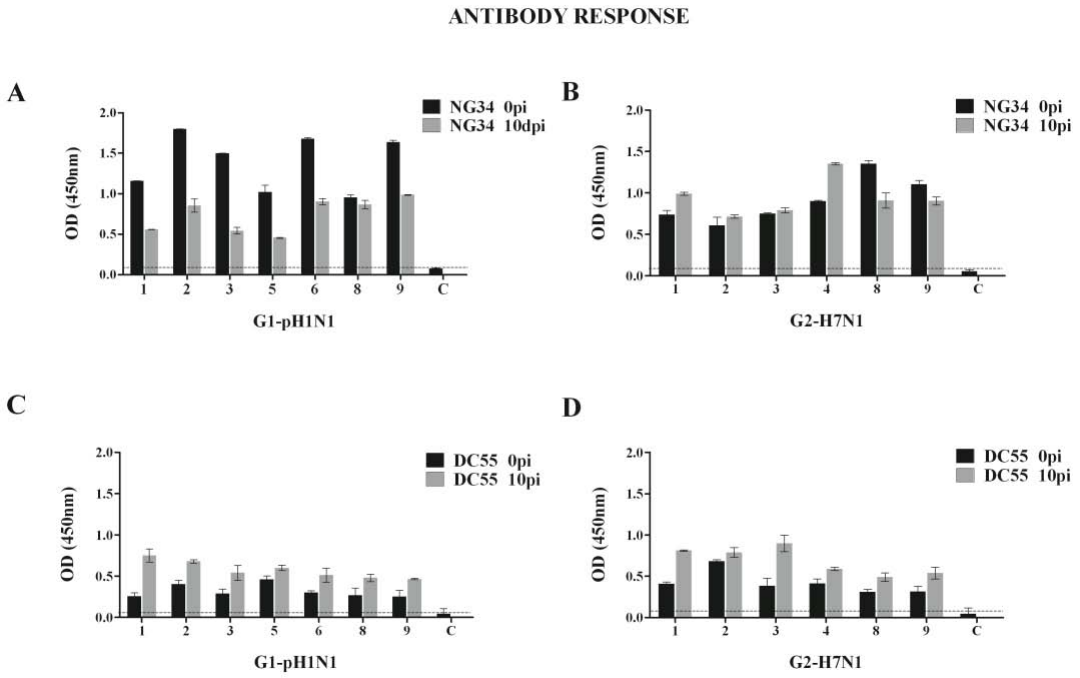

Figure 1.6. NG34+DC89 induce NG34 and DC55 peptide-specific antibodies. Sera from individual mice were obtained at day 0 and 10 days after the challenge with either pH1N1 or H7N1 viruses. Sera (1:100 dilution) from surviving mice of the G1, G2 groups and negative control were tested by ELISA using either the NG34-peptide ELISA (A and B) or a DC55-peptide ELISA (C and D)

In contrast with the VIN1-peptide used in our previous study (Vergara-Alert et al., 2012), immunization with the newly designed formulation, NG34+DC89, induced nAbs. Each individual serum sample was tested against both IV strains used for the challenge. In G1A, neutralizing antibody titres against pH1N1 virus ranged from >40 (cut-off) to 1:160-5120, and HI titres ranged between 1:16-:512. Two out of nine (22%) sera were found negative (HI <40) against pH1N1 (Figure 6A and 6B). On the other hand, neutralizing antibody titres against H7N1 virus ranged from >40 to 1:40-160, and 4/9 (44%) sera were found to be negative against H7N1. HI titres ranged from 1:8-128 and 2/9 (22%) sera were found <40 against H7N1 (Figure 1.7A and 1.7B). In G2A, neutralizing antibody titres against pH1N1 virus ranged from <40 to 1:80-2560 and HI titres ranged from 1:32-256 and 2/9 (22%) sera were found <40 against pH1N1 (Figure 1.7C). While, neutralizing antibody titres against H7N1 virus ranged from >40 to 160 and 4/9 (44%) sera were found to be negative against H7N1and HI titres ranged from 1: 8-128 and 3/9 (27%)sera were found <40 against H7N1 (Figure 1.7C). As expected, no detectable neutralizing antibody titres were found in sera from control mice (G1B and G2B). Each individual serum sample was tested against both challenge strains (expressed as medians and reported on box-and Whisker plots in Figure 1.7) were also analysed using nonparametric Mann-Whitney test revealing statistical differences between challenge strains pH1N1 and H7N1 (P<0,0001) in G1A. In addition, statistically significant differences were found in G2A between the two challenge strains (P< 0,001).

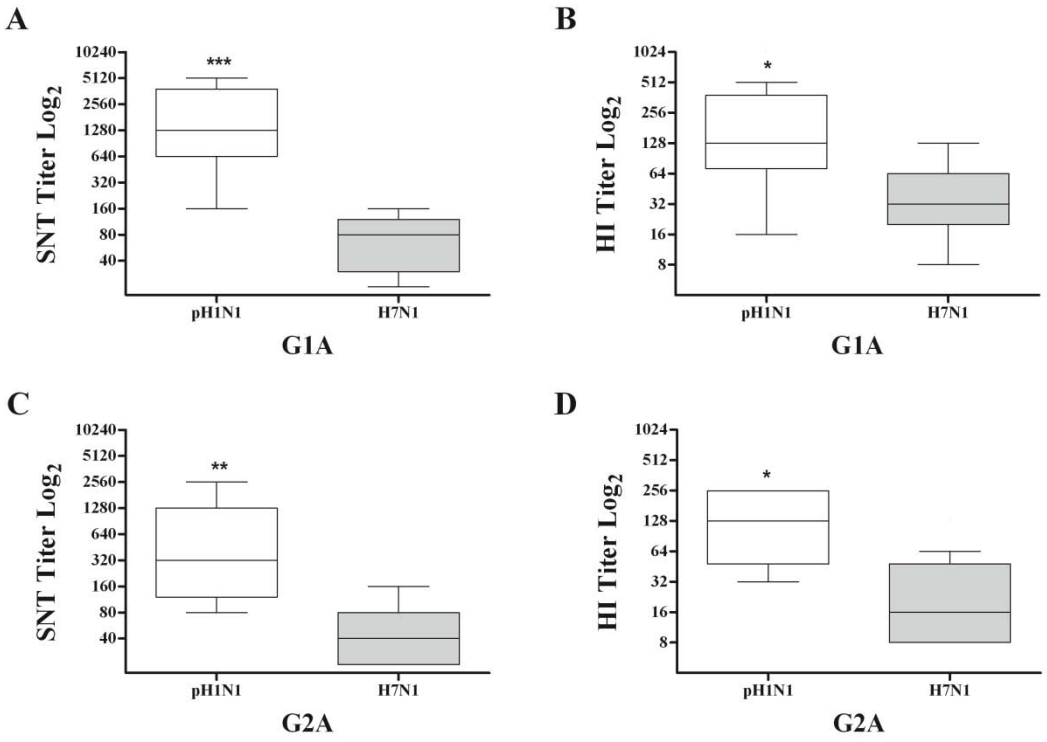

Figure 1.7. Immunization with NG34+DC89 induce neutralizing antibodies and hemagglutinin inhibition titres against challenge virus. SNT and HI titres obtained with sera of mice from G1A before challenge (A and B). SNT and HI titres obtained with sera of mice from G2A before challenge(C and D). Average values are shown as lines. Significant differences *($p<0.05$), ** ($p<0.001$), *** ($p<0.0001$).

The neutralizing and protective potential of these antibodies correlated with their potential to recognize both the pH1N1 and H7N1 viruses in vitro. Thus, sera from NG34+DC89-mice were capable to specifically recognize pH1N1 and H7N1 infected-MDCK cells as shown by IF (Figure 8A and 8B). As expected, sera from negative control animals showed no reaction (Figure 1.8E). Importantly, every single cell infected by pH1N1 and H7N1 viruses was also recognized by the specific-NP monoclonal antibody (Figure 1.8C and 1.8D) confirming the specificity of the reactions.

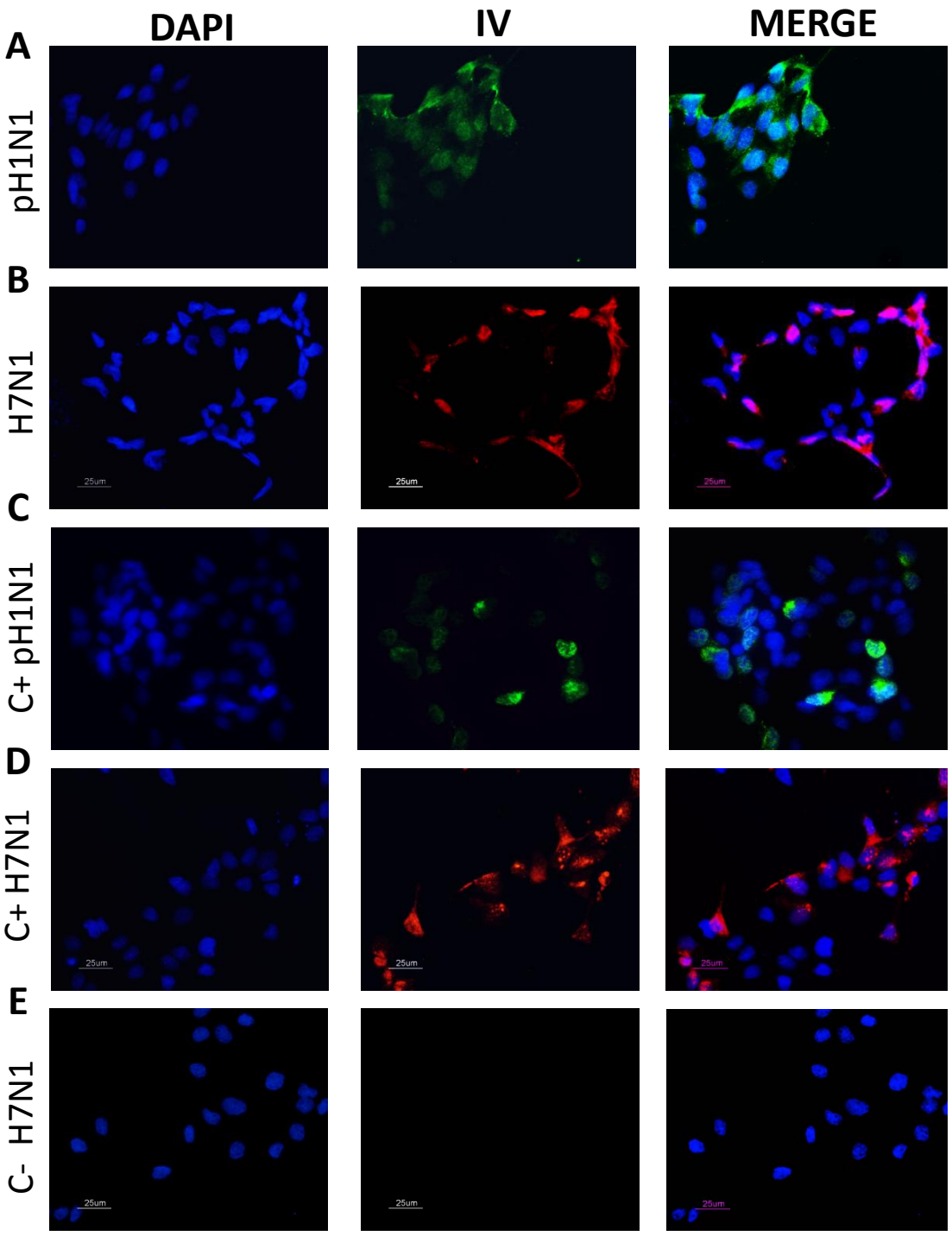

Figure 1.8. NG34+DC89 sera recognize pH1N1 and H7N1 viruses *in vitro*. Indirect immunofluorescence of pH1N1 (A and C) and H7N1 (B, D and E) infected-MDCK cells at 16 dpi using as primary antibody: serum from a representative mouse vaccinated with NG34+DC89 (A and B), serum from a control mouse (E). Anti-NP monoclonal antibodies against the NP protein (I and J), was used as control for the assay.

Mucosal antibodies in bronchoalveolar lavage (BAL)

The presence of specific IgA antibodies in BAL were detected in 6 out of 13 mice which survived, and the last immunization significantly increased the level of antigen-specific antibodies, at least as shown by control animals (Figure 1.9A and 1.9B). Almost all surviving mice displayed antibodies as shown in G1 (Figure 1.9A) and compared to G2 (Figure 1.9B). Again, the induction of specific IgAs against the NG34 peptide was higher optical density (0.8) than for the DC55 peptide**.**

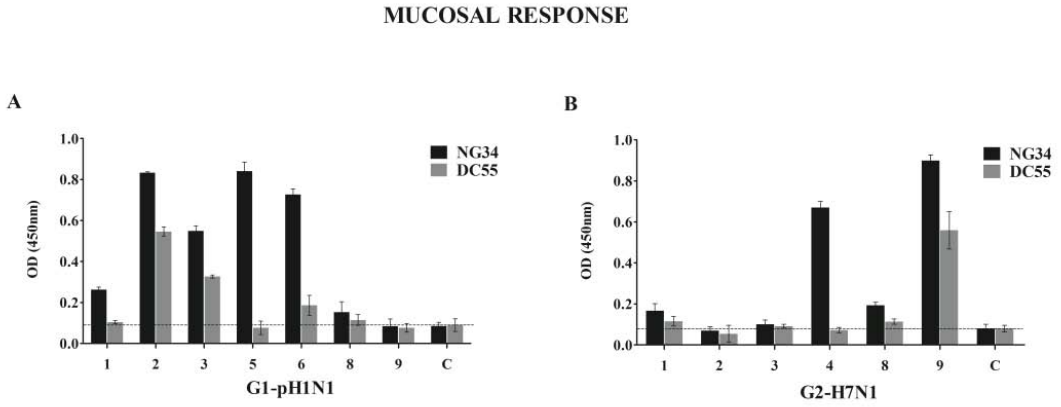

Figure 1.9.Mucosal response from vaccinated mice which survived after IV infections. Inductions of peptide-specific IgA in BAL from G1 (A) and G2(B). Error bars represent standard deviations. Samples were analysed in duplicate**.**

1.4. DISCUSSION

Currently, most efforts have been focused on the development of broadly protective universal IV vaccines that can provide a neutralizing immune response against drifted seasonal IV strains, and also against potential pandemic viruses (Krammer and Palese, 2015). Recent studies report that antibodies with broad neutralizing activity against multiple IV strains or subtypes bind to the stalk of the viral HA, suggesting that a vaccine based on this region could elicit a broadly protective immune response (Corti et al., 2011; Ekiert et al., 2009; Pica et al., 2012; Steel et

al., 2010; Sui et al., 2009; Vareckova et al., 2003; Wang et al., 2010a). However, here we describe NG34+DC89 synthetic peptides derived from the globular domain HA1 as multivalent vaccine candidates against IV. Although, some studies indicated that antibodies recognizing HA1 do not cross-react with the HA of other subtypes (Gocník et al., 2008). We have shown that NG34+DC89 construct is able to elicit cross-reactive antibodies against H1- and H7- IV subtypes in mice.

The fact that NG34+DC89 peptides are based on the sequence from an H1 IV subtype could lead to elicit an immune response broadly reactive with other HA subtypes such as H7, as revealed on analysis by (Veljkovic et al., 2009b). This information is also represented by the IS frequency component (Figure 1.2). Therefore, we can speculate that the NG34-elicited antibodies might recognize both pH1N1 and H7N1, as previously shown ISM (Vergara-Alert et al., 2012). We assume that the highly conserved domain from HA1 plays an important role in A/H1N1 and A/H7N1 receptor interaction and might result in virus neutralization. Therefore, HA1-peptides might confer protection against influenza infection. Although, DC89 elicited specific antibody response in vaccinated animals, this response was relatively low and it might be attributed to DC55, as shown in Figures 6C and 6D. DC89 peptide also elicited low cross-reactivity with H7, as detected by S/N (Figure 1.2F). Therefore, we assume that due to low specificity in IS frequency component between H7N1 and DC89, antibodies elicited by DC89 are not so specific and probably in order to achieve neutralization it would be necessary to increase the antibodies concentration. These findings correlated with SNT and HI assay, where it was shown that the H1-antibody responses were higher compared to H7-antibodies in vaccinated animals (Figure 1.7). Animals that did not show antibodies titres by HI and SNT tests were not protected to IV infection. Interestingly, the specific NG34-antibody responses were boosted after H7N1 challenge but the titres decreased after pH1N1 challenge. These results might correlate with the efficacy by which the antibodies induced are immediately sequestered after H1N1 in vivo challenge, sharing almost identical sequences. Sequence divergence between the vaccine peptides and H7N1 could be enough to promote the antibody boosting while avoiding the formation of antibody

antigen solid complexes (Depner et al., 1995; Gocník et al., 2008). The neutralizing and virus titration data seems to confirm this hypothesis.

A critical factor for success of the immunization is the route of administration (Kaufmann, 2006). Currently, inactivated influenza vaccines are administered by intramuscular route which induce a strong systemic humoral responses but not mucosal immune response that is important in the first line of defense against influenza (Clements et al., 1986; Renegar et al., 2004; Wagner et al., 1987; Wang et al., 2004). In contrast, live influenza vaccines delivered by intranasal route stimulate the production of both nasal mucosal IgA and IgG Abs and plasma IgG. However, other studies only reported mucosal IgA antibodies upon intranasal vaccination (Liu et al., 2013; Van Riet et al., 2012). Several studies support the importance of mucosal IgA responses in protection against multiple IV strains and disease in human and mouse (Arulanandam et al., 2001; Benton et al., 2001; Zhang et al., 2002). A recent work suggests that IgA is more important than IgG in the protection of the upper respiratory tract, whereas IgG is more important than IgA in the protection of the lungs (Renegar et al., 2004). Here, we demonstrate that a peptide vaccine administered by intraperitoneal route is able to elicit IgA and IgG which confer protection against IV infection. The protection afforded correlate with the presence of neutralizing antibodies in serum and elevated levels of NG34-specific antibody in BAL (Figure 9). Moreover, an absence of lesions and viral RNA in lungs was also reported (Figure 1.4A and 1.4B). The intraperitoneal route contributed to deliver the NG34+DC89 peptides directly into the peritoneal cavity, which is the anatomic site of B-cells entry which are activated in peripheral lymph nodes. This process could enhance the efficient induction of antibodies of the IgG and IgA isotype (Berberich et al., 2007; Hardy and Hayakawa, 2001; Ogra et al., 2001).

Our results herein with homologous and heterologous viral challenge indicate that NG34+DC89 might be a potential candidate that could further be optimized or adapted to desired vaccine preparation or immunity (cellular or humoral) and improve in other species, thus obtaining a stronger immunity and higher antibody titers (before infection), that could

confer better protection against all types of influenza viruses, including those emergent pandemic strains (Du et al., 2010; Watanabe et al., 2013; Watanabe et al., 2012).

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CHAPTER 2

Study II: Neutralizing antibodies against heterosubtypic influenza strains after HA1 peptides immunization in pigs

2.1. INTRODUCTION

Seasonal outbreaks by influenza viruses have constantly challenged the animal and human health during the last two centuries. Such scenario has been recently illustrated by the emerging 2009 pandemic H1N1 (pH1N1) virus infection. According to the World Health Organization (WHO), this pandemics lead to a minimum of 18,449 human deaths. Influenza infections in pigs also imply an important public health risk. Pigs have been suggested as 'mixing vessels' in genetic reassortment events, since they are susceptible to both human and avian influenza viruses (Kundin, 1970; Kida et al., 1994; Brown et al., 1998; Alexander and Brown, 2000; Trebbien et al., 2011). Human influenza virus vaccines are regularly updated with contemporary strains (Pedersen et al., 2014). Therefore, the dynamics of frequent and unpredictable changes of the surface glycoproteins (hemagglutinin and neuraminidase) continues to complicate control of influenza A viruses (IAV) (Taubenberger and Kash, 2010). These antigenic variations occur in a way that antibodies, which are effective against a particular strain of the virus, may not be able to neutralize viruses that subsequently circulates in the same population. Unfortunately, mismatch between formulated vaccines and the prevalent strains indeed (Taubenberger and Kash, 2010) happens and causes severe illness and economic burden (Carrat and Flahault, 2007).

The use of inactivated seasonal vaccines is a common practice in pigs and can effectively protect against homologous viruses (Vincent et al., 2008). However, their ability to protect against heterologous challenge viruses has been erratic (Macklin et al., 1998; Vincent et al., 2008) and several studies suggested an association between mismatched inactivated vaccines and vaccine-associated enhanced respiratory disease (VAERD) (Vincent et al., 2008; Gauger et al., 2011, 2012). For example, in a heterologous vaccination-challenge experiment in this animal model, H1N2 vaccinated pigs developed severe clinical disease, enhanced lung consolidation and potentiated microscopic lesions compared to non-vaccinated controls, upon pH1N1 infection (Gauger et al., 2011). It is of

note that the vaccine did not prevent infection, and neutralizing antibodies in serum did not cross-react with the challenge virus. Though the validity of the association is clear, the exact mechanism remains to be elucidated. Also, it is highly relevant to observe that the VAERD effect appears to be specifically connected to some—but not all (Van Reeth et al., 2001; Kyriakis et al., 2010) whole virus inactivated vaccine strains, and it is not triggered in heterologous challenge experiments following immunization with a live-attenuated influenza (Vincent et al., 2012), a non-replicating adenovirus five vector-based- (Braucher et al., 2012) or a split pandemic H5N1 vaccine.

To date, several next generation vaccines for swine influenza viruses (SIV) have been developed and tested in pigs, but none of them have reached the market (Reeth and Ma, 2013). Hence, novel approaches are being considered as the design of highly conserved epitopes and to make it immunogenic sufficient enough for inducing protective immunity (Lee et al., 2014; Soema et al., 2015). Further, the conserved epitopes can be presented in a variety of platforms including soluble proteins with adjuvant, subunit or domain epitopes fused to a carrier protein, virus-like particles (VLPs) and nanoparticles (Ma et al., 2013; Petukhova et al., 2013; Wang et al., 2014a; b). Currently, M2 and HA proteins has been reported as targets to derive conserved epitopes (immunogens) to include in vaccine formulation (Hikono et al., 2012; Vergara-Alert et al., 2012; Oxford, 2013; Pica and Palese, 2013; Hefferon, 2014). M2e is conserved among influenza A viruses, and has minor changes in the membrane-proximal region (Ito et al., 1991; Mozdzanowska et al., 2003; Schotsaert et al., 2009; Wang et al., 2014b). However, M2e-specific immune responses elicited by seasonal vaccines or viral infection are low due to its low immunogenicity resulting from its relatively low epitope density and smaller size compared to the other two surface antigens HA and neuraminidase (NA), which may shield M2e from the host immune system. Most M2e-based vaccine binds non-neutralizing antibodies that provide some, albeit limited, protection (Zhou et al., 2010). Although epidemiological evidence supports the development of $CD8^+$ T-cell vaccines to influenza A viruses (Epstein, 2006), in animal models, the effectiveness of $CD8^+$ T cells in protecting against influenza A

viruses remains controversial; while some investigators reported induction of protection (Ulmer et al., 1998), others reported lack of efficacy (Lawson et al., 1994) or even exacerbation of disease following viral challenge (Heinen et al., 2002). This also indicates that other conserved epitopes that can induce neutralizing antibody responses should be combined with M2e to develop a fully protective universal influenza vaccine. Heinen et al. 2002, have demonstrated in pigs, the antibody response against M2e, and the M2e+NP DNA vaccine additionally induced an influenza virus-specific lymphoproliferation response, but no protection after challenge with a swine influenza virus (H1N1)(Heinen et al., 2002). Other authors have reported similar poor protection against nasal virus shedding in pigs vaccinated with a recombinant M2 protein vaccine (Kitikoon et al., 2009).

HA, on other hand, is the major influenza antigen inducing neutralizing antibody responses during vaccination and viral infection (Zhang et al., 2014). Loeffen et al. 2011 have demonstrated a vaccination with a soluble recombinant HA trimer protects pigs against a challenge with pH1N1 virus and developed neutralizing antibodies against the homologous virus, which were cross-reactive with a European avian-like H1N1 SIV, but not with H1N2 (Loeffen et al., 2011). Such HA trimers are supposed to be better vaccine candidates than HA monomers, because they resemble the natural HA more closely and thus induce higher levels of neutralizing antibodies (Wei et al. 2008). Other authors have reported protection of weaned pigs by vaccination with human adenovirus 5 recombinant viruses expressing the HA and the nucleoprotein (NP) of H3N2 SIV. These pigs showed complete protection with (HA+NP) and nearly complete protection (HA only) against nasal shedding and lung lesions (Wesley et al., 2004). Based on this information some authors have described the use of conserved peptides from HA2 in protection against influenza viruses of the structurally divergent subtypes H3N2, H1N1, and H5N1 in mice (Wang et al., 2010). Vergara-Alert et al., 2010 has reported that vaccinated pigs with the HA subunit 1 produce broad humoral and T-Cell responses against homologous virus. In addition, the induced antibodies were able to recognize in vitro heterologous influenza viruses (Vergara-Alert et al., 2012).

We used Informational Spectrum Method (ISM) bioinformatics platform (Veljkovic et al., 2008) to select immunogenic HA1 peptides from H1 influenza virus and tested their potential to induce protective immune response in conventional pigs against influenza viruses. Our results indicate that the peptides selected in this work could be potential immunogenic targets and may be useful in the development of new vaccine formulations.

2.2. MATERIALS AND METHODS

Cell culture and viruses

For the present study, five strains of influenza virus were used, this includes; two avian strains: LPAI virus H5N2 subtype (A/Anasplatyrhynchos/2420/2010) and HPAI virus H7N1 subtype isolate (A/chicken/FPV/Rostock/1934) [GenBank AAA43150-AAA43257-AAA64422], two swine strains; 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] andone human strain; H1N1 subtype (A/Catalonia/63/2009) (hereafter referred to as pH1N1) [GenBank GQ464405-GQ464411 and GQ168897]. The H5N2 LPAIV strain was obtained from the ongoing surveillance program carried out in Catalonia, northeastern Spain. Stocks of avianinfluenza viruses were produced in 9-day-old embryonated specific pathogen free (SPF) chicken eggs. The allantoic fluid was harvested at 48 hours post inoculation, aliquoted and stored at -80°C until use. Virus was diluted tenfold in phosphate buffer saline (PBS) for titration in 9-day-old embryonated chicken eggs. The 50% egg lethal dose (ELD) for H7N1 subtype, and the 50% egg infective dose (EID), was determined using the Reed and Muench method (L. J. Reed and Muench, 1938; Villegas, 1998). pH1N1and stocks of swine influenza viruses were propagated at 37.5°C in the allantoic cavities of 11 day-old embryonated chicken eggs originating

from a commercial specific-pathogen-free (SPF) flock. The infectious virus titre was determined in Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) cells and measured as tissue culture infectious dose 50% (TCID50) by the Reed and Muench method (L. J. Reed and Muench, 1938; Villegas, 1998)

Bioinformatic analysis and peptides synthesis

Based on ISM predictions (Veljkovic et al., 2009a; Veljkovic et al., 2009b), four different peptides were designed and used to immunize conventional pigs. The selected peptides were conserved and mapped to the flanking region of the HA1 protein within the VIN1 or VIN2 domain. VIN1 is located within the site E between residues 42 and 75, one of the five major antigenic domains of the HA molecule (Veljkovic et al., 2009b). VIN2 is located in the C-terminus of the protein which encompasses residues 286–326 of the HA molecule (Veljkovic et al., 2009a). NG34-peptide was in VIN1 and SS35-peptide was in VIN2. Other two peptides DC55 and RA22 were randomly selected within HA1 protein based on ISM in order to compare immunogenicity. These peptides (NG34, DC55, RA22 and SS35) were derived from the HA1 protein of the human strains. A first peptide NG34 was derived from A/Catalonia/63/2009 (H1N1) strain [GenBank: ACS36215]. Three peptides (DC55, RA22 and SS35) from A/California/04/2009 (H1N1) IV strain [GenBank: ACS45035]. All peptides were chemically synthesized (GL Biochem Ltd, Shanghai). To analyse and align the amino acid sequences of four HA1-peptides synthesized and HA proteins of influenza A viruses used in this study, a BioEdit Sequence Alignment Editor was employed. All these strain sequences are available in GenBank.

Influenza Nucleoprotein (NP)-specific ELISA

A competitive enzyme-linked immunosorbent assay (C-ELISA) was carried out to examine the presence of specific antibodies against influenza NP

using a commercially available C-ELISA kit (ID-VET, Montpellier, France) as described by (Zhou et al., 1998).

Experimental design

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-5796). Fourteen 7week-oldconventional pigs (Landrace X Pietrain) were housed in the experimental farm facilities of the *Universitat Autònoma de Barcelona (UAB)* during forty-seven days; animals received food and water *ad libitum*. All animals were negative for serum specific antibodies against influenza NP as determined by ELISA test. The pigs were divided into seven groups (2 animals each group, table 1), and vaccinated three times with either single peptide or combinations of 2-3 peptides. Final concentration of peptides used for all vaccinations was constant (15 µg /pig). First vaccination was administered with complete Freund's adjuvant followed by incomplete Freund's adjuvant (second vaccination 19 days later) and without adjuvant (last vaccination, on day 40), by intramuscular (i.m) route (Table 2.1). Control animals (2 pigs) received saline and were kept in similar conditions. Sera samples were taken before each vaccination on days 0, 19 and 40. In addition, nasal swabs and sera sample were also collected at sacrifice on day 47. Nasal swabs were placed in 1 mL of PBS and frozen at -80ºC until use. Sera and nasal swabs were used to detect specific antibody responses, respectively. All pigs were euthanized with an intravenous overdose of sodium pentobarbital at 47 days.

Table 2.1. Experimental design.

GROUP	Animals	1st VACCINATION Complete Freund Adjuvant 0 day	2nd VACCINATION Incomplete Freund Adjuvant 19 day	3rd VACCINATION No Adjuvant 40 day	SACRIFICE Sodium pentobarbital 47 day
G1	1-2	Saline solution	Saline solution	Saline solution	Sodiumpentobarbital
G2	3-4	NG34	NG34	NG34	Sodiumpentobarbital
G3	5-6	DC55+RA22	DC55+RA22	DC55+RA22	Sodiumpentobarbital
G4	7-8	SS35	SS35	SS35	Sodiumpentobarbital
G5	9-10	NG34+DC55	NG34+DC55	NG34+DC55	Sodiumpentobarbital
G6	11-12	NG34+SS35	NG34+SS35	NG34+SS35	Sodiumpentobarbital
G7	13-14	NG34+DC55+SS35	NG34+DC55+SS35	NG34+DC55+SS35	Sodiumpentobarbital

IgG and IgA Antibody detection

An indirect ELISA was developed to measure the peptide-specific antibodies in serum samples from vaccinated pigs following the protocol previously described by our group (Vergara-Alert et al., 2012), with some modifications. Briefly, 96 well plates (Costar Corning Inc.) were coated with 5µg/ml of each peptide in coating buffer (sodium carbonate-bicarbonate 0.05M) and incubated overnight at 4°C. After blocking with 3% bovine serum albumin (BSA) in PBS-Tween20 (PBS-Tw) for 1h at 37°C, plates were incubated for 2h at 37°C with pig sera at 1:100 dilution. After washing three times with PBS-Tw, anti-pig IgG (whole molecule)-Peroxidase (Sigma) diluted 1:20,000 was added to wells followed by 45 min incubation at 37°C. Following another round of washing PBS-Tw, 50µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution were added to the wells and allowed to develop for 5–10 min at room temperature (RT), protected from the light. Finally, the reaction was stopped with 50µl/well of 1M sulphuric acid and measured by optical absorbance at 450 nm. For the detection of IgA in swabs undiluted material was used and goat anti-pig IgA peroxidase

conjugate (AbD Serotec®) diluted 1:10,000 was applied as secondary antibody in the ELISA.

Seroneutralization (SN) assay

Serum samples were heated to 56°C for 30 min to inactivate complement and other unspecific inhibitors. Titres were reported as the reciprocal of the highest dilution of serum that completely neutralized viral growth. All sera were titrated in duplicates until a dilution up to 1:2,560 in 96-well, flat-bottomed plates (Costar Corning Inc.) in a final volume of 50µl. After dilution, 100 $TCID_{50}$/50µL of the trypsin treated (10µg/ml; Sigma-Aldrich) pdmH1N1 virus or H7N1 were added to each well and incubated for 2h at 37°C in a 5% CO_2 atmosphere. The virus and serum mixtures were then added to 96-well plates containing confluent MDCK cells (5×10^4 cells/well) and incubated for 7 days. The antibody titre was calculated by cytopathic effect.

Hemagglutination inhibition (HI) assay

The HI test was used to measure antibody titres from serum samples. The HI test was performed following the standard procedures (World Organisation for Animal Health, 2010) using chicken red blood cells (RBC) and 4 hemagglutination units of either pH1N1, H7N1 HPAIV, H5N2 LPAIV, SwH1N1 and SwH3N2 influenza viruses. To avoid unspecific inhibitions, sera from individuals were treated prior to use. Briefly, one volume of serum samples was treated overnight at 37°C with four volumes of Receptor Destroying Enzyme (Sigma) solution (100 U/ml). Next day, serum samples were incubated for 30 min at 56°C after the addition of five volumes 1.5% sodium citrate. Finally, one volume of a 50% suspension of RBC was added and incubated for 1h at 4°C. Known positive and negative sera were used as controls. HI titres of ≥32 were considered positive.

2.3. RESULTS

Peptide selection, alignment and sequences identity

Four peptides were selected from the HA1 domain of H1 virus subtypes. NG34 peptide belongs to VIN1 region and corresponds to positions 101–134 from the A/Catalonia/63/2009 (H1N1) virus. SS35 peptide was selected from the VIN2 region and corresponds to positions 306-340 from the A/California/04/2009 virus. In addition, two peptides DC55 and RA22 were randomly selected. Both DC55 (positions 18-72) and RA22 (positions 330-351) peptides were derived from the A/California/04/2009 virus. All four selected peptides were aligned with 2 strains of H1 (A/California and A/Catalonia) and two amino acids different were found in positions 101 and 338 as shown in Figure 2.1.

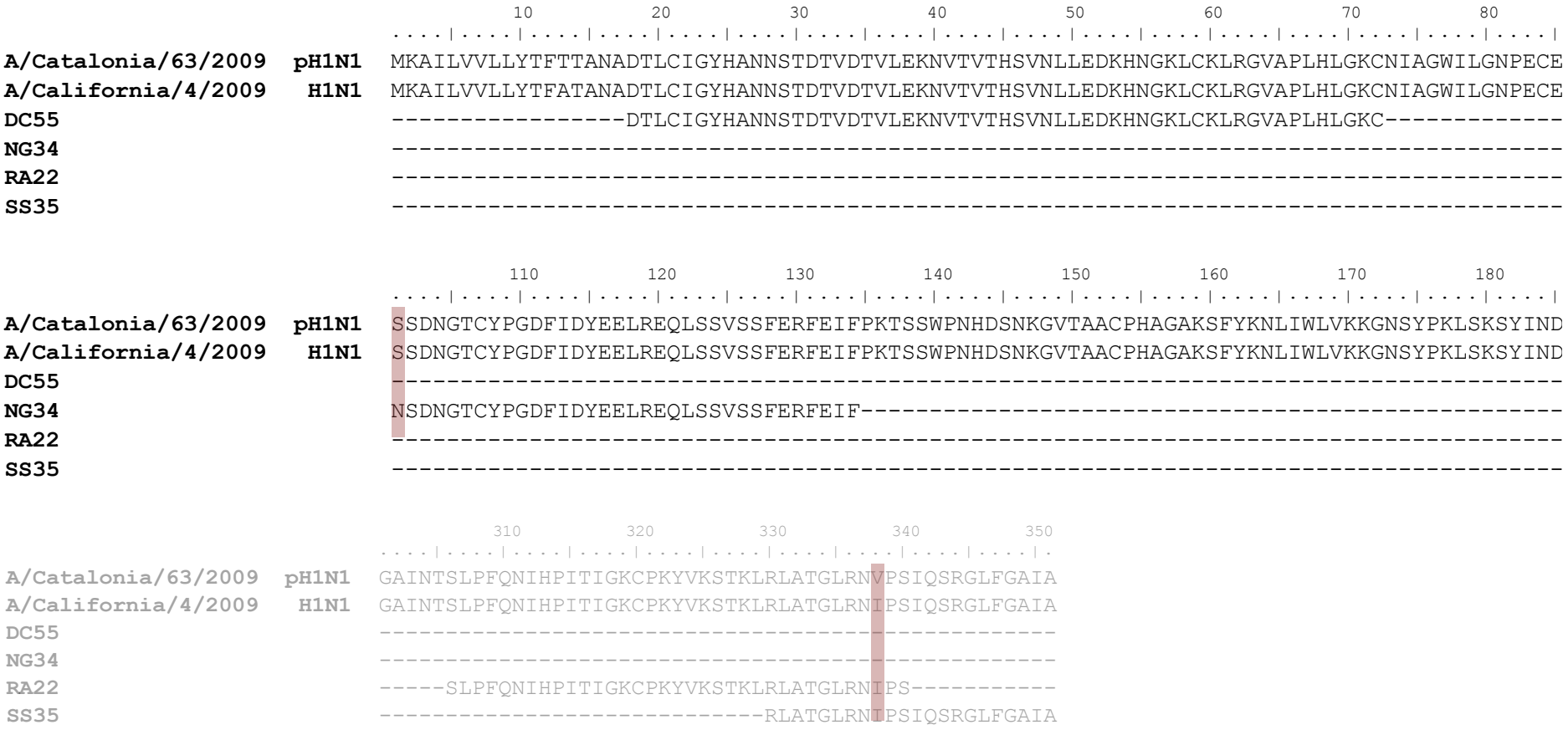

Figure 2.1 Amino acid sequences from HA1-peptides used for immunization compared to the homologue sequence of the HA from Influenza A viruses (H1N1). The amino acid differences between sequences are marked

Furthermore, the sequence identity between HA1-peptides and influenza A viruses were compared as shown in Table 2.2 and Figure 2.2. All selected peptides (NG34, DC55, SS35 and RA22) had an identity between 80 and 97% with the pH1N1 and SwH1N1 viruses. They also displayed certain identity (59-74%) with H5N2 LPAIV virus. The identity between selected four peptides and H7N1 HPAIV virus was less than 60%; specifically, RA22 and SS35 (54% and 57%) showed a higher identity than NG34 and DC55 (29% and 31%). All selected HA1-peptides also displayed an identity between 26% and 68% with those sequences from the SwH3N2 virus, in which RA22, NG34, DC55 and SS35 peptides had an identity of 68%, 26%, 31% and 57%, respectively.

```
                                  110         120         130
                                  ....|....|....|....|....|....|....
A/Catalonia/63/2009 (pH1N1)       SSDNGTCYPGDFIDYEELREQLSSVSSFERFEIF
A/chicken/FVP/Rostock/1934 (H7    REG.DV....K.VNE.A..QI.RGSGGIDKETMG
A/duck/Denmark/65047/04 (H5N2)    NPV..L......N.....KHL...TNH..KIQ.I
A/swine/Spain/001/2010  (H3N2)    .KAFSN...Y.VPE.TS..SLIA.SGTL.FTNED
A/swine/Spain/003/2010  (H1N1)    LVIW.VHH.PTDS.QQT.YQNNHTYI.VGSSKYY
NG34                              N.............................

                                    20          30          40          50          60          70
                                  ..|....|....|....|....|....|....|....|....|....|....|....|..
A/Catalonia/63/2009 (pH1N1)       DTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGKLCKLRGVAPLHLGKC
A/chicken/FVP/Rostock/1934 (H7    A.ETVERTNIPKICSKGKRTTDLGQCGLLGTITGPPQCDQFLEFSADLIIERREG
A/duck/Denmark/65047/04 (H5N2)    .QI..........EQ...IM........AQDI..KA......S.N..K..I.RD.
A/swine/Spain/003/2010  (H1N1)    EV.V.WGVHHPP..SDQQT.YQ.NHTYI..G--SS.YYQRFTPEIVAR.KVREQA
A/swine/Spain/001/2010  (H3N2)    A.ELVQNFSMGKICQNPHRILDGANC.LIDS..G.P.CDGFQNEKWDLFIERS.A
DC55                              ..................................................

                                  330         340         350
                                  |....|....|....|....|....|
A/Catalonia/63/2009  pH1N1        RLATGLRNVPS----IQSRGLFGAIA
A/chicken/FVP/Rostock/1934 H7N    L....MK...EPSKKRKK........
A/duck/Denmark/65047/04 H5N2      V.........Q----KET........
A/swine/Spain/3/2009   H1N1       .M......I..----...........
A/swine/Spain/001/2010 H3N2       -------------------------
RA22                              ........I..----...........

                                      310         320         330         340
                                  ....|....|....|....|....|....|....|....|
A/Catalonia/63/2009  pH1N1        SLPFQNIHPITIGKCPKYVKSTKLRLATGLRNVPS
A/chicken/FVP/Rostock/1934 H7N    R......NSRAV....R...QES.L....MK...E
A/duck/Denmark/65047/04 H5N2      .M..H....L...E.......DR.V.........Q
A/swine/Spain/3/2009   H1N1       N.....V......E.........Q..M......I..
A/swine/Spain/001/2010 H3N2       ----------------------------------
SS35                              ................................I..
```

Figure 2.2 Comparison of amino acid sequence of HA1-peptides and HAs of influenza A viruses. The amino acid identities between sequences are represented in dots

Table 2.2. Identity analysis of selected peptides compared to reference isolates.

STRAIN	SHORT NAME	NG34	DC55	RA22	SS35
A/Catalonia/63/2009	pH1N1	97%	100%	95%	97%
A/chicken/FVP/Rostock/1934	H7N1	29%	31%	54%	57%
A/duck/Denmark/65047/04	H5N2	59%	67%	73%	74%
A/swine/Spain/3/2009	SwH1N1	85%	82%	95%	86%
A/swine/Spain/14/2011	SwH3N2	26%	31%	68%	57%

Immunological cross-reactivity of selected peptides

The immunological cross-reactivity between peptides and HA1 proteins of the virus strains used in vitro was evaluated using ISM. Each value between peptide and HA1 protein (see Table 2.3) is a measure of similarity (S/N) and represent only one peak of the common frequency (Appendix I), which correlate with the same biological or biochemical function between proteins. SS35 peptide, compared to the rest of the peptides, showed a high specificity of cross-reactivity with all Influenza A virus strains used in this study. NG34 peptide showed a high specificity of cross-reactivity with pH1N1 > H3N2 > H5N2 virus strains. NG34 also reacted with H7N1 > SwH1N2, albeit with relatively lower specificity. DC55 peptide showed a high specificity of cross-reactivity with pH1N1 ≥ H5N2 compared to CIS with H3N2 > H7N1 ≥ SwH1N1. RA22 peptide showed a higher specificity of cross-reactivity with H7N1 > pH1N1 > H3N2 compared to H5N2 > SwH1N1.

Table 2.3 Specificity of the cross-reactivity represented by S/N between peptides used for vaccination and HA1 protein of the virus strains used for in this study

HA1-PEPTIDE	STRAIN	S/N in CIS (Peptide x HA1)
NG34	pH1N1	23.120
	H7N1	16.053
	H5N2	19.313
	SwH1N1	15.854
	SwH3N2	22.927
DC55	pH1N1	19.834
	H7N1	13.120
	H5N2	19.829
	SwH1N1	13.339
	SwH3N2	15.485
RA22	pH1N1	19.342
	H7N1	22.500
	H5N2	13.404
	SwH1N1	15.336
	SwH3N2	19.995
SS35	pH1N1	38.312
	H7N1	25.063
	H5N2	22.533
	SwH1N1	33.265
	SwH3N2	36.918

HA1 peptide-specific humoral response against influenza A viruses

In order to visualize the IgG antibody response in pigs vaccinated, each serum sample was evaluated for its capacity to recognise selected HA1-peptides using ELISA (Figure 2.3). As expected, no detectable levels of peptide-specific antibodies were found in sera from control pigs (No. 1 and 2). One of the two pigs vaccinated with NG34-peptide (No. 3) had lower levels of antibodies after three vaccinations (Figure 3A, 3B and 3C), in contrast pig No. 4, elicited high-levels of specific antibodies against NG34. However, both pigs No3 and No.4 induced cross-reactive antibodies against SS35. Pigs vaccinated with DC55+RA22 (pig No. 5) elicited specific antibodies to DC55 and RA22 after 1st vaccination (Figure 2.3A), while Pig No 6 had lower levels of DC55 and RA22 antibodies after three vaccinations (Figure 2.3C). Animals vaccinated with SS35 (pigs No. 7 and 8) showed a strong level of anti-SS35 antibodies after 1st vaccination and elicited cross-reactive antibodies against NG34 after 2nd vaccination (Figure 2.3B). Animals vaccinated with NG34+DC55 (pigs No 9 and 10) showed specific antibodies against NG34 and elicited a strong level of anti-DC55 antibodies. Interestingly, both pigs No. 9 and No. 10 showed cross-reactivity with SS35. Animals vaccinated with NG34+SS35 (pigs No 11 and 12) showed specific antibodies against NG34 induced high level of SS35-antibodies (Figure 2.3C). Animals vaccinated with NG34+DC55+SS35 (pigs No 13 and 14) elicited a strong level of anti-DC55 and anti-SS35 antibodies. Only pig No.13 elicited lower level against NG34 (Figure 2.3C) in contrast pig No 14 showed high-level specific antibodies against NG34

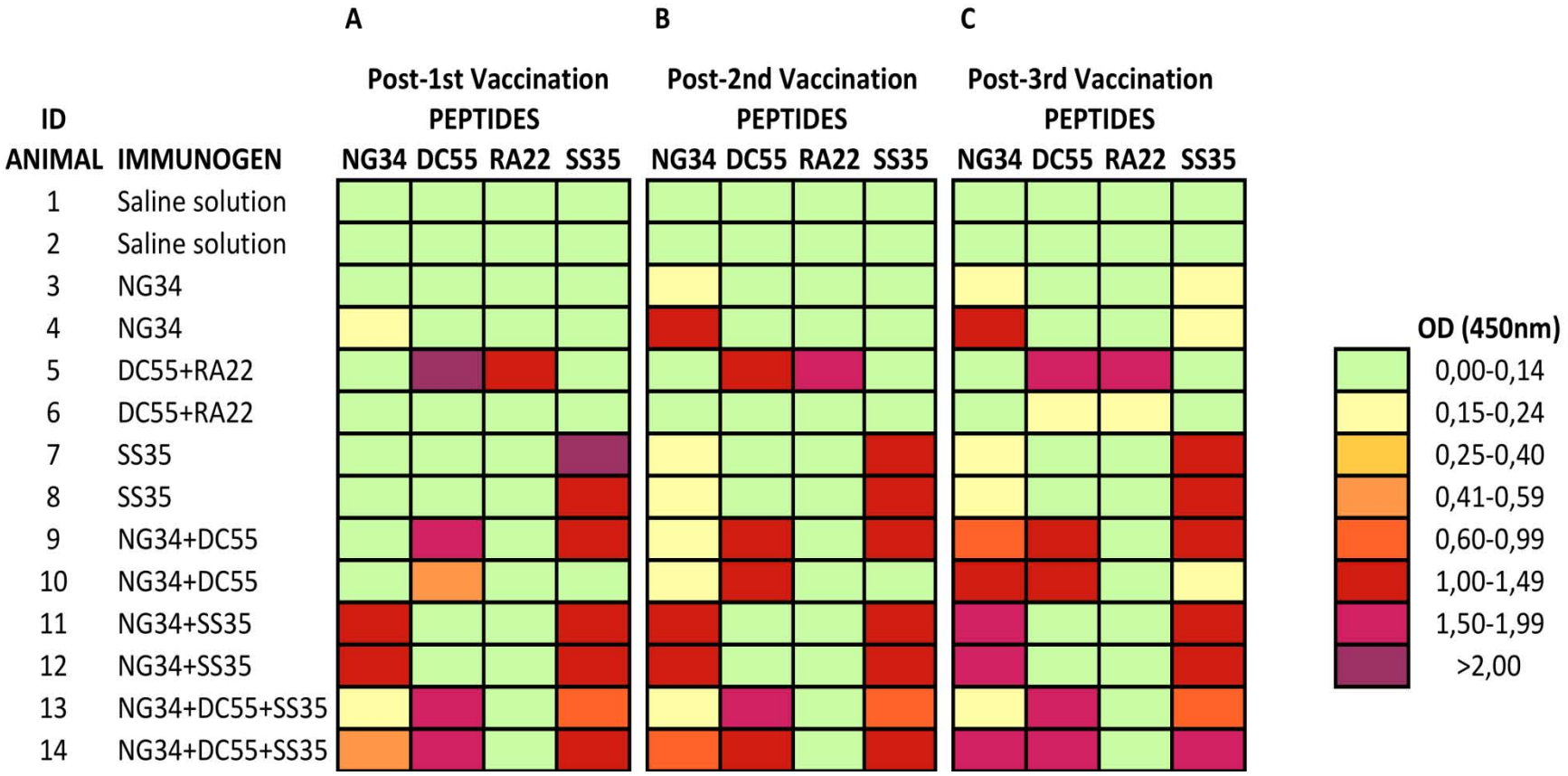

Figure 2.3. Humoral response after vaccinations. (A to C) IgG- specific antibodies in serum samples after vaccination. The darker colours were related to higher O.D while lighter colours corresponds to negative or lower antibody response. Reaction was detected with anti-pig IgG (whole molecule)-peroxidase conjugate.

Mucosal immune response in nasal secretions

Nasal swabs were collected at 7 days post-3rd vaccination from all pigs to confirm the presence of IgA antibodies by ELISA test. The presence of specific IgA antibodies was detected in all vaccinated pigs (Figure 2.4). As expected, no detectable levels of peptide-specific antibodies were found in swabs from control pigs (No. 1 and 2). Pig No. 3 not elicited antibody response against NG34 compared to pigs No. 4. Pig Nº 5 vaccinated with DC55+RA22, showed specific antibodies against both peptides while pig No. 6, which elicited only DC55-antibodies. Animals vaccinated with SS35 (pigs No. 7 and 8) induced specific antibodies against the SS35 peptide. Animals vaccinated with NG34+DC55 (pigs No. 9 and 10) generated a low antibody response to NG34 and DC55. Similarly, pig No. 11 vaccinated with NG34+SS35 elicited a lower NG34 antibody response and did not show SS35 antibody response compared to pig No. 12. Finally, animals vaccinated with NG34+DC55+SS35 showed moderate level of antibodies against NG34, relatively weak response to DC55 no anti-SS35 antibodies (pig No 13). Otherwise, pig No. 14 showed a moderate level of antibodies against NG34 and SS35 peptides, and a lower level against the DC55

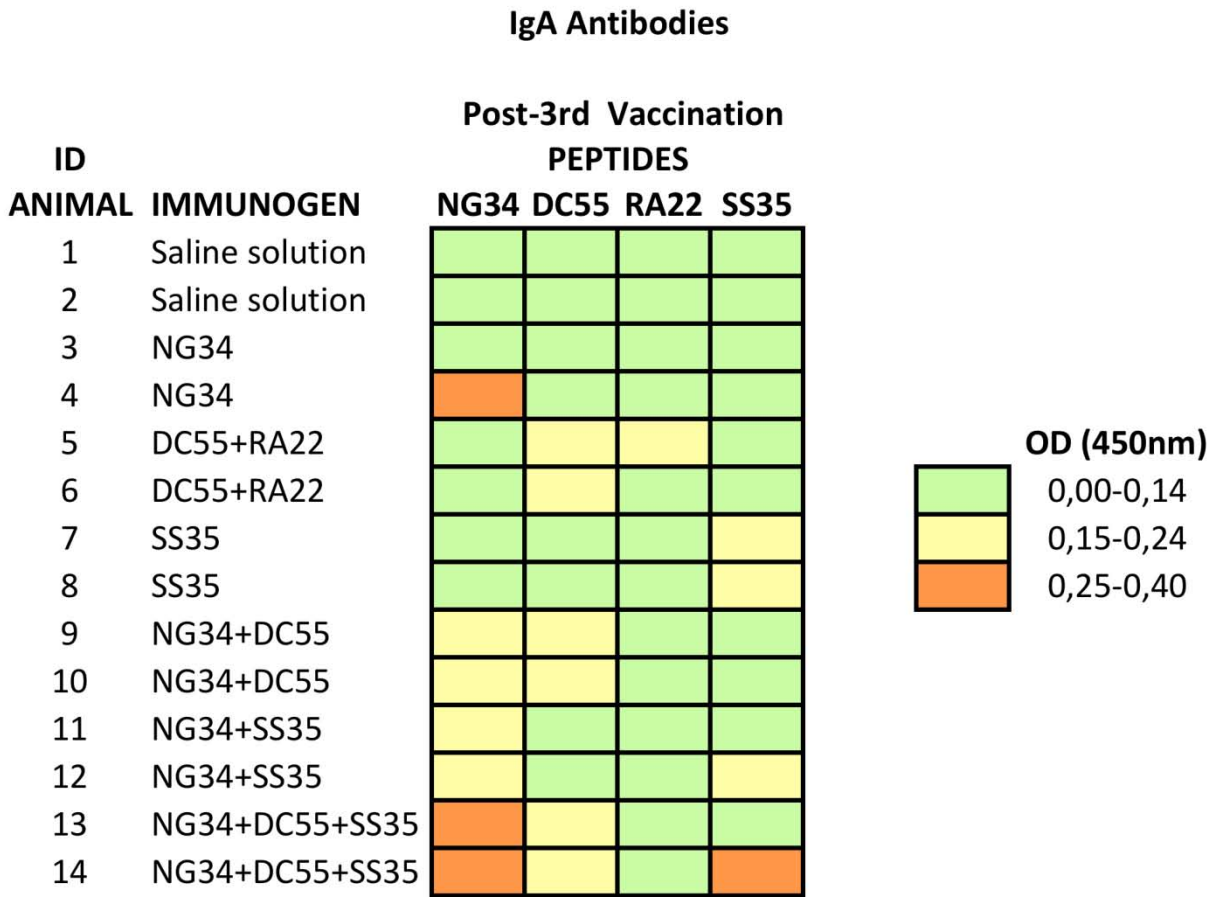

Figure 2.4. Mucosal response post-3rd vaccination. Specific IgA antibodies in nasal swabs post-3rd vaccination. The darker colours were related to higher O.D. while lighter colours were represented negative or lower O.D. Reaction was detected with goat anti-pig IgA peroxidase conjugate.

Pigs vaccinated with HA1 peptides elicit neutralizing and hemagglutination-inhibiting antibody responses against different subtypes of Influenza A virus

Humoral protection against influenza virus is predominantly mediated by antibodies against HA. Thus, serum samples were examined for the presence of antibodies capable of neutralizing different influenza viruses and inhibit hemagglutination (Table 2.4). Based on SN test, sera from pigs No. 1 and 2 showed low neutralizing antibodies titres of 1:10 or 1:20; essentially no reactivity was found against IAV that were tested in vitro. Positivity cut-off for neutralization was considered with a titre at ≥ 1:40. All 12

vaccinated pigs (100%) displayed neutralizing antibodies against the pH1N1 virus, in which 50% pigs (No. 4, 7, 8, 9, 10, and 13) induced a neutralizing antibody titre of ≥ 1:160 and 50% pigs (No. 3, 5, 6, 11, 12 and 14) got a titre ranging from 1:40-1:80. Similarly, neutralizing antibodies against SwH1N1 were detected, in which 41% of pigs (No. 4, 7, 12, 13 and 14) had SN titre of 1:160, as shown in Table 2.4.

To explore the potential ability of HA1-peptides to generate heterosubtypic immune responses, neutralizing antibody responses were evaluated not only against H1 subtypes but also against H7, H5 and H3 subtype viruses. SN titres were detected in 66% of vaccinated pigs and ranged from 1:80-1:160 against H7N1; pigs (No. 3, 5, 6 and 9) had SN titres of 1:40. SN titres against H5N2 ranged from 1:40-1:320, in which 41% of vaccinated pigs had titres of 1:160-1:320 (pigs No. 4, 7, 10, 11 and 13). Additionally, SN titres against H3N2 were detected in all vaccinated pigs, in which 25% pigs (No. 7, 13 and 14) displayed titres of 1:160 and 75% (pigs 3, 5, 6, 8, 9, 10, 11 and 12) had titres ranging from 1.40-1:80 (Table 2.4).

By HI test, only an antibody titre of ≥1:32 was considered as positive. Serum samples from all vaccinated pigs were positive for pH1N1 virus; the range of positive titres was 1:32–1:128, and 50% of pigs (No. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 14) elicited high HI titres (1:64). All vaccinated pigs also reacted against SwH1N1 virus (titre of 1:32). Two out of twelve (17%) serum samples from vaccinated pigs were negative against H7N1 virus (pigs No. 5 and 6) and 83% (pigs No. 4, 7, 8, 9, 10, 11, 12, 13 and 14) had a range of positive titres ranging 1:32-1:64. HI titres against H5N2 were detected in 75% of the vaccinated pigs (No.4, 7, 8, 9, 10, 11, 12, 13 and 14), which ranged from 1:32-1:64. Additionally, HI titres against H3N2 were detected in 58% of the pigs (No. 3, 4, 7, 9, 11, 13 and 14), with values of presented titres ≥1:32 (Table 2.4)

Table 2.4. Panel of vaccinated pig sera tested by two serological assays: HI and SN.

ANTIBODY TITER											
Animal ID	**Immunogen**	**pH1N1**		**H7N1**		**H5N2**		**SwH1N1**		**SwH3N2**	
		HI[a]	**SN[b]**	**HI[a]**	**SN[b]**	**HI[a]**	**SN[b]**	**HI[a]**	**SN[b]**	**HI[a]**	**SN[b]**
1	Saline solution	<16	<20	<16	<20	<16	<20	<16	<20	<16	<20
2	Saline solution	<16	<20	<16	<20	<16	<20	<16	<20	<16	<20
3	NG34	32	40	64	40	16	40	32	40	32	40
4	NG34	64	80	32	80	32	160	32	160	32	80
5	DC55+RA22	32	80	16	40	16	40	32	80	16	40
6	DC55+RA22	32	40	16	40	16	40	32	40	16	40
7	SS35	64	320	32	160	32	320	32	160	32	160
8	SS35	64	80	32	80	32	80	32	80	16	80
9	NG34+DC55	64	160	64	40	32	40	32	80	32	40
10	NG34+DC55	64	80	64	80	32	320	32	80	16	40
11	NG34+SS35	32	320	64	80	32	160	32	80	32	40
12	NG34+SS35	32	640	64	160	32	40	32	160	16	80
13	NG34+DC55+SS35	128	160	64	80	64	160	32	160	32	160
14	NG34+DC55+SS35	32	320	64	80	32	80	32	160	32	160

Sera were collected from vaccinated pigs at 12 days after 3rd vaccination
[a] HI Hemagglutinin inhibition test
[b] SN Seroneutralization test
HI titres ≥32 were considered positive
SN titres ≥40 were considered positive

2.4 DISCUSSION

The idea of a multivalent influenza vaccine is which can confer protection against different subtypes. In this study, we used the ISM bioinformatic tool to select four (NG34, DC55, RA22 and SS35) peptides from within the hemagglutinin subunit 1 protein (HA1) from H1 viruses and was evaluated their immunogenicity in conventional farm pigs against homologous and heterologous viruses of influenza. We show here that the peptide immunization not only induces antibodies that can be blocking the virus entry to cell and inhibiting hemagglutinin against homologous viruses,

but also heterologous viruses like H7N1 and H5N2 and most important circulating H3N2 virus.

Here we demonstrate, that similar information encoded by primary structures of HA1 protein of viruses and peptides seem to be correlated with their immunological cross-reactivity that can be reflected in a common biological property such as the induction of a specific cross-reactive antibody response. As shown in Figure 2.3, the anti-NG34 serum was able to cross-react with SS35 peptide and vice versa, whereas anti-DC55+RA22 serum did not cross-reaction with other peptides. Although RA22 peptide sharing 10 common amino acids with SS35. Similar results, were reported by Krsmanovic et al., 1998 on base of ISM, using a set of synthetic peptides, distinct in amino acid sequences, can induce polyclonal antibodies, which cross-react with proteins of the human immunodeficiency virus type 1 (HIV-1), in New Zealand White rabbits (Krsmanovic et al., 1998).

Our findings in pigs are similar to our previous studies in the mouse model (Chapter 1). Here as well, mice immune to a HA1-peptides vaccine showed specific anti-HA1 antibodies able to neutralizing and inhibit hemagglutination against homologous and heterologous virus like H7N1. Although heterosubtypic protection has been frequently studied in mice, the pig model offers specific advantages for studying protection between influenza viruses.

Pigs are natural hosts for a variety of genetically and antigenically diverse H1 and H3 viruses. However to date, few studies in pigs (Heinen et al., 2001; Reeth and Ma, 2013), ferrets (Yetter et al., 1980; Laurie et al., 2010)and mice (Liang et al., 1994; Kreijtz et al., 2007) that show a complete protection between H1 and H3 viruses has been reported. Heinen et al, 2001 reported that no cross-reactive HI antibodies from SIV H1N1-infected pigs were observed with SIV H3N2 or vice versa after primary infection (Heinen et al., 2001). Later, Van Reeth et al, 2003 have demonstrated protection against a H1N2 swine influenza virus in pigs previously infected with H1N1 and H3N2 subtypes, but they generally lacked HI and SN antibodies against H1N2 (Van Reeth et al., 2003).

Antibodies inhibiting the H1N2 NA were, in contrast, consistently observed in these pigs, despite the clear distinction between NA antibodies induced by H1N2 or H3N2 infection (Van Reeth et al., 2003). Although antibodies against NA contribute to decrease the amount of virus released from infected cells, but it is unable to neutralise the virus and prevent initiation of infection such as the HA antibodies (Johansson et al., 1989; Mozdzanowska et al., 1999). However, recently Khurana et al., 2013 have reported that vaccinating pigs with whole inactivated H1N2 virus vaccine induced anti-HA2 antibodies that promote virus fusion and enhance influenza virus respiratory disease (Khurana et al., 2013). The study presented not only show that using HA-peptides selected from HA1 protein can induce cross-reactive antibodies against H1 SIV, pH1N1, H3N2, H7N1 and H5N2 subtypes; but also promote secretory IgA-specific antibodies in nasal swabs. Thus, well-matched IgG and IgA specific antibodies against the HA could potentially prevent an influenza virus infection, and may contribute in clearing the virus from the lungs, as reported previously (Waffarn and Baumgarth, 2011; Vergara-Alert et al., 2012). Therefore the secretory IgA production in the nasal cavity could participate as principal mediator of the upper respiratory tract immunity, whereas IgG from serum could contribute mostly to lower respiratory tract immunity. However, substantial IgA levels have also been found in BAL fluids of pigs, and in lung lysates of mice, suggesting that antibodies may also be locally produced in the lung (Wang et al., 2004; Kitikoon et al., 2006; Khatri et al., 2010) .

In the present experiment, the immunogenic effect of HA1-peptides for IgA levels at nasal swabs and the SN or HI cross-reactive antibodies generated after immunization in pigs would suggest that humoral responses with cross-neutralizing activity in sera could play a major role in conferring protective heterosubtypic immunity. However, the protective effect of these peptides must be tested in different natural hosts of the virus, followed by a heterologous challenge. For that reason our hypothesis is also being tested in chicken that is critical for the reduction of potential cross-species adaptation and spread of influenza viruses, which will minimize the risk of animals being the source of the future pandemics.

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CHAPTER 3

Study III: Synthetic peptides from the hemagglutinin of influenza viruses confer partial protection against highly pathogenic A/H7N1 virus in a free-range chicken model

3.1 INTRODUCTION

Severe outbreaks of HPAIV in birds have resulted in human deaths, raising concern about its potential for adaptation to humans. In addition, the virus has affected the poultry industry, leading to culling of flocks and major economic losses (Uiprasertkul et al., 2005; Peiris et al., 2007; Malik Peiris, 2009). Currently, the most effective means of controlling a potential pandemic virus in humans is vaccination, and vaccines are also an effective tool in the control of avian influenza in poultry when combined with adequate biosafety and monitoring. The majority of currently available influenza vaccines for poultry are inactivated whole-virus vaccines (Swayne, 2006; Rao et al., 2009; Toro et al., 2010). However, it is important to note that depending on individual responses and the homology between the vaccine strain and the hemagglutinin (HA) of the challenge strain, the vaccine may only protect against disease and birds may shed variable quantities of virus (Swayne, 2006; Rao et al., 2009; Rahn et al., 2015). Although highly efficacious, these vaccines are hindered by long production times, biosafety concerns, inflexibility in quickly altering antigenic composition, and limited breadth of protection (Rao et al., 2009). Thus, research efforts have been accelerated to develop new production methods and alternative strategies to reduce production time while continuing to elicit efficient immune responses. Thus far, the hallmark for influenza vaccine efficacy has been the induction of an adequate level of virus-neutralizing antibodies in serum (Hobson et al., 1972; Small et al., 1976). These antibodies are primarily directed against some conserved viral proteins or domains, including the M2 extracellular (M2e) domain, the HA stalk domain (HA2) and the HA globular domain (HA1) (Gerhard et al., 2006; Fiers et al., 2009; Wu et al., 2012; Pica and Palese, 2013; Jang and Seong, 2014).

Most of the studies have reported that the M2e could be a candidate antigen for developing universal influenza A vaccines (Fiers et al., 2004; Schotsaert et al., 2009; Nayak et al., 2010; Kim et al., 2013; Swinkels et

al., 2013). However, there is evidence that serum antibody responses to M2e are low following experimental infection of animals (Feng et al., 2006; Schotsaert et al., 2013). M2e-based immunity is infection-permissive and does not eliminate disease symptoms in animal models (Mozdzanowska et al., 1999; Fiers et al., 2009; Kang et al., 2012; Lee et al., 2014). Use of conserved HA-based vaccines is an attractive alternative for the induction of broad-spectrum immunity against multiple strains of influenza, and to decrease both morbidity and mortality. Thus, some studies demonstrated that the HA2 domain was considerably more conserved than the HA1 domain (Both et al., 1983; Gocník et al., 2008; Bommakanti et al., 2010; Fan et al., 2015) and HA2 was able to induce a specific antibody response during natural infection in human and mice (Kostolansky et al., 2002). However, the immune response primarily targets the HA1, and the immunogenicity of the HA2 is very weak(Gerhard, 2001; Steel et al., 2010), with low levels of antibodies, which are not thought to contribute to neutralization of the virus (Wilson et al., 1981; Gerhard, 2001; Staneková et al., 2012). Experimental studies on HA2-peptide conjugated to the keyhole limpet hemocyanin (KLH) have demonstrated reduction in mortality after challenge with homologous or heterologous viruses in mice, but it did not protect against body weight loss (Bommakanti et al., 2010; Staneková et al., 2011). Similarly, some authors reported that the use of headless HA2 VLP vaccines provided full protection against death and partial protection against disease following lethal viral challenge (Steel et al., 2010). In contrast, recombinant vaccinia virus expressing HA2 did not confer protection against homologous virus, nevertheless resulted in an increase in mice survival and faster elimination of virus from the lungs (Gocník et al., 2008). In a chicken influenza infection model, induction of serum-neutralizing antibodies and complete protection against homologous virus with a Newcastle disease virus (NDV)-vectored vaccine expressing HA have been reported(Nayak et al., 2010) Other studies using a polypeptide of HA2 conjugated to KLH reported the induction of antibodies that could confer partial protection against heterosubtypes viruses (Wang et al., 2010a; b).

In fact, there are no effective vaccines capable of inducing a broad-immunity against multiple strains of influenza virus that couldprotect

against both morbidity and mortality. Based on the bioinformatic tool Informational Spectrum Method (ISM), we focused our efforts on developing a broadly protective influenza vaccine. This theoretical prediction allows the selection of highly conserved peptide sequences with the potential to elicit broader immune responses than conventional vaccines (Veljkovic et al., 2009b). The Informational spectrum method (ISM) is a virtual spectroscopy method for investigation of protein-protein interactions and structure-function analysis of proteins. Physical and mathematical basis of the ISM was described in details elsewhere (Cosic et al., 1986; Cosic, 2012). Briefly, protein sequences are transformed into signals by assignment of numerical values for each amino acid. These values correspond to electron-ion interaction potential (EIIP) (Veljković and Slavić, 1972) corresponding with the electronic properties of amino acids which are essential parameters determining the properties of biological molecules. The result is a series of frequencies and their amplitudes (the informational spectrum, IS). The obtained frequencies correspond to the distribution of structural motifs with defined physico-chemical characteristics determining the biological function of the sequence. When comparing proteins which share the same biological function, the ISM allows detection of code/frequency pairs in IS which are specific for their common biological properties (Veljkovic et al., 1985). This common characteristic of sequences is represented by peaks, a consensus informational spectrum (CIS) of proteins. Significance of information is determined by the signal-to-noise ratio (S/N), representing the ratio between the signal intensity at one particular IS frequency and main value of the whole spectrum. A higher S/N value at the characteristic frequency (F) in CS/CIS of two or more proteins suggests a higher specificity of cross-reactivity (Veljkovic et al., 1985, 2008, 2009a; b). Confirming the theoretical predictions, our group has reported that immunization of conventional pigs with the ISM-predicted HA1peptide NF34 induced humoral and T-cell responses (Vergara-Alert et al., 2012). However, antibodies observed in that study were not able to neutralize the challenge virus, although 50% protection was obtained (Vergara-Alert et al., 2012). In order to achieve neutralizing antibodies, the NF34peptide was modified by changing two amino acids (E103D and K130R; here referred as NG34). Tested in mice (Chapter 1) and in pigs (Chapter 2), immunization with

NG34 elicited humoral and mucosal immune responses and elicited antibodies able to neutralize and inhibit the hemagglutination of homologous and heterologous virus.

In the present study, we evaluated two conserved peptides NG34 and SS35 selected from the HA1 of H1 influenza virus subtype for their ability to protect free-range chickens against H7N1 HPAIV infection. Free-range chickens were vaccinated with these two peptides and the antibody response was evaluated in sera. The elicited antibodies recognized heterologous virus subtypes like LPAIV H5N2. Our data indicate that NG34 peptide is more immunogenic and contributes to elicit protective immune response in chicken. On the other hand, SS35 peptide neither induced a detectable level of serum-inhibiting hemagglutination antibodies nor protected free-range chickens from the HPAIV lethal challenge.

3.3. MATERIALS AND METHODS

Viruses

For the present study, two isolates of Influenza virus A were used: HPAIV A/Chicken/Italy/5093/1999 (H7N1) was isolated during the 1999–2000 Italian epidemic (Capua et al., 2000), and LPAIV A/*Anasplatyrhynchos*/2420/2010 (H5N2) was obtained during the course of the surveillance program for avian influenza viruses (AIV) in wild birds in Catalonia, North-East Spain. Virus stocks were produced in the allantoic fluid of 9-day-old specific pathogen free (SPF) embryonated chicken eggs at 37ºC for 72 h. The allantoic fluids were harvested, aliquoted and stored at -80°C until use. The infectious virus titre was determined in SPF embryonated chicken eggs and titres were measured as median embryo lethal dose (ELD_{50}) for both viruses by following the Reed and Muench method (L. J. Reed and Muench, 1938; Villegas, 1998).

Bioinformatic analysis and peptide synthesis

Two peptides were selected based on ISM predictions (Veljkovic et al., 2009a; b) and were used to immunize free-range chickens. NG34 and SS35 peptides are highly conserved and mapped to the flanking region of the HA1 from A/Catalonia/63/2009 (H1N1) strain [GenBank: ACU31124] and A/California/04/2009 (H1N1) strain [GenBank: ACS45035]. The peptides were produced by GL Biochem (Shanghai) Ltd. To analyse and align the amino acid sequences of the peptides and HA proteins of IAVs used (H7N1 and H5N2 LPAIV available in GenBank), we employed Mega 6 Integrated software for Molecular Evolutionary (see Table 3.2).

Animals

One-day-old free-range chickens were housed in the experimental farm facilities of the *Universitat Autònoma de Barcelona (UAB).* Before vaccination, at 9-days-old serum samples from all individuals were confirmed to be seronegative against AIV by a competitive ELISA test (C-ELISA) (IDVET, Montpellier, France) as described by (Zhou et al., 1998). Before infection, chickens were transferred to the *ABSL-3 facilities of the Centre de Recerca en Sanitat Animal (CReSA-Barcelona).* Each experimental group was housed in a different negative pressured isolator with HEPA-filtered air. Food and water were provided *ad libitum* throughout the experiment. 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).

Experimental design

Forty free-range chickens were randomly separated into eight groups (n= 5 animals/group). Animals were vaccinated three times, with their corresponding peptide and adjuvant, as described in Table 3.1. Initial vaccination in G1, G2, G3 and G4, with the peptide was performed with higher concentration (15µg) and followed with decreasing concentrations (7.5µg and 3.75µg, respectively) in later vaccinations. Vaccinations were performed by subcutaneous administration (in the base of the neck, final volume 250µL/animal). At infection, all free-range chickens from G1 to G7 were intranasally inoculated with $10^{4.5}$ ELD_{50} of the H7N1 virus in a final volume of 50µL. Animals from group G8 were sacrificed five days before infection and were used as negative control group.

Table 3.1 Experimental design

Group	Nº Animals	1st VACCINATION 15µg/chicken 9-days-old	2nd VACCINATION 7.5µg/chicken 30-days-old	3rd VACCINATION 3.5µg/chicken 45-days-old	INFECTION 50µL/chicken 80 -days-old
G1	5	NG34 + Standard	NG34 + Standard	NG34 + Standard	H7N1
G2	5	NG34 + Montanide	NG34 + Montanide	NG34 + Montanide	H7N1
G3	5	SS35 + Standard	SS35 + Standard	SS35 + Standard	H7N1
G4	5	SS35 + Montanide	SS35 + Montanide	SS35 + Montanide	H7N1
G5	5	PBS + Standard	PBS + Standard	PBS + Standard	H7N1
G6	5	PBS + Montanide	PBS + Montanide	PBS + Montanide	H7N1
G7	5	PBS	PBS	PBS	H7N1
G8	5	PBS	PBS	PBS	-

Abbreviations: Standard= Standard water-in-mineral oil adjuvant; Montanide= Montanide™ ISA 71R VG adjuvant; PBS = phosphate buffer saline.

Sampling

Blood from 9-days-old free-range chickens was collected before vaccination and after each vaccination (at 30, 45 and 57-days-old). In

addition, at 5 days before infection (75-days-old) and at 10 days post-infection (dpi) blood samples were also collected. Serum was obtained from the blood and was used to detect specific humoral response (Figure 3.1). From each animal, feather pulp (FP) samples, oropharyngeal (OS) and cloacal (CS) swabs were collected at 0, 4, 7 and 10 dpi, in order to measure viral shedding (Figure 3.1). All free-range chickens were monitored daily after challenge for clinical signs and scored following the OIE system (World Organisation for Animal Health et al., 2015): healthy (0), sick (1), severely sick (2), moribund or dead (3). Sick animals were considered those showing one of the following clinical signs: respiratory involvement, depression, diarrhea, and cyanosis of the exposed skin or wattles, edema of the face and/or head and nervous signs. Severely sick chicken were considered those showing more than one of the above-mentioned clinical signs. The mean clinical score and mortality rate (mean death time, MDT) were calculated. According to ethical procedures, animals presenting severe clinical symptoms were euthanized with intravenous administration of sodium pentobarbital (100mg/kg, Dolethal®, Vétoquinol, France).

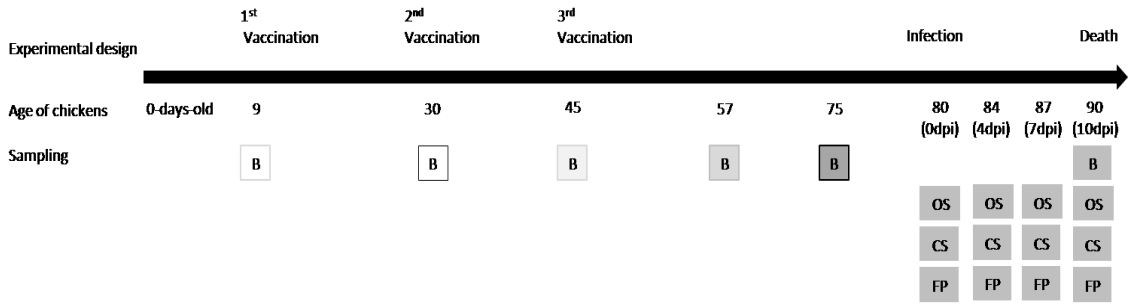

Figure 3.1 Experimental design, age of the chickens and sampling. Abbreviations: B= blood; OS= oropharyngeal swabs; CS= cloacal swabs; FP= feather pulp. DPI= Days post infection

Viral RNA detection by RRT-qPCR

Viral RNA quantification using one step RRT-PCR was performed in FP, OS and CS, which were collected in sterile Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, S.A., UK) with 100 units ml^{-1} penicillin-streptomycin. Viral RNA was extracted with Nucleospin viral RNA virus kit (Macherey-Nagel, 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 by Spackman et al. (2002). Amplification conditions were as described previously by Busquets et al. (2010) in a 7500 Fast & 7500 Real-Time PCR System (Life Technologies, S.A, UK) using 3µl of eluted RNA in a total volume of 20µl. The detection limit of the assay was six viral RNA copies of in vitro-transcribed RNA per reaction, which was equivalent to 2.60 $\log_{10}$ viral RNA copies/sample.

ELISA IgY and IgA detection against peptides

A peptide-based ELISA was developed to measure the presence of specific antibodies in serum and OS samples from vaccinated free-range chickens following the protocol previously described by our group (Vergara-Alert et al., 2012), with some modifications. Briefly, for measurement of IgY, plates were coated with 5µg/ml of each peptide (100µl/well) in coating buffer (sodium bicarbonate 0.1 M) and incubated overnight at 4°C. After blocking with 3% bovine serum albumin (BSA)/PBS for 1h at 37ºC, sera from free-range chicken (dilution 1:100) were incubated for 2h at 37°C for the evaluation of IgY. Plates were then washed four times with 0.1% Tween20-PBS (PBS-Tw), and HRP-conjugated goat-anti-chicken IgY (abcam ab97135) diluted 1:5,000 was added to the wells and incubated for45 min at 37ºC. After washing the plates four times with PBS-Tw, 50µl/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich)

were added and allowed to develop for 8-10 min at RT. Plates were read at 450 nm. For IgA measurement, coated-plates were incubated with OS (undiluted sample) and then incubated with 1:10,000 HRP-conjugated goat-anti-chicken IgA (abcamab112817), followed by TMB.

Antibody detection against whole virus

Disposable flat-bottom high-binding ELISA plates (Costar-3590, Corning NY) were coated with 7.3x106 ELD50/ml of UV-inactivated H7N1 HPAIV in coating buffer (sodium bicarbonate 0.1 M) overnight at 4ºC. After blocking with 1% casein/PBS for 1h at 37ºC, serum samples from free-range chickens from 0 and 10dpi were added to the wells diluted at 1:50, followed by 1h incubation at 37ºC. Plates were washed four times with PBS-Tw, and HRP-conjugated goat-anti-chicken IgY (abcam ab97135) diluted 1:5,000 was added to the wells followed by 30 min incubation at 37ºC. After washing the plates four times with PBS-Tw, positive reactions were developed with TMB and plates were read at 450 nm.

Seroneutralization (SN) assay

Serum samples were heated at 56°C for 30 min to inactivate complement and other unspecific inhibitors. Titres were reported as the reciprocal of the highest dilution of serum that completely neutralized viral growth. All sera were titrated in duplicates until a dilution of up to 1:2,560 in 96-well, flat-bottomed plates (Costar Corning Inc.) in a final volume of 50µl. After dilution, 100 $TCID_{50}$/50µL of the H7N1 virus or H5N2 were added to each well and incubated for 2h at 37°C in a 5% CO_2 atmosphere. The virus and serum mixtures were then added to 96-well plates containing

confluent MDCK cells (5×10^4cells/well) and incubated for 7 days. The antibody titre was calculated by the cytopathic effect

Hemagglutination Inhibition (HI) assay

Serum samples were also analysed for the presence of antibodies against specific H7-subtype by HI test. The assay was performed according to the international standard procedure as described previously by the World Organisation for Animal Health (2015) for testing avian sera using chicken red blood cells and 4 hemagglutination units of H7N1 HPAIV and H5N2 LPAIV. To avoid non-specific positive reactions, sera were pre-treated by adsorption with chicken red blood cells and heat-treated at 56°C for 30 min. Positive and negative sera were included as controls.

Indirect Immunofluorescence (IF)

A total of 3×10^4 MDCK cells cultured on glass coverslips in six-well plates were infected with H7N1 HPAIV and H5N2 LPAIV at a MOI of 0.1. After16hof incubation, infected cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then blocked with 3% BSA in PBS for 1h and incubated with the sera from the free-range chickens (1:100) for 1h in the blocking solution at RT. After three washes with PBS, the cells were incubated with FITC-labelled anti-Chicken IgY antibody (1:200 in PBS) (Gallus Immunotech) for 1h at RT. A mouse anti-NP monoclonal HB65 (1:300) was used as control for the infection, and reaction was detected with a Cy3-labelled anti-mouse antibody. Finally, nuclei were counter stained with Hoechst 33258 (Sigma-Aldrich) (1:500 in PBS) for 10 minutes at RT, and coverslips were mounted with Vectaschield. Fluorescence images were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan).

The images were processed using the Image Jv1.45l software (http://rsb.info.nih.gov/ij).

3.4. RESULTS

Bioinformatic analysis and peptides synthesis

To identify the common information encoded by the primary structures of NG34 or SS35 peptides and HA1 proteins of influenza strains (H7N1 HPAIV and H5N2 LPAIV), cross-spectral analysis of these viral proteins was performed. In this analysis we included complete HA1 sequences from H7N1 HPAIV and H5N2 LPAIV, which are available in the GenBank databases. Figure 3.2 show that the consensus IS of synthetic peptides and HA1 protein of the virus strains contains only one common peak, which represents cross-reactivity between proteins. Common peak for NG34 and SS35 and HA1 of the challenging strain H7N1 HPAIV is shown at the characteristic frequencies of F(0.244) and F(0.285) in their consensus informational spectra (CIS) (Figure 3.2A and 3.2B). Likewise, cross-reactivity with HA1 H5N2 LPAIV was also analysed. Characteristic frequencies of NG34 F(0.246) and SS35 F(0.289) are shown below (Figure 3.2C and 3.2D).

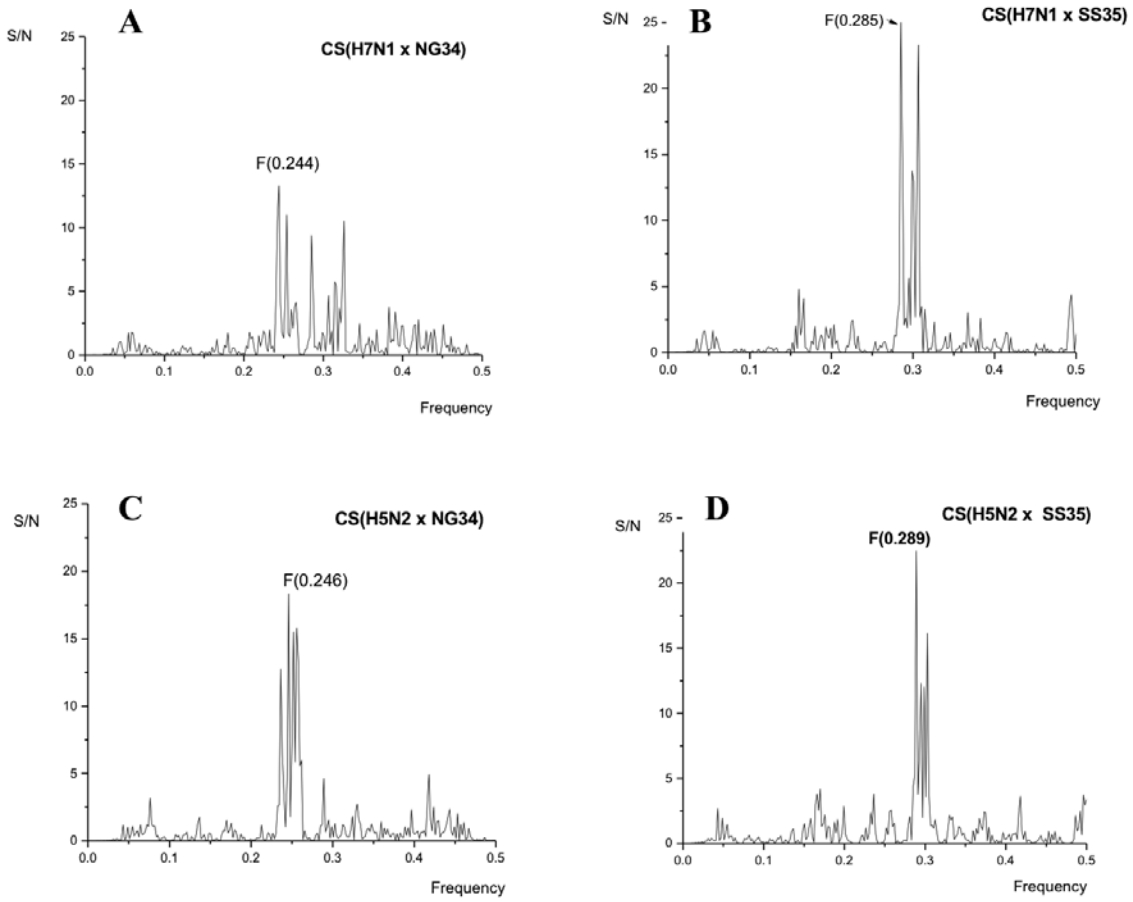

Figure 3.2 (A) NG34 and HA1 of the H7N1, (B) SS35 and HA1 of the H7N1, (C) NG34 and HA1 of the H5N2, (D) SS35 and HA1 of the H5N2. Specificity of cross-reactivity represented by S/N between peptides.

Additionally, a comparative analysis between the amino acid sequences from the NG34 and SS35 peptides, used for immunization, and the sequences of the challenging strain H7N1 HPAIV, and H5N2 LPAIV were performed (Table 1). NG34 peptide sequence identity is approximately 29% to the corresponding sequence from H7N1 HPAIV, while the SS35 sequence shares 63% homology with the same virus. Conversely, the identity between H5N2 LPAIV and NG34 is 59%, and 77% when compared to SS35

.

Table 3.2 Amino acid sequences from the NG34 and SS35 peptides used for immunization compared to the heterologous sequence of the HA receptor recognition domain of the H7N1 HPAIV and H5N2 LPAIV

STRAIN	SHORT NAME	RESIDUES	SEQUENCES	IDENTITY
A/Catalonia/63/2009 H1N1	NG34	101-134	NSDNGTCYPGDFI**D**YEELREQLSSVSSFERFEIF	
A/Chicken/Italy/5093/1999	H7N1	99-132	REGSDV**CYPGKF**VNE**E**A**LR**QI**L**RESGGIDKEAMG	29%
A/duck/Denmark/65047/04	H5N2	100-133	**N**PV**NG**L**CYPGDF**N**DYEEL**KHL**LSS**TNH**FE**KIQ**I**-	59%
A/California/04/2009 (H1N1)	SS35	306-340	SLPFQNIHPITIGKCPKYVKSTKLRLATGLRNIPS	
A/Chicken/Italy/5093/1999	H7N1	300-333	-**LPFQNI**NSRAV**GKCPRYVK**QES**L**L**LATG**MK**NV**P**-**	63%
A/duck/Denmark/65047/04	H5N2	304-307	**S**M**PF**H**NIHP**L**TIG**E**CPKYVKS**DR**L**V**LATGLRN**V**P**Q	77%

The amino acid identities are represented in bold type.

Vaccination with HA-based peptides induces specific antibodies that recognize H7N1 HPAIV and H5N2 LPAIV

We developed indirect ELISAs to analyse the immunogenicity of NG34 and SS35 peptides used in this study (Figure 3.3 and Figure 3.4). All free-range chickens from G1 (NG34+Standar W/O) and G2 (NG34+Montanide) elicited specific antibodies after the first vaccination (Figure 3.3A). In addition, all free-range chickens showed an increase in antibodies at 8 days after the last vaccination (Figure 3.3A). Interestingly, these animals also showed specific-antibodies against SS355 (Figure 3.3B). In G3 (SS35+Standar W/O) only one animal (Nº 11) elicited high levels of specific antibodies after the first vaccination (Figure 3.4A). The rest of animals showed antibodies only after the second and third vaccination (Figure 3.4A). On the other hand, in G4 (SS35+Montanide), only one animal (Nº 17) showed high levels

of detectable antibodies after the last vaccination (Figure 3.4A). All vaccinated animals with SS35 also showed antibodies against NG34 (Figure 3.4B). At 5 days before infection, NG34 and SS35 antibodies were slightly decreased in most of the animals. (Figure 3.3 and Figure 3.4).

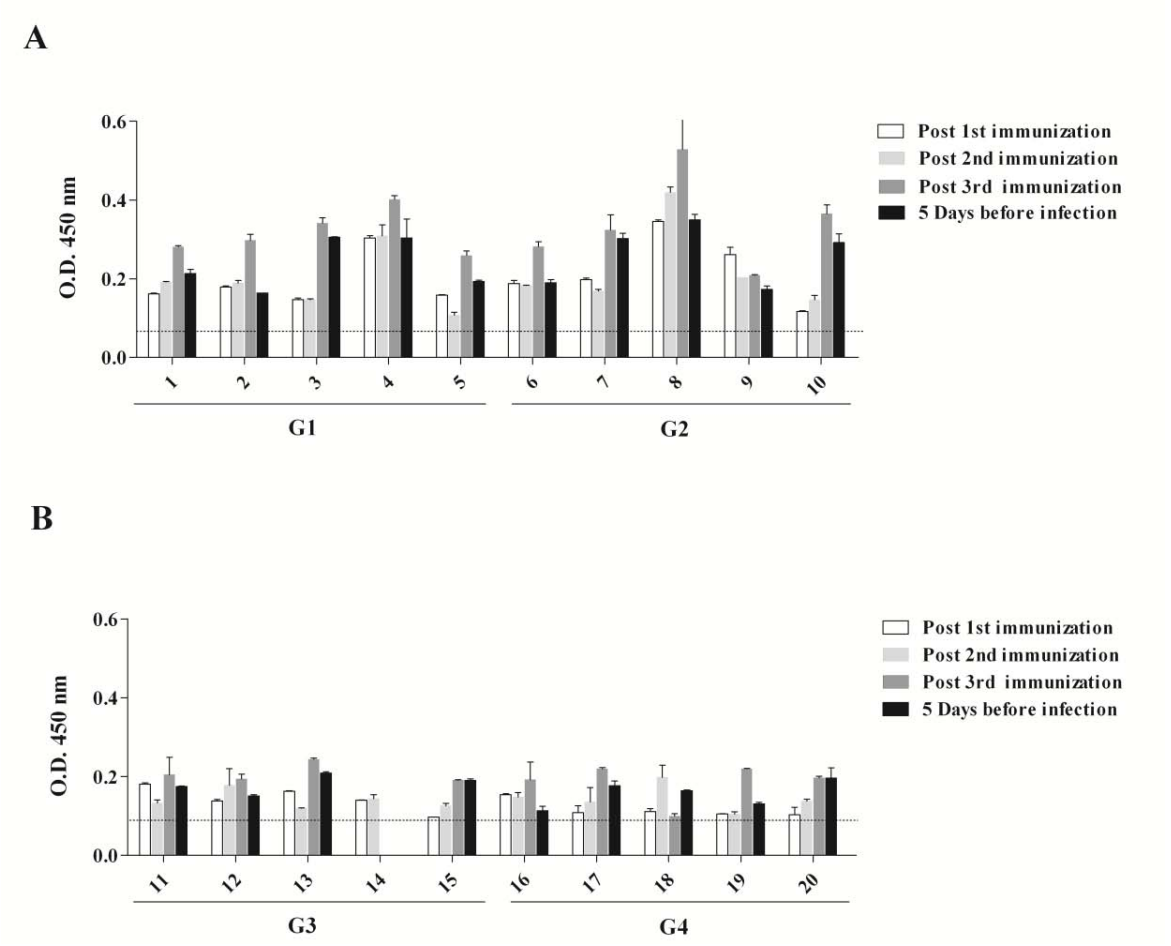

Figure 3.3 Vaccination with NG34 peptide induce cross-reacting antibodies. NG34 peptide antibodies in sera from vaccinated-chickens with NG34 (A) and S35 (B) by ELISA. G1 is from vaccinated animals with NG34+Standar W/O and G2 is from vaccinated animals with NG34+Montanide. Animal Nº14 died after second immunization.

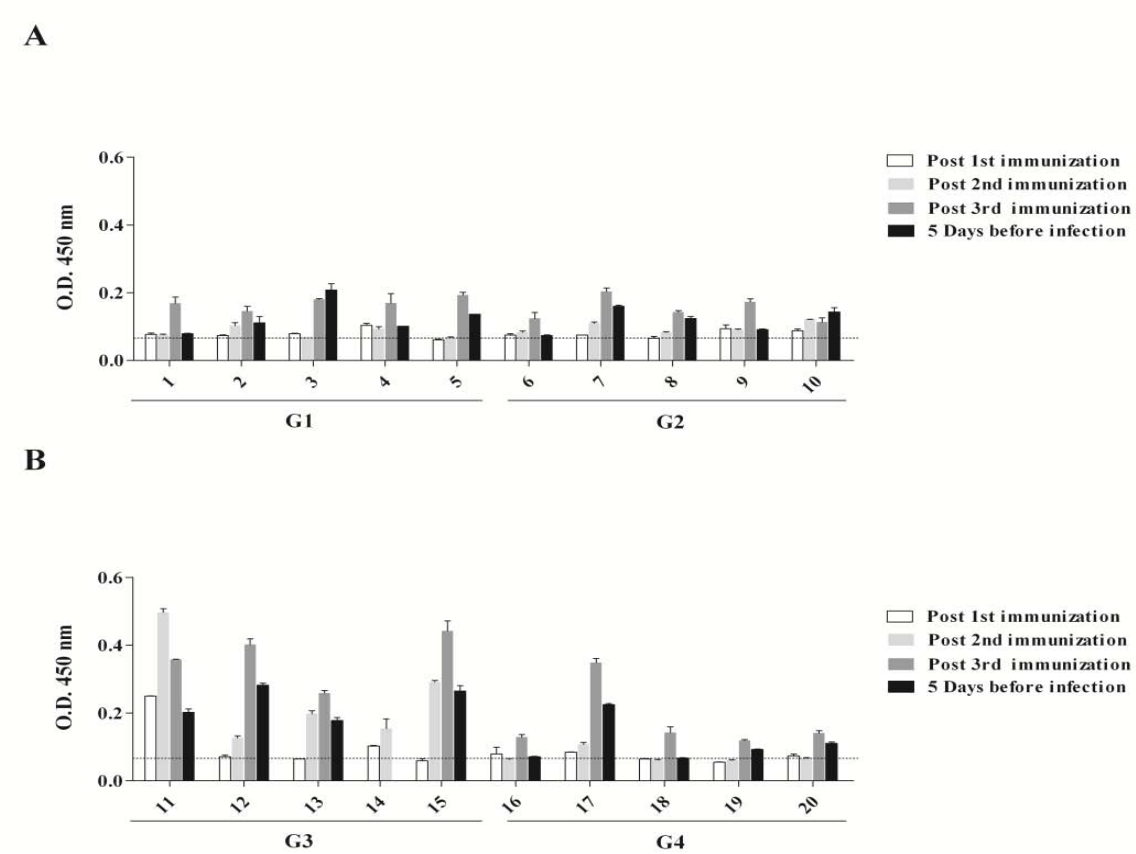

Figure 3.4 Vaccination with SS35 peptide induce cross-reacting antibodies. NG34 peptide antibodies in sera from vaccinated-chickens with NG34 (A) and S35 (B) by ELISA. G1 is from vaccinated animals with SS35+Standar W/O and G2 is from vaccinated animals with SS35+Montanide.

Nonetheless, sera obtained before the infection from chickens vaccinated with NG34 or SS35 did recognize the H7N1 HPAIV and H5N2 LPAIV, as shown by indirect IF (Figure 3.5B, 3.5C, 3.5E and 3.5F). As expected, sera from control animals showed no reaction (Figure 3.5G). Importantly, every single cell infected by H7N1 HPAIV and H5N2 LPAIV viruses was also recognized by the specific-NP monoclonal antibody (Figure 3.5A and 3.5D) confirming the specificity of the reactions.

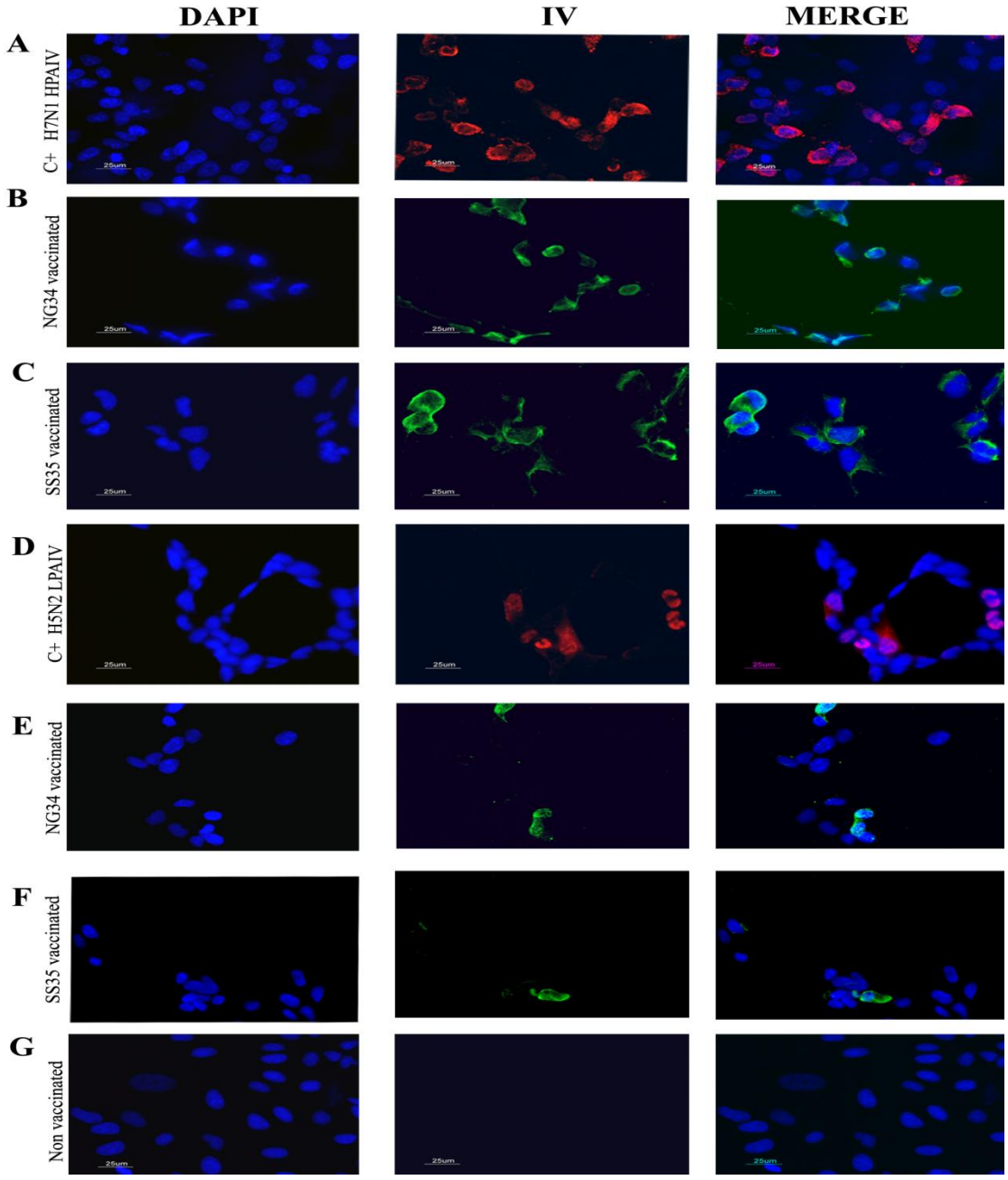

Figure 3.5 NG34 and SS35 specific antibodies are able to recognize HPAIV H7N1 and H5N2 LPAIV *in vitro*. Indirect immunofluorescence of HPAIV H7N1 (A, B, C and G) and LPAIV H5N2 (D, E and F) infected MDCK cells at 16hpi using as primary antibody serum sample from a vaccinated chicken with NG34 (B and E) and a vaccinated chicken with SS35 (C and F). Anti-NP monoclonal antibodies against the NP protein (A and D), used as control for assay, as secondary antibody a Cy3 (red signal) conjugated anti-mouse antibody were used. A serum sample from an adjuvant control (G).

Vaccination with NG34 peptide reduces the clinical signs after lethal infection with H7N1

Thirty-five-days after the last vaccination, free-range chickens were subjected to intranasal challenge with a lethal dose of $10^{4.5}$ EID_{50}/50uL of H7N1 HPAIV. One out of five (20%) vaccinated-chicken from G1 (NG34+Standard) survived the infection (Figure 5A). Likewise, two out of five (40%) vaccinated-animals from G2 (NG34+Montanide) survived (Figure 3.6B). The rest of the animals from G1 and G2 died between days 3 and 5 post-infection. All animals from control groups G5 (PBS+Standard), G6 (PBS+Montanide) and G7 (PBS alone) (Figure 3.6) and animals from SS35 vaccine groups G3 (SS35+Standar) and G4 (SS35+Montanide) succumbed to infection between days 3 and 5dpi (Figure 3.6C and 3.6D). Apathy, depression, petechial haemorrhages on the comb, wattles and legs were presentin almost all animals (19/25). No lesions were observed in vaccinated animals that survived (Nº3, Nº7 and Nº10) confirming the protection against H7N1 HPAIV induced by the NG34 peptide-vaccination (Figure 3.6A and 3.65B).

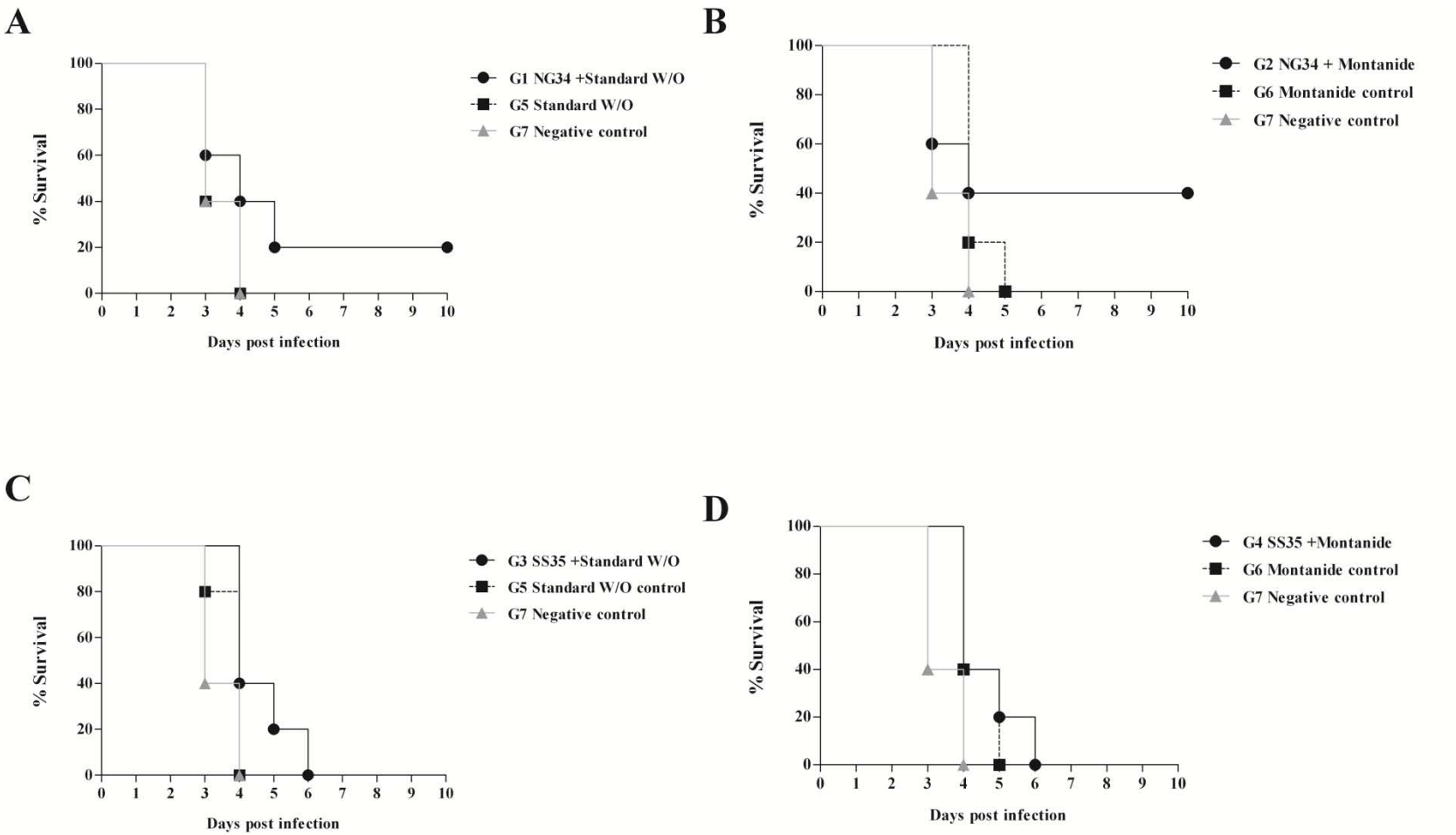

Figure 3.6 Survival rates of chickens vaccinated with HA peptides after challenge with H7N1 HPAIV. Survival curves (in percentage) of free-range chickens from: A. (G1, vaccinated with NG34+Standard W/O) B. (G2, NG34+Montanide Isa71R VG); C. (G3, SS35+ Standard W/O) and D. (G4, SS35+Montanide Isa71R VG). Controls G5 (PBS+Standard W/O), G6 (PBS+Montanide Isa71R VG), and G7 (PBS alone) are included in all figures.

NG34 antibodies specifically neutralize and inhibit the hemagglutination activity of H7N1 HPAIV and H5N2 LPAIV viruses

Sera from vaccinated and control free-range chickens were used to evaluate the capability to neutralize the virus and to inhibit the hemagglutination activity of H7N1 HPAIV and H5N2 LPAIV. In this study the titre cut-off for SN was considered ≥40. At 5 days before challenge, vaccinated animals with NG34 (G1 and G2) showed a neutralizing antibody titre of 1:40-80 against the H7N1 virus and 1:20-40against H5N2. Vaccinated animals with SS35 (G3 and G4) displayed antibody titres against H7N1 virus between 1:20 and 1:80 (Table 3.3), and 1:20-40 against H5N2). This titre however its not sufficient to protect against infection. All animals died at day 4 after infection. Three NG34 peptide vaccinated animals that survived (animal Nº3 from G1, animals Nº7 and Nº10 from G2) displayed titres up to 1:640 against the H7N1 and 1:320 against the H5N2 viruses. Controls G5, G6 and G7 did not show SN activity against H7N1 and H5N2 viruses, as expected (Table 3.3).

The cut-off for HI was established at titre ≥8. At 5 days before challenge, vaccinated animals with NG34+Standard (G1, Nº3animal) did not show HI activity against H7N1 or H5N2. In addition, none of the vaccinated chicken with SS35 (G3 and G4) showed any H7 and H5 hemagglutination inhibitory activity at 5 day before challenge. Vaccinated chickens with NG34+Montanide (G2, Nº7 and Nº10animals) showed low HI antibody titres (1:8) against H7N1, but did not show HI titres against H5N2 at 5 days before challenge(Table 3.3). On the other hand, sera collected from the three surviving animals (Nº3, Nº7 and Nº10) at 10days after infection, inhibited the hemagglutination by H7N1 and H5N2, indicating the antigenic boost during the infection (Table 3.3). Controls G5, G6 and G7 did not show HI activity against H7N1 and H5N2 viruses, as expected (Table 3.3).

Table 3 HI and SN titres obtained against H7N1 HPAIV and H5N2 LPAIV strains with serum from chicken at 5 days before infection (5dbi) and at 10 days post-infection (10dpi)

GROUP[a]	HI TITRE[b]				SN TITRE [c]			
	5 dbi		10 dpi		5 dbi		10 dpi	
Animal identification	H7N1[d]	H5N2[e]	H7N1[d]	H5N2[e]	H7N1[d]	H5N2[e]	H7N1[d]	H5N2[e]
G1 (NG34+Standard)								
1	<4	<4	†	†	40	40	†	†
2	<4	<4	†	†	40	40	†	†
3	<4	<4	128	32	80	40	640	320
4	<4	<4	†	†	80	40	†	†
5	<4	<4	†	†	40	40	†	†
G2 (NG34+Montanide)								
6	<4	<4	†	†	40	40	†	†
7	8	<4	64	32	40	40	640	320
8	<4	<4	†	†	80	40	†	†
9	<4	<4	†	†	40	20	†	†
10	8	<4	64	64	40	40	320	160
G3 (S335+Standard)								
11	<4	<4	†	†	80	20	†	†
12	<4	<4	†	†	40	40	†	†
13	<4	<4	†	†	40	40	†	†
14	†	†	†	†	†	†	†	†
15	<4	<4	†	†	40	40	†	†
G4 (SS35+Montanide)								
16	<4	<4	†	†	20	20	†	†
17	<4	<4	†	†	40	20	†	†
18	<4	<4	†	†	20	20	†	†
19	<4	<4	†	†	20	20	†	†
20	<4	<4	†	†	20	20	†	†
G5 (PBS+Standard)								
21	<4	<4	†	†	<20	<20	†	†
22	<4	<4	†	†	<20	<20	†	†
23	<4	<4	†	†	<20	<20	†	†
24	<4	<4	†	†	<20	<20	†	†
25	<4	<4	†	†	<20	<20	†	†
G6 (PBS+Montanide)								
26	<4	<4	†	†	<20	<20	†	†
27	<4	<4	†	†	<20	<20	†	†
28	<4	<4	†	†	<20	<20	†	†
29	<4	<4	†	†	<20	<20	†	†
30	<4	<4	†	†	<20	<20	†	†
G7 (PBS alone)								
31	<4	<4	†	†	<20	<20	†	†
32	<4	<4	†	†	<20	<20	†	†
33	<4	<4	†	†	<20	<20	†	†
34	<4	<4	†	†	<20	<20	†	†
35	<4	<4	†	†	<20	<20	†	†

[a] After vaccination as indicated for each group free-range chickens were challenged intranasally with A/Chicken/Italy/5093/1999 (H7N1) ($10^{4.5}$ of ELD_{50}).[b] HI titres ≥8 were considered positive.
†= SN and HI assays not done due to death by H7N1 HPAIV-infection.

To further confirm the elicited humoral response in surviving vaccinated chickens, we also compared the specific antibodies against NG34 and whole virus (H7N1 HPAIV) by ELISA at 5 day before infection and 10 days after infection (10dpi) (Figure 3.7). The specific antibodies against NG34 yielded similar results compared to that obtained against whole virus. There is no significant difference between the two antigens (NG34 and H7N1 virus) at 0dpi. Nevertheless, after challenge with H7N1 HPAIV, an increase in specific antibody response against NG34 was observed in surviving vaccinated animals at 10dpi.

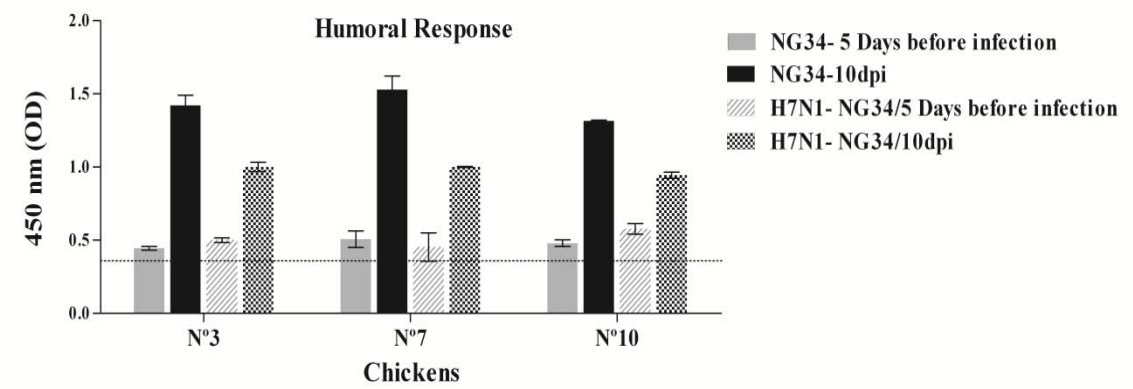

Figure 3.7 NG34 elicited humoral response in surviving vaccinated free-range chickens. Sera obtained at 5 days before infection and 10 days post-infection from three NG34-vaccinated free-range chickens that survived a lethal challenge (Nº3, Nº7 and Nº10) were tested for binding to NG34 and whole virus (HPAIV H7N1) by ELISA. Reaction was with HRP-conjugated goat-anti-chicken IgY. Error bars represent standard deviations of undiluted sample. Samples were analysed in duplicate and the values of the duplicates were almost similar.

Also, the surviving vaccinated animals (Nº3, Nº7 and Nº10) showed the presence of specific IgA antibodies against NG34 was also observed in oropharyngeal swabs at 5 days before infection. Likewise, lower levels of cross-reaction against SS35 was also detected (Figure 3.8).

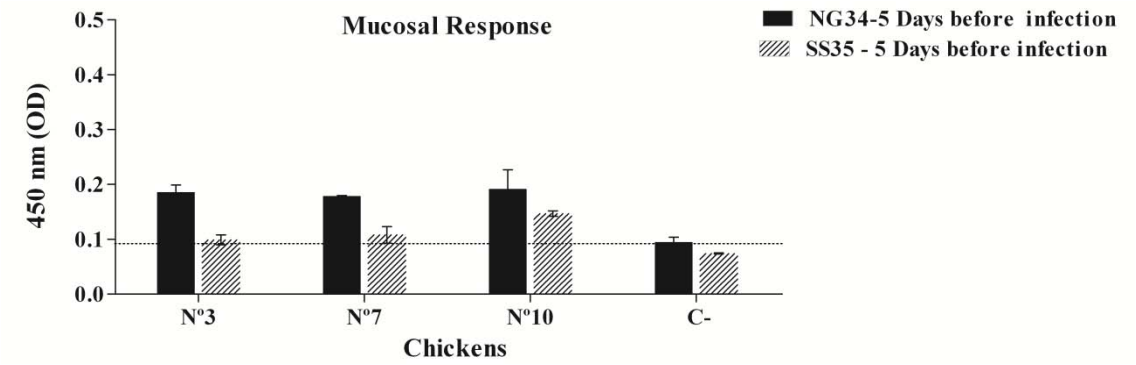

Figure 3.8 NG34 elicited mucosal response in surviving vaccinated free-range chickens. IgA-specific antibodies in oropharyngeal swabs from the three vaccinated free-range chickens that survived the lethal challenge and negative control at 5 days before infection were tested by ELISA. Reaction was with HRP-conjugated goat-anti-chicken IgA. Error bars represent standard deviations of undiluted sample. Samples were analysed in duplicate and the values of the duplicates were almost similar.

NG34 peptide vaccination reduces H7N1 HPAIV shedding

FP, OS and CS were collected on days 4, 7 and 10 after H7N1 HPAIV challenge to test viral shedding. Animals that survived the challenge (Nº3 from G1, and Nº7 and Nº10 from G2) exhibited viral shedding at the selected time points (4, 7 and 10dpi). Interestingly, surviving animals were able to reduce gradually, and significantly, the viral load of H7N1 HPAIV in FP, as compared to control groups (G5, G6 and G7) (Figure 3.9A). Animal Nº3 showed a significant reduction in viral load in FP after 7dpi onwards ($p<0.05$) (Figure 3.9A). Also, this tendency was detected in OS (Figure 3.9B). Interestingly, in animal Nº7 no viral RNA was detected at 10dpi in CS, while in the other animals that survived relatively low levels of viral genome was detected (Figure 3.9C).

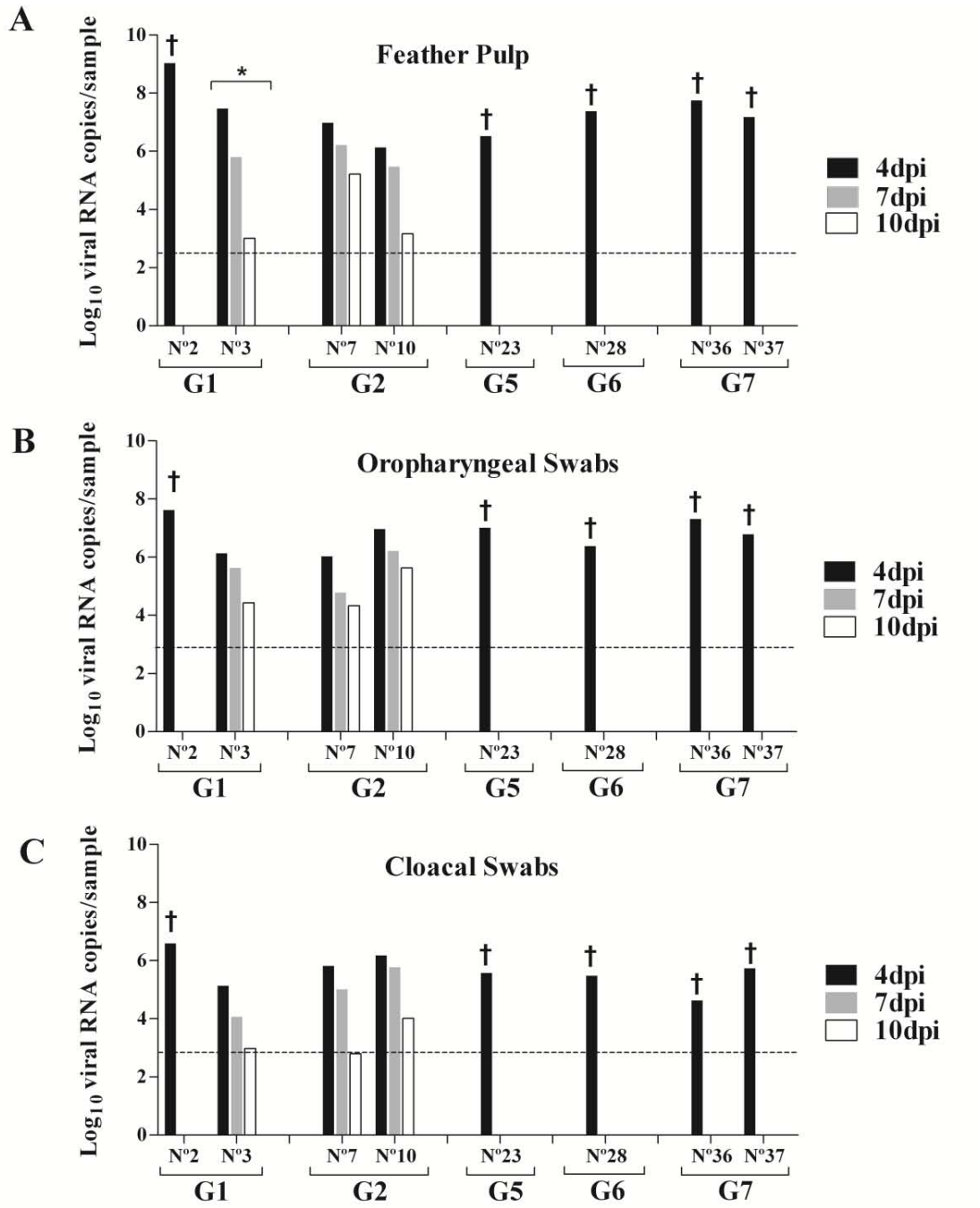

Figure 3.9 Viral RNA shedding detected by RRT-PCR in free-range chickens experimentally challenged with AIV. Viral RNA detected by RT-qPCR expressed as log_{10} viral RNA copies per sample of feather pulp (A), oropharyngeal swabs (B); and cloacal swabs (C) collected at days 4, 7 and 10 post-infection. No samples were available from G3 and G4, since animals died before the sampling. Limit of detection indicated by the dashed line (2.60 log_{10} viral RNA copies/sample). Asterisk (*) indicates statistical significant differences (P> 0.05). Cross (†) indicates animals that died after 4dpi.

3.4. DISCUSSION

In this study we show that vaccination of free-range chickens with highly conserved HA1 peptides (NG34 and SS35) elicited antibody response and conferred partial protection against a lethal challenge with HPAIV H7N1. We demonstrated that immunization with NG34 and SS35 peptides is able to induce peptide-specific antibody, which recognize heterologous H5N2 and H7N1 virus *in vitro*. Also, these antibodies were able to inhibit hemagglutination and to neutralize heterologous influenza viruses like H5N2 and H7N1.The observed cross-reactivity and recognition of heterologous antigens may in part be attributed to the information encoded by the primary structures of peptides (NG34 and SS35) and HA1 proteins of the H7N1 HPAIV and H5N2 LPAIV as represented by the common frequency component in their informational spectra shown in Figure 1. According to ISM analysis, this information represents the long-range component of the peptide–virus interaction between HA1 and a putative ligand, such as a receptor or an antibody (Krsmanovic et al., 1998; Veljkovic et al., 2003, 2004, 2009a; b; Vergara-Alert et al., 2012). Veljkovic et al., 2003 have reported previously that the informational spectra similarity between proteins is more important for their immunological cross-reactivity than their sequence similarity, as demonstrated in an experimental study with human immunodeficiency virus type 1. Based on ISM analysis, 2 mimetic peptides (NTM and VIP) were designed as potential candidates for prevention and treatment of (HIV-1), taking into account the similar frequency component in their informational spectra rather than difference in their primary structures (Veljkovic et al., 2003).Thus, we hypothesised that a similar mechanism by recognition and interaction between HA of the H7N1 HPAIV and H5N2 LPAIV and its target (HA1-peptides) could correlate with specific antibody response.

We used each synthetic peptide (NG34 and SS35) individually to evaluate the immune response and their protection potential against H7N1 HPAIV challenge in free-range chickens. NG34 seemed to be more immunogenic than SS35 in free-range chickens, but both peptides were able to induce a serum antibody response and recognize the H7N1 and H5N2 viruses *in vitro*. Moreover, we demonstrated that sera from surviving animals (previously vaccinated with NG34, Nº7 and Nº10) exhibited neutralizing antibodies and low but detectable HI activity before HPAIV challenge, possibly involved in eliminating clinical symptom and reducing virus shedding (viral load reduction of almost 2 logs in FP, OS and CS).Curiously, serum from one surviving vaccinated animal (Nº3), which showed no detectable HI activity, was also able to reduce H7N1 HPAIV shedding. Our results suggest that the low-level HI titre at 0days before challenge is boosted by the infection with the virus; inducing antibodies that are able to neutralize the virus, as it have been shown at 10dpi. Different mechanisms could explain the protection observed in this animal, for example induction of T-cell response that may eliminate influenza virus infected cells (Hillaire et al., 2011; Vergara-Alert et al., 2012). This could be caused by HA, which acts as a minor target for subtype-specific cytotoxic T lymphocytes (Zinkernagel and Doherty, 1979; Braciale et al., 1984; Bennink et al., 1986). In addition, IgA response was detected in Nº3, Nº7 and Nº10 animals, which could also contribute to survival. The mucosal immune response probably also has a role in protection from the HPAI infection because the initial exposure to the virus is through a mucosal surface(R. H. Waldman and R. H. Waldman, F. M. Wigley, P. A. Small, 1970; Tamura et al., 1990). In several studies in mouse (Chen and Quinnan, 1988; Tamura et al., 1988) and human(Murphy and Clements, 1989; Moldoveanu et al., 1995), the degree of protection against influenza correlates better with the level of secretory than serum antibodies (Shvartsman and Zykov, 1976; Chen and Quinnan, 1988; Murphy and Clements, 1989; Waldman and Bergmann, 1989).Our results also

support that high levels of secretory IgA could correlate with protected animals against challenge (Figure 5B).

The immunological cross-reactivity between SS35-peptide and H7N1 correlated very well since SS35 specific antibodies were observed in SS35 vaccinated animals prior to challenge. However, these antibodies did not inhibit hemagglutination. Surprisingly, taking into account previous predictions by ISM and recent results *in vivo* (in pig immunization studies) using the same influenza virus strains, SS35peptide was able to induce neutralizing antibodies and hemagglutinin inhibitory antibodies against different subtypes influenza. The observed differences in the ability of NG34 and SS35 antibodies to inhibit hemagglutination could probably be attributed to the replicative determinants of influenza viruses in different hosts (avian and human), in particular the preferences of sialic acid–galactose receptors(Rogers and Paulson, 1983; Couceiro et al., 1993; Ito and Kawaoka, 2000). It has been suggested that hemagglutinins from various influenza viruses differ in the ability to recognize naturally occurring sialic acid–galactose structures(Choppin and Tamm, 1960; Choppin I., 1960; Carroll et al., 1981; Paulson, 1985).One of the most striking examples is the difference in hemagglutinin receptor specificity between human and avian strains of influenza A virus(Rogers and Paulson, 1983; Rogers et al., 1983a; b; Rogers and Dsouza, 1989). Human strains preferentially agglutinate erythrocytes that contain terminal NeuAcα2,6Gal sequences, while the hemagglutinin of avian strains preferentially binds erythrocytes bearing NeuAcα2,3Gal sequences (Rogers et al., 1983a). Therefore, we assume that SS35 could have affinity to NeuAcα2,6Gal receptor, but not NeuAcα2,3Gal, whereas NG34 may show affinity to both receptors. These results suggest that the lack of a suitable receptor accounts for the inefficient protection of SS35-peptide in chickens. More experiments are needed to demonstrate this hypothesis, such as the possibility of using cell cultures blocking NeuAcα2,3Gal or NeuAcα2,6Gal receptors by

NG34 or SS35 antibodies before infection or using 3D analysis of antibody-antigen complexes to verify the binding.

Peptides generate an antibody response generally less immunogenic than live or killed whole microorganism and therefore require carrier molecules and/or adjuvants for the induction of a robust immune response (Petrovsky and Aguilar, 2004; Mohan et al., 2013). In this study, we used two adjuvants (Montanide^{TM} 71R VG and Standard W/O) and demonstrated no significant difference between the immune response induced. In agreement, Montanide^{TM} 71R VG has been described as a robust, safe and flexible adjuvant for the production of avian influenza vaccines and other poultry vaccines(Vg et al., 2013).Additionally, Liu et al.2011have also reported the use of Montanide in SPF chickens vaccinated with a recombinant H5N3 vaccine, which induced protective antibodies compared with other adjuvants, such as mineral oil (Liu et al. 2011).

Unfortunately, in this study, with such an aggressive challenge model in which a lethal dose was employed, most of the animals died. A high cross-reactivity between NG34peptide and H7N1 HPAIV correlated to survival of a few animals (n=3/10). Although, the number of surviving animals was relatively small, our results indicate that vaccination with NG34 conferred protection against H7N1, since sera collected from these animals before challenge showed a good recognition of homologous and heterologous influenza viruses. Moreover, the results of this study may be limited by a small sample size and relatively low statistical power. Consequently, we cannot demonstrate the protection efficacy of NG34 and SS35. However, to our knowledge, so far in poultry no HA1-peptide vaccines have been described. Our findings are encouraging and could help usingHA1-peptides in future universal vaccine formulations against influenza outbreaks or even pandemics.

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PART III
GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

In the framework of control programs aimed to eradicate viral diseases in animal health, the vaccines play an important role, being directly involved in the reduction and prevention of viral transmission. Consequently, there is an increasing the demand for novel vaccine, more safe and efficient and capable to induce a greater reduction of virus replication (van Oirschot, 1999).

In this context, the influenza vaccines development remains an important challenge nowadays, due to relatively long production times, moderate efficacy and the hurdle of antigenic drift and shift of viral immunogen (Jang and Seong, 2014; Soema et al., 2015). The current seasonal influenza vaccines induce neutralizing antibodies against the viral membrane surface proteins hemagglutinin (HA) and neuraminidase (NA). Due to antigenic shift and drift of HA and NA genes, neutralizing antibodies elicited by influenza vaccines lack cross-reactivity against non-matching influenza strains. While seasonal adjustments to the vaccine strains are made to cope with this problem, it is not as convenient and fast as a potential cross-protective influenza vaccine (Gerhard et al., 2006; Osterhaus et al., 2011; Pica and Palese, 2013; Wang et al., 2013; Jang and Seong, 2014).

The aforementioned limitations of current influenza vaccines may be resolved through the implementation of new technologies in the field of influenza production and vaccine formulation. Focusing on regions of viral proteins that are highly conserved across virus subtypes, vaccine strategies involving the HA1, HA2 and M2 proteins, and multivalent approaches have provided broad-based protection in animal models and show much promise (Okuno et al., 1993; Steel et al., 2010; Wang et al., 2010; Lee et al., 2014).

Currently, most efforts have been focused on the development of broadly protective universal IV vaccines that can provide a neutralizing immune

response against drifted seasonal IV strains, and also against potential pandemic viruses (Krammer and Palese, 2015).

Recent studies report that antibodies with broad neutralizing activity against multiple IV strains or subtypes bind to the stalk of the viral HA, suggesting that a vaccine based on this region could elicit a broadly protective immune response (Corti et al., 2011; Ekiert et al., 2009; Pica et al., 2012; Steel et al., 2010; Sui et al., 2009; Vareckova et al., 2003; Wang et al., 2010a).

Considering these precedents the objective of this thesis was to select conserved peptides from the hemagglutinin subunit 1 (HA1) of influenza viruses and evaluate their effective protection in different species. Here we describe NG34+DC89 synthetic peptides derived from the globular domain HA1 as multivalent vaccine candidates against IV. Although, some studies indicated that antibodies recognizing HA1 do not cross-react with the HA of other subtypes (Gocník et al., 2008). We have shown that NG34+DC89 construct is able to elicit cross-reactive antibodies against H1- and H7- IV subtypes in mice. Therefore, we can speculate that the NG34-elicited antibodies might recognize both pH1N1 and H7N1, as previously shown ISM (Vergara-Alert et al., 2012). We assume that the highly conserved domain from HA1 plays an important role in A/H1N1 and A/H7N1 receptor interaction and might result in virus neutralization. Therefore, HA1-peptides might confer protection against influenza infection. These findings correlated with SNT and HI assay, where it was shown that the H1-antibody responses were higher compared to H7-antibodies in vaccinated animals (Figure 1.7). Animals that did not show antibodies titres by HI and SNT tests were not protected to IV infection. Interestingly, the specific NG34-antibody responses were boosted after H7N1 challenge but the titres decreased after pH1N1 challenge. These results might correlate with the efficacy by which the antibodies induced are immediately sequestered after H1N1 in vivo challenge, sharing almost identical sequences. Sequence divergence between the vaccine peptides and H7N1 could be enough to promote the antibody boosting while avoiding the formation of antibody-antigen solid complexes (Depner et al., 1995; Gocník et al., 2008). The neutralizing and virus titration data seems to confirm this hypothesis.

Our results herein with homologous and heterologous viral challenge indicate that NG34+DC89 might be a potential candidate that could further be optimized or adapted to desired vaccine preparation or immunity (cellular or humoral) and improve in other species, thus obtaining a stronger immunity and higher antibody titers (before infection), that could confer better protection against all types of influenza viruses, including those emergent pandemic strains (Du et al., 2010; Watanabe et al., 2013; Watanabe et al., 2012).

On the other hand, the ideal multivalent influenza vaccine will confer protection against different virus subtypes. In this regards, we used the ISM bioinformatic tool to select four (NG34, DC55, RA22 and SS35) peptides from within the hemagglutinin subunit 1 protein (HA1) from H1 viruses. The immunogenicity of these peptides was evaluated in conventional farm pigs against homologous and heterologous viruses of influenza. In this study we show here that the peptide immunization not only induces antibodies that can block viral entry into the cell and inhibit hemagglutination of homologous viruses, but also heterologous viruses, as H7N1 and H5N2, and importantly the circulating H3N2 virus.

Pigs are natural hosts for a variety of genetically and antigenically diverse H1 and H3 viruses. However to date, few studies in pigs (Heinen et al., 2001; Reeth and Ma, 2013), ferrets (Yetter et al., 1980; Laurie et al., 2010) and mice (Liang et al., 1994; Kreijtz et al., 2007) have reported a complete protection between H1 and H3 viruses. Heinen et al. (2001) reported that no cross-reactive HI antibodies from SIV H1N1-infected pigs were observed with SIV H3N2 or vice versa after primary infection (Heinen et al., 2001). Later, Van Reeth et al. (2003) demonstrated protection against a H1N2 swine influenza virus in pigs previously infected with H1N1 and H3N2 subtypes, independently of HI and SN antibodies against H1N2 (Van Reeth et al., 2003). Antibodies inhibiting the H1N2 NA were, in contrast, consistently observed in these pigs, despite the clear distinction between NA antibodies induced by H1N2 or H3N2 infection (Van Reeth et al., 2003). Although antibodies against NA contribute to decrease the

amount of virus released from infected cells, they are unable to neutralise the virus and prevent initiation of infection like the HA antibodies. Our results not only show that HA-peptides selected from HA1 protein can induce cross-reactive antibodies against H1 SIV, pH1N1, H3N2, H7N1 and H5N2 subtypes; but also promote secretory IgA-specific antibodies in nasal swabs. Thus, well-matched IgG and IgA specific antibodies against the HA could potentially prevent an influenza virus infection, and may contribute to clearing the virus from the lungs, as reported previously (Waffarn and Baumgarth, 2011; Vergara-Alert et al., 2012). Therefore, the secretory IgA production in the nasal cavity may participate as principal mediator of the upper respiratory tract immunity, whereas IgG from serum may contribute mostly to lower respiratory tract immunity. However, substantial IgA levels have also been found in BAL fluids of pigs, and in lung lysates of mice, suggesting that IgA antibodies may also be locally produced in the lung (Wang et al., 2004; Kitikoon et al., 2006; Khatri et al., 2010) .

The immunogenic effect of HA1-peptides for IgA levels at nasal swabs and the SN or HI cross-reactive antibodies generated after immunization in pigs would suggest that humoral responses with cross-neutralizing activity in sera could play a major role in conferring protective heterosubtypic immunity. However, the protective effect of these peptides must be tested in different natural hosts of the virus, followed by homologous and heterologous challenges. For that reason our hypothesis is also being tested in chicken, which is critical for the reduction of potential cross-species adaptation and spread of influenza viruses, and will minimize the risk of animals being the source of future pandemics.

We also have studied the effect of the highly conserved HA1 peptides (NG34 and SS35) in free-range chickens. The vaccinated animals elicited antibody response and were partial protected against a lethal challenge with HPAIV H7N1. The immunization of animals with NG34 and SS35 peptides is able to induce peptide-specific antibody, which recognize heterologous H5N2 and H7N1 virus *in vitro*. Also, these antibodies were able to inhibit hemagglutination and to neutralize heterologous influenza viruses like H5N2 and H7N1. The observed cross-reactivity and recognition of heterologous

antigens may in part be attributed to the information encoded by the primary structures of peptides (NG34 and SS35) and HA1 proteins of the H7N1 HPAIV and H5N2 LPAIV as represented by the common frequency component in their informational spectra shown in Figure 1. According to ISM analysis, this information represents the long-range component of the peptide–virus interaction between HA1 and a putative ligand, such as a receptor or an antibody (Krsmanovic et al., 1998; Veljkovic et al., 2003, 2004, 2009a; b; Vergara-Alert et al., 2012).

Moreover, we demonstrated that sera from surviving chickens exhibited neutralizing antibodies and low but detectable HI activity before HPAIV challenge, possibly involved in eliminating clinical symptom and reducing virus shedding (viral load reduction of almost 2 logs in FP, OS and CS). Our results suggest that the low-level HI titre at 0days before challenge is boosted by the infection with the virus; inducing antibodies that are able to neutralize the virus, as it have been shown at 10dpi. Different mechanisms could explain the protection observed in this animal, for example induction of T-cell response that may eliminate influenza virus infected cells (Hillaire et al., 2011; Vergara-Alert et al., 2012). This could be caused by HA, which acts as a minor target for subtype-specific cytotoxic T lymphocytes (Zinkernagel and Doherty, 1979; Braciale et al., 1984; Bennink et al., 1986). In addition, IgA response was detected in some surviving animals, which could also contribute to survival. The mucosal immune response probably also has a role in protection from the HPAI infection because the initial exposure to the virus is through a mucosal surface(R. H. Waldman and R. H. Waldman, F. M. Wigley, P. A. Small, 1970; Tamura et al., 1990). In several studies in mouse (Chen and Quinnan, 1988; Tamura et al., 1988) and human(Murphy and Clements, 1989; Moldoveanu et al., 1995), the degree of protection against influenza correlates better with the level of secretory than serum antibodies (Shvartsman and Zykov, 1976; Chen and Quinnan, 1988; Murphy and Clements, 1989; Waldman and Bergmann, 1989). Our results also support that high levels of secretory IgA could correlate with protected animals against challenge (Figure 5B). In this surviving animals, peptide vaccine candidates generate an antibody response generally less immunogenic than live or killed whole

microorganism and therefore require carrier molecules and/or adjuvants for the induction of a robust immune response (Petrovsky and Aguilar, 2004; Mohan et al., 2013).

In the present thesis has been designed, characterized and validated a multivalent vaccine candidates capable of conferring an immune response against influenza virus in three different animal species. The resulting immune response after vaccination with this vaccine prototype can be associated with promising levels of virological and clinical protection in chickens, mice and pigs.

The experiments performed have involved a limited number of animals. Some essential aspects of the immune responses induced by the vaccine candidates remain unknown, as well as some of the mechanisms involved in the partial protection obtained in the three animal models used. Possibly, using other antigenic presentation strategies could improve the protection levels obtained. However, the finding of a multivalent strategy, capable of conferring an acceptable protection level in three different species is promising for the future development of an optimal broad-spectrum vaccine against influenza virus.

CONCLUSIONS

1. All studied HA1 peptides are immunogenic in all the animal models tested and induced humoral and mucosal immune response.

2. NG34+DC89 vaccination elicited cross-reactive antibodies against the pandemic H1N1 2009 (pH1N1) virus and the H7N1 highly pathogenic influenza virus (HPAIV) in mice.

3. Vaccination with NG34+DC89 peptides confer partial protection against the pandemic H1N1 2009 (pH1N1) virus and a H7N1 highly pathogenic influenza virus (HPAIV) in mice.

4. The NG34, DC55, RA22 and SS35 peptides in vaccinated pigs induced specific HA-antibodies capable to neutralize and inhibit homologous and heterologous viruses like the two avian strains H7N1 HPAIV and H5N2 LPAIV and the circulating H3N2.

5. NG34 and SS35 peptides induced specific antibodies in free-range chicken that recognized heterologous viruses, as H7N1 HPAIV and H5N2 (LPAIV).

6. Free-range chickens vaccinated with NG34 were partially protected against lethal challenge with H7N1 HPAIV. In contrast, animals vaccinated with SS35 were not protected.

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