ABSTRACT

Title of Document: MOLECULAR PATHOGENESIS OF INFLUENZA IN SWINE AND ENGINEERING OF NOVEL RECOMBINANT INFLUENZA VIRUSES.

**Lindomar Jose Pena, Ph.D., 2011**

Directed By: **Dr. Daniel R. Perez,**
**Associate Professor**
**Department of Veterinary Medicine**

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae* and represent major pathogens of both humans and animals. Swine influenza virus is an important pathogen that affects not only the swine industry, but also represents a constant threat to the turkey industry and is of particular concern to public health. In North America, H3N2 triple reassortant (TR) IAVs first emerged in 1998 and have since become endemic in swine populations. In the first part of this dissertation, we focused on the role of surface glycoproteins and PB1-F2 to unravel their roles in the virulence of TR IAVs in this important natural host. We found that surface glycoproteins are necessary and sufficient for the lung pathology, whereas the internal genes play a major role in the febrile response induced by TR H3N2 IAVs in swine. With respect to PB1-F2, we found that PB1-F2 exerts pleiotropic effects in the swine host, which are expressed in a strain-dependent manner. Pathogenicity studies in swine revealed that the presence of PB1-F2 leads the following effects in context of three TR strains tested: no effect in the context of sw/99 strain; increases the virulence of pH1N1; and decreases the virulence of ty/04. Next, we developed temperature-sensitive live attenuated influenza vaccines for use in swine and shown that these vaccines are safe and

efficacious against aggressive intratracheal challenge with pH1N1. Lastly, we rearranged the genome of an avian H9N2 influenza virus to generate replication-competent influenza virus vectors that provide a robust system for expression and delivery of foreign genes. As a proof-of-principle, we expressed the hemagglutinin from a prototypical highly pathogenic avian influenza virus (HPAIV) H5N1 and shown that this vectored H5 vaccine retained its safety properties in avian and mammalian species, and induced excellent protection against aggressive HPAIV H5N1 challenges in both mice and ferrets. Taken together, these studies have advanced our understanding of molecular basis of pathogenesis of influenza in the swine host and have contributed to the development of improved vaccines and influenza-based vectors with potential applications in both human and veterinary medicine.

# MOLECULAR PATHOGENESIS OF INFLUENZA IN SWINE AND ENGINEERING OF NOVEL RECOMBINANT INFLUENZA VIRUSES

By

Lindomar Jose Pena

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

Advisory Committee:
Professor Daniel R. Perez, Chair
Professor Siba K. Samal
Professor Xiaoping Zhu
Professor Yanjin Zhang
Professor Jeffrey J. DeStefano

# Foreword

***Always trust***

*Don't lose your faith amidst the shadows of the world.*
*Even if your feet bleed, keep going, raising your faith to the celestial light above you.*
*Be faithful and work.*
*Strive for kindness and wait patiently.*
*Everything goes by and everything is renovated on Earth, but what comes from Heaven will remain forever.*
*Out of all miserable, the unhappiest ones are those who lost faith in God and in themselves, because the worst misfortune is to be deprived of faith and continue living.*
*Therefore look up and walk ahead.*
*Strive and serve. Learn and step ahead.*
*Dawn shines beyond the night.*
*Today the storm might tighten your heart and torment your ideal, inflicting distress and threatening with death.*
*Don't forget, however, that tomorrow will be another day.*

***By Chico Xavier***

# Dedication

I dedicate this dissertation to my father, Domingos (*in memoriam*); my mother, Maria; my brother, Adriano; and my sisters, Edilene (*in memoriam*) and Darlene.

You are my fortress, my love and my reason to live.
I love you from the bottom of my heart.

# Acknowledgements

First and foremost I would like to thank my doctoral advisor, Dr. Daniel Perez, for his guidance and assistance in each step during this journey. I also would like to thank him for sharing his knowledge and research interests with me and for giving me the opportunity to be part of his research team. I want to thank him for his help in experimental design, data interpretation, and scientific writing. I thank him for encouraging me to always pursue research questions that will move the field forward. I would not have been able to achieve this great honor without his financial, personal, and intellectual support. Thank you for always being there for anything I needed.

I also would like to thank Drs. Jeffrey DeStefano, Siba Samal, Xiaoping Zhu, and Yanjin Zhang for their time, support, and advice as my graduate advisory committee.

I thank specially Dr. Siba Samal, chairman of the Department of Veterinary Medicine, for his support, advice, and his soothing words always when I needed them. I will be forever grateful to all your efforts to make my stay more pleasant at VetMed.

I would like to thank Dr. Marvin Grubman, lead scientist at the USDA-Plum Island Animal Disease Center, for his friendship, encouragement, and for giving me the necessary confidence to follow this path. I will never forget the 25 months I worked in your laboratory; each day was a beautiful and rewarding experience. I thank him for being a constant presence in my life even after my departure from Plum Island. Thank you for showing me that to be a top scientist, one must not forget to be a good human being by cultivating good values, such as ethics and respect to others and eradicating the detrimental ones, such as competitiveness and selfishness. I want to thank him for always treating me fairly, respecting me, trusting me, and for being an example of scientist and human being that I hope to one day provide an equally excellent model for every person I meet.

Additional appreciation goes to my collaborators and contributors at the USDA-National Animal Disease Center (NADC), where I did part of my Ph.D. work. I would like to thank Amy L. Vincent, Kelly Larger, Marcus Kehrli, Alessio Lorusso,

Crystal L. Loving, Jamie Henningson, Michelle Harland, Philip C. Gauger, Hillary Horst, and the NADC animal caretakers for all their contributions to my dissertation. Thank you for hosting me, for training me, and allowing me to experience one of the most pleasant moments of my Ph.D. studies.

I want to express my appreciation to all past and current lab members for their valuable help and support during this project. A thank you also goes out to all my fellow graduate students who have made this time easier. Further thanks go to all postdoctoral fellows, staff and faculty members of the Department of Veterinary Medicine for their support, generosity, advice and pleasant company.

I want to thank Skip Haskins for his friendship and for voluntarily teaching me English during two years. Thank you for dedicating your Saturday mornings to teach me your language and embracing me as a member of your family. Without your help I wouldn't have been able to pass the GRE and TOEFL examinations, which were fundamental for my admittance into the Veterinary Medical Sciences (VMSC) program.

It is also a pleasure to thank Edmilson, Neide and their family for their friendship, support and for incorporating me into their family during my stay in the US. I also would like to thank all my friends for their encouragement and support. Thank you for always being there for me.

I would like to thank Ana Silvia for her love, companionship, serenity, and high moral values. I want to thank you for all the moments we spent together. You have reiterated on me the values and principles with which I have built my character and personality. I love you very much!

Most importantly, none of this would have been possible without the love, sacrifice and support of my family. I want to thank my father, Domingos *(in memoriam*), for his example, constant advices, integrity, and for fomenting on me the passion for animals. I thank my mother, Maria, for her unconditional love and example of strength and sweetness. You both showed me to never limit myself and never give up on my values and principles. A special word of appreciation goes to my brother, Adriano, for his love, friendship, support, encouragement, and for taking care of our parents while I was abroad pursuing my studies. I am very thankful to my

sisters, Edilene (*in memoriam*) and Darlene, for their love, support, and motivation. Also, I thank my aunt Efigênia for her constant presence in our lives and my niece Mariana for bringing more happiness to our family. I love you all from the bottom of my heart.

I thank Chico Xavier whom, through his books and life example, helped me to believe in the power of faith and to continue on the righteous path. Finally, I thank God and Jesus Christ for not letting me to lose faith during the times of torments and injustices and for being my anchor to help me overcome all the obstacles with determination and composure.

Thanks to all of you who made my dream come true.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1: Introduction

## *1.1. General Introduction*

Influenza A viruses belong to the family *Orthomyxoviridae* and represent major pathogens of both humans and animals. There are five different genera in the Orthomyxoviridae family: the *Influenzavirus A*, *B* and *C*; *Thogotovirus*; and *Isavirus*. Influenza A viruses are enveloped viruses with 80-120 nm in diameter and its nucleocapsid form a helical symmetry. Its genome contains eight single-stranded RNA segments of negative polarity (vRNA), which encode 10-12 viral proteins. Each viral RNA segments exists as a viral ribonucleoprotein complex (vRNPs), in which the RNA is coated with NP and forms a helical hairpin that is bound on one end by the hererotrimeric polymerase complex formed by PB2, PB1, and PA. For each genomic viral RNA segment, the coding region is flanked by non-coding sequences on both ends. The 5' and 3'terminal sequences of the vRNA molecules together constitute the promoter structure, in a stepwise interaction with viral RNA polymerase complex (Sorrell, Ramirez-Nieto et al. 2007).

The virus envelope is derived from the host cellular plasma membrane and it is covered with about 500 projecting glycoprotein spikes with hemagglutinating and neuraminidase activities. These activities correspond to the two major surface viral glycoproteins: the hemagglutinin (HA) and neuraminidase (NA), present as homotrimers and homotetramers, respectively. To date, 16 HA and 9 NA subtypes have been described. These surface glycoproteins are the main antigenic determinants of the virus. The HA is the viral ligand that recognizes sialic acid receptors present on glycoproteins and glycolipids on the cells laying the mucosa of the respiratory and intestinal tract. Binding to host receptors is the first step in the viral life cycle and this property makes the HA a major contributor to the host range of influenza viruses. Receptor-bound viruses enter into the cell by endocytosis followed by acid-activated membrane fusion in endosomes. When the endosomal pH reaches 5.0, the viral HA protein undergoes a conformational change that exposes the fusion peptide on the HA and causes it to fuse with the viral envelope. When this occurs, the viral ribonucleoprotein particles (vRNPs) are released from matrix protein 1 (M1) and released into the cytoplasm. RNPs are then imported into the nucleus, where transcription and

replication of the influenza genome occurs. New viral RNA is encapsidated in the capsid protein, and together with new M1 is then transported to budding sites at the plasma membrane where HA and NA have been incorporated into the cell membrane. Progeny virions are formed and released by budding. The sialidase activity of the NA, which disrupts the interaction between the HA and sialic acid receptors promotes the release and spread of progeny viral particles following their production by infected cells (Palese and Shaw 2007).

In cells infected with certain strains of influenza, a nonstructural protein named PB1-F2 can be detected (Chen, Calvo et al. 2001). This protein is 87-101-amino acid long and it is coded by an alternate (+1) open reading frame within the PB1 gene (segment 2). PB1-F2 is not required for viral replication in ovo or in tissue culture. Translation of PB1-F2 is thought to be mediated by ribosomal scanning of the PB1 mRNA (Chen, Calvo et al. 2001). PB1-F2 expression causes apoptotic-cell death in immune cells (mainly monocyte-derived) and contributes to viral pathogenicity by virtue of inhibiting mechanisms of viral clearance (Zamarin, Ortigoza et al. 2006; McAuley, Hornung et al. 2007). Additionally, the presence of PB1-F2 in both the 1918 and the mouse-adapted PR8 influenza A virus enhances secondary bacterial pneumonia in the mouse model (McAuley, Hornung et al. 2007). However, two recent studies investigating the role of PB1-F2 in the pathogenesis of clinically relevant viruses and the pandemic H1N1 concluded that expression of PB1-F2 has minimum effects in the virulence of influenza viruses in animal models (Hai, Schmolke et al. ; McAuley, Zhang et al. 2010). From these studies, it appears that the role of PB1-F2 in virulence of influenza viruses is cell-type and virus strain specific. The contribution of PB1-F2 to virulence of influenza viruses in the swine host is completely unknown.

Swine influenza virus (SIV) is an infectious disease which affects not only the swine industry but also represents a constant threat to the turkey industry and is of particular concern to public health. H3N2 triple reassortant (TR) swine influenza viruses first emerged in the United States in 1998 and have since become endemic in swine populations. A unique feature shared by all of these TR influenza viruses is the maintenance of the so-called triple reassortant internal gene (TRIG) cassette, which consists of the avian-like PB2 and PA genes, the human-like PB1 gene and the classical swine NP, M and NS genes. Fever, sneezing, coughing, and abortion rates up to 7% in breeding sows characterize the disease caused by these viruses. Since 2003-2004, however, the H3N2 TR swine influenza viruses have emerged and spread within commercial turkey operations in the US, causing drastic declines in egg production. Although virulence determinants for avian and human influenza viruses are well-characterized, little is known about the molecular markers that modulate the

pathogenesis of influenza viruses in swine. Therefore, there is a need to understand the molecular basis of pathogenesis in this important natural host (Olsen 2002; Vincent, Ma et al. 2008).

In the swine industry, influenza is controlled mainly by biosecurity and vaccination. Currently commercially available swine influenza vaccines are based on inactivated whole virus preparations of the H1N1 and H3N2 subtypes. The use of vaccination has become a common practice to control swine influenza in the US. Nonetheless, these commercially available vaccines provide limited protection against the antigenically diverse influenza viruses that circulate in North American pigs, and consequently, forced swine producers to use autogenous inactivated vaccines with the hope of achieving better antigenic matching. However, it is important to note that the use of inactivated vaccines has been associated with enhanced pneumonia when immunized pigs were challenged with divergent viruses (Vincent, Ma et al. 2008). Even though temperature-sensitive live attenuated influenza vaccines (LAIV) have been shown to be safe and efficacious for humans and horses, this attenuation approach have not yet been developed for pigs. Thus, the development of LAIV for swine has the potential to circumvent the drawbacks associated with commercial vaccines and improve both the homotypic and heterosubtypic protection of pigs against this important swine pathogen.

The generation of viral vectors for the delivery of foreign proteins and biologically active molecules remains an attractive approach with broad applications in the infectious disease field. Since the development of reverse genetic methods for segmented negative sense RNA viruses, influenza has also been considered potential vaccine vectors. The use of influenza viruses as vector for delivery of foreign genes with immunoprophylactic and therapeutic applications has several advantages: (a) influenza viruses are known to induce strong cellular and humoral immune responses both at the systemic and the mucosal levels; (b) Because the virus has an RNA genome without a DNA phase in its replication cycle, safety concerns regarding chromosomal integration of viral genes into the host are virtually inexistent.(c) influenza viruses have 16 different HA and 9 different NA, most of which only circulate in wild birds and against which humans and domestic animals don't have pre-existing immunity (Kittel, Sereinig et al. 2004; Martínez-Sobrido and García-Sastre 2007). Thus, using influenza viruses with different HA/NA combinations can circumvent the problem of boost vaccinations in populations that have pre-existing immunity against the vector; (d) influenza viruses are very well characterized and attenuated strains are used as live vaccines for humans and livestock. Additionally, engineering of highly attenuated influenza viruses can be easily accomplished by introducing some of the several attenuating mutations

that have already been mapped in the viral genome.(e) expression of foreign gene under the control of the NS gene segment promoters allows early and high levels of expression of the transgene of interest. However, all the influenza virus vectors developed to date have been either unstable or have tolerated only insertions of short peptides. Among all the viral proteins, only the HA and NA have been successfully engineered for stable expression of foreign epitopes (Martínez-Sobrido and García-Sastre 2007). Nevertheless, neither HA nor NA glycoproteins are optimal targets for the insertion of foreign sequences because of insert size constraints and the fact that booster immunizations would be affected by pre-existing immunity to the vector. Thus, both the instability of the transgene and the insert size limitation has precluded the progress in the development of influenza virus as a vector for delivery of foreign genes.

## *1.2. Research Objectives and Outline*

The main goal of my dissertation research is to dissect virulence determinates of influenza virus for swine and develop novel live attenuated influenza vaccines. With respect to the first objective, we focused on the role of surface glycoproteins and PB1-F2 to study, for the first time, the role of these genes in virulence of triple reassortant influenza viruses in pigs. These studies will advance the scientific knowledge in the influenza field and will hopefully shed light for the rational development of vaccines against this important swine disease. Regarding novel vaccines, we have developed a temperature sensitive (*ts*) live attenuated vaccine for use in pigs and also rearranged the genome of an avian influenza virus to develop a vector-influenza vaccine against the highly pathogenic avian influenza virus H5N1. Reverse genetics technology will be used to introduce attenuation markers in the polymerase genes of a swine-like TR H3N2 influenza virus, A/turkey/Ohio/313053/04 (H3N2) (ty/04), with the ultimate goal of developing LAIV against epidemiologically important viruses for swine. Lastly, we have developed a novel strategy for engineering influenza viruses that stably expresses foreign genes. Influenza viruses generated using this technology have the potential for generating improved vaccines against influenza and other pathogenic agents as well to facilitate studies regarding influenza pathogenesis in several biological systems.

The research objectives of my dissertation are as follows:

1- **Role of surface glycoproteins in the virulence of triple reassortant H3N2 influenza viruses in swine**

   A) Development of reverse genetics system for generation of triple reassortant H3N2 influenza A viruses from cloned cDNAs

   B) Development of a swine model of infection to study the virulence of H3N2 triple reassortant influenza A viruses

   C) Role of surface proteins in the lung pathology of triple reassortant H3N2 influenza viruses in swine

2- **Role of PB1-F2 in the virulence of triple reassortant H3N2 and pandemic H1N1 influenza viruses in swine**

   A) Construction and rescue of PB1-F2 recombinant viruses

   B) Effect of PB1-F2 expression in modulating polymerase activity

   C) Use of porcine respiratory explants to study the interaction of influenza with the respiratory tract

   D) Pathogenesis studies in swine

3- **Modifications in the polymerase genes of a swine-like triple reassortant influenza virus to generate live attenuated vaccines for humans and livestock**

   A) Construction and phenotypic characterization of LAIV in vitro

   B) Safety and efficacy of LAIV against H1N1pdm in mice and swine

4- **Shuffling of the influenza genome to engineer vectors stably expressing foreign genes**

   A) Engineering of influenza virus vectors by genome rearrangement

   B) Characterization of influenza virus vectors expressing foreign genes

   C) Safety studies in chickens

   D) Evaluate the safety and the efficacy of an H9N2 vector expressing the H5 HA against a highly pathogenic H5N1 challenge in both mice and ferrets

# Chapter 2: Literature Review

## *2.1. Influenza Virus*

### 2.1.1. Discovery of the influenza A viruses

The first written description of infection by Influenza A virus probably occurred in 1878, when Perroncito described "fowl plague" as a highly pathogenic disease affecting chickens in Italy (Perroncito 1878). This was the first document that differentiated fowl plague and fowl cholera based on clinical disease. A few years later, in 1901, Centanni and Savonuzzi, demonstrated that fowl plague (i.e. highly pathogenic avian influenza virus) was caused by a filterable agent (prototype strain A/Brescia/1902 (H7N7)) (Centanni and Savonuzzi 1901). Influenza in swine was first recognized as an epizootic disease in 1918, concomitant with the worst recorded human influenza pandemic. The viral etiology of the disease in pigs was determined for the first time in 1930 by Richard Shope, who conclusively demonstrated the filterability of agent responsible for the respiratory disease in pigs and isolated, for the first time, an influenza A virus (prototype strain: A/swine/Iowa/15/1930 (H1N1)) (Shope 1931; Shope 1931). Human influenza was first identified as a viral disease in 1933 by Smith and co-workers (prototype strain A/WS/33 (H1N1)) (Smith 1933). However, It was not until 1955 that it was demonstrated that the genomic composition of the fowl plague virus was essentially identical to the human influenza virus (Schafer 1955). By then, it had already been shown that human influenza viruses shared many biological properties with their avian counterparts, including the ability to grow in embryonated chicken eggs and agglutinate red blood cells (Hirst 1941). Influenza's ability to agglutinate erythrocytes is a feature very exploited in diagnostic tests. Equine influenza virus was first isolated in the former Checoslovaquia in 1956 (prototype strain: A/equine/Prague/1/56 (H7N7)) (Sovinova, Tumova et al. 1958) and, later, the more epidemiologically relevant H3N8 subtype of equine influenza virus was first isolated in 1963 during an outbreak of this disease in Miami (prototype strain: A/equine/Miami/1/63) (Waddell, Teigland et al. 1963). In a remarkable host shift event, an H3N8 of equine-origin caused severe outbreaks of respiratory disease in dogs in 2004 (Crawford, Dubovi et al. 2005). Since then, canine influenza virus (prototype strain A/canine/Florida/43/2004 (H3N8)) has become enzootic in this animal population (Harder and Vahlenkamp 2010).

### 2.1.2. Classification and nomenclature

Influenza A virus (IAV) belongs to the family *Orthomyxoviridae*, a family of enveloped viruses whose members contain six to eight segmented, single-strand RNA genomes with negative orientation. The name of the family is derived from the Greek *orthos*, which means correct or straight, and *myxa*, which means mucus. The family of *Orthomyxoviridae* comprises five different genera: the *Influenzavirus A, Influenzavirus B, Influenzavirus C*, *Thogotovirus,* and *Isovirus*. Recently, three novel viruses (Quaranfil, Johnston Atoll, and Lake Chad viruses) have been proposed to form a novel genus of the family *Orthomyxoviridae* (Presti, Zhao et al. 2009). This recommendation was based on phylogenetic analyses, virion ultrastructural features, and the unique end-terminal sequences identified in these viruses. Influenza viruses type A, B and C are distinguished according to antigenic differences between their internal nucleocapsid (NP) and matrix (M) proteins. Type A viruses (eight RNA segments) display broad host range and these viruses can infect many avian and mammalian species. These viruses are responsible for worldwide respiratory disease epidemics in humans and are associated with thousands of deaths annually. Type B viruses (eight RNA segments) are mainly human pathogens, although infection has been described in seals (Osterhaus, Rimmelzwaan et al. 2000). Type C viruses (seven RNA segments) can affect humans and swine, but infection is usually of low consequence (Muraki and Hongo 2010). The members of the *Thogotovirus* genus are a group of arboviruses that are transmitted by ticks, which includes the thogoto virus (six RNA segments), Dhori virus (seven RNA segments), and a variant known as Batken virus. Thogoto virus has been associated with febrile encephalic disease in humans (Siebler, Haller et al. 1996). Araguari virus (six RNA segments), is a less studied member of the genus *Thogotovirus* that was isolated form a marsupial (*Philander opossum*) in the Brazilian Amazon rainforest (Da Silva, Da Rosa et al. 2005). Infectious salmon anemia virus is the only member of the *Isovirus* genus (eight RNA segments) and causes devastating disease in farmed Atlantic salmon. Infectious salmon anemia is one of just 12 viral diseases of fish reportable to the OIE, the World Organization for Animal Health (Clouthier, Rector et al. 2002).

Nomenclature system for different influenza virus is based on: (1) host of origin, (2) geographic origin, (3) strain number, (4) year of isolation, and (5) antigenic subtype. For human strains, the host is omitted (Palese and Shaw 2007). For example, the nomenclature A/turkey/Ohio/313053/04 (H3N2) represents a type A virus isolated from a turkey in Ohio as

a virus strain number 313053 in the year of 2004. According to its HA and NA composition, the virus is classified as H3N2 subtype.

### 2.1.3. Morphology and genome organization

Influenza A virus (IAV) particles are pleomorphic, although filamentous virions are the most predominantly observed during initial virus isolation of field samples. Laboratory-adapted propagated in eggs or cell cultures have more regular spherical morphology (Fig. 2.1). IAV are surrounded by a host-derived lipid bilayer envelope, with protruding spikes formed by the two outer glycoproteins: hemagglutinin (HA) and neuraminidase (NA), and a small number of the M2 ion channel protein (Nayak, Hui et al. 2004). The M1 protein plays a pivotal role in virus architecture (Bourmakina and Garcia-Sastre 2003; Elleman and Barclay 2004). Deletion of the cytoplasmic tail of M2, HA, or NA also alters virion morphology from spherical/elliptical to irregular shape (Jin, Leser et al. 1997; Iwatsuki-Horimoto, Horimoto et al. 2006). The interactions among M1, M2, HA, and NA are important for the formation and preservation of the characteristic virion shapes. Morphological alterations in virions can be caused by artifacts introduced during sample preparation for negative staining, such as air drying (Nermut and Frank 1971), low pH (Ruigrok, Hewat et al. 1992), and ultracentrifugation (Sugita, Noda et al. 2011).

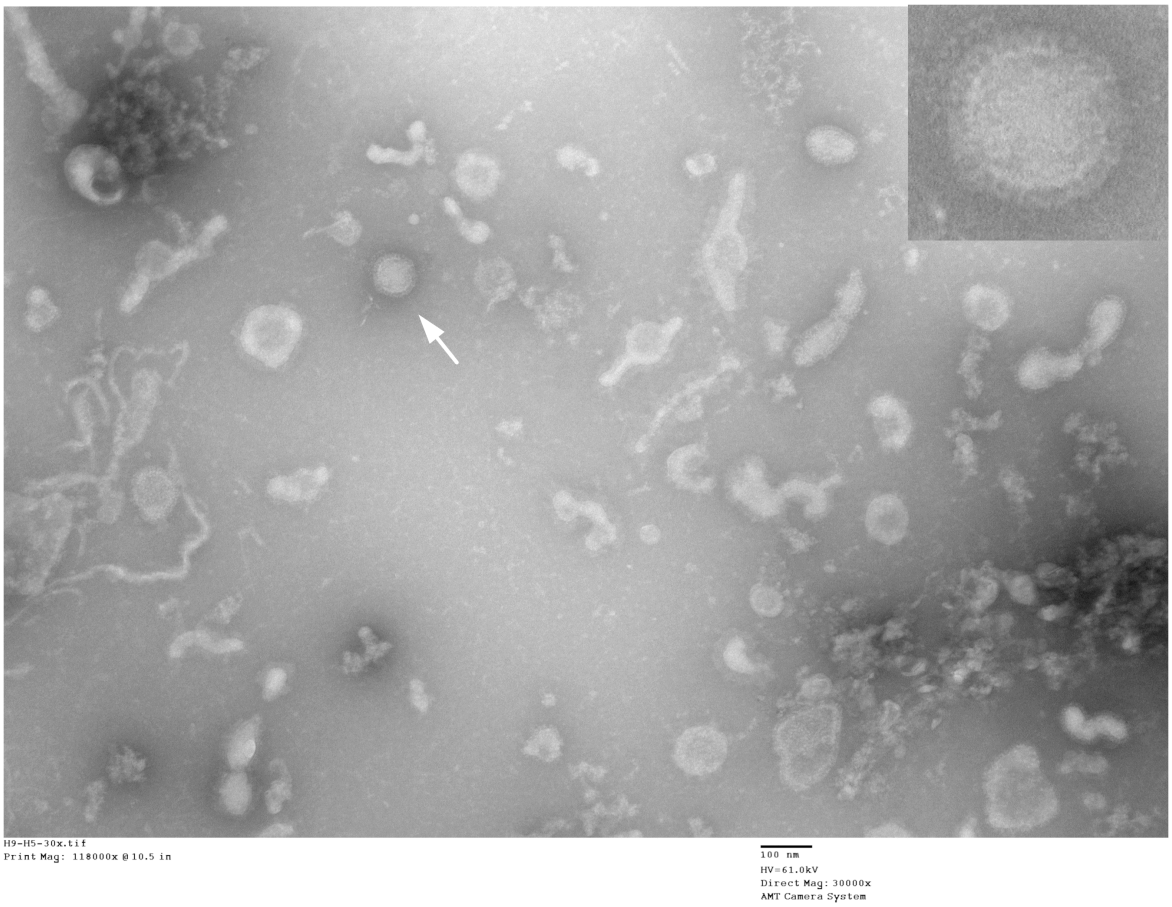



**Figure 2.1.Transmission electron microscopy of influenza A virus particles**. The avian influenza virus strain A/Guinea Fowl/Hong Kong/WF10/99 (H9N2) was propagated in embryonated eggs, purified and negatively stained with 2% phosphotungstic acid (PTA). The arrow indicates a spherical virion (inset). The lipid bilayer is not visible in the intact particles, but HA and NA glycoprotein spikes are seen as electron-dense areas.

The HA is a type I transmembrane protein that forms homotrimers in mature virions. Each monomer consists of an outer HA1 subunit covalently linked through disulfide bonds to a membrane-anchoring HA2 subunit (Cross, Burleigh et al. 2001). The HA protein is a multifunctional protein responsible for binding to receptors on host cells, membrane fusion, and induction of neutralizing antibodies. Each virion has approximately 500 radial projections of glycoproteins spikes in its envelope (Ruigrok, Andree et al. 1984). The HA is a major envelope protein that accounts for about 80% of the spikes (the NA forms the remaining 20% of the spikes) (Cross, Burleigh et al. 2001).

The NA is a type II transmembrane protein that is assembled as homotetramers in the virions. The sialidase activity of NA removes sialic acids from sialyloligosaccharides in the host cells and is critical for the release and spread of progeny virions at the final stage of infection (Gong, Xu et al. 2007). Recently, it was shown that NA also plays an important role early during the viral infection, presumably by removal of mucus covering the airways epithelium (Matrosovich, Matrosovich et al. 2004). The receptor-destroying activity of the NA serves to counter the receptor-binding activity of the HA. Although mutant influenza virus lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice, compensatory mutations are introduced in the HA receptor-binding pocket to reduce the virus's affinity for cellular receptors (Hughes, Matrosovich et al. 2000). Furthermore, the balance between the HA and NA activities are crucial for influenza virus replication, virulence, and transmission (Mitnaul, Matrosovich et al. 2000; Hulse, Webster et al. 2004; Lu, Zhou et al. 2005; Yen, Liang et al. 2011). Influenza A viruses are classified into subtypes based on two surface antigens, the HA and NA glycoproteins. To date, 16 HA and 9 NA subtypes have been described (Fouchier, Munster et al. 2005). Wild aquatic birds are considered the natural reservoirs of all influenza A viruses, in which all the HA and NA subtypes have been found. In these birds, influenza viruses usually replicate in the intestinal tract, cause no disease, and transmit by fecal contamination of water. These viruses occasionally establish stable lineages in terrestrial birds as well as mammalian species such as humans, swine, horses, and dogs (Webster, Bean et al. 1992; Fouchier, Munster et al. 2005; Gibbs and Anderson 2010).

The M2 protein, a type III transmembrane protein, is the third virus membrane integral protein, which is found in the form of homotetramers (Lamb, Zebedee et al. 1985; Lear 2003). M2 is a minor component of the envelope, but plays a crucial role during uncoating through its proton pump activity (Lear 2003). The inner core of the virion consists

of M1 matrix protein and ribonucleoprotein (RNP) with helical symmetry. The matrix protein (M1) is the most abundant protein in the IAV virion and is located in the inner surface of the lipid bilayer. By coating the inner face of the envelope, M1 gives stability to the interior structure of the emerging viral particle. Furthermore, M1 interacts with the cytoplasmic tails of the viral integral membrane proteins HA, NA, M2 and serves as a docking site for the recruitment of the viral RNPs (Ali, Avalos et al. 2000; Nayak, Hui et al. 2004; Chen, Leser et al. 2008; Rossman and Lamb 2011).

Each of the eight viral RNP is composed of the RNA polymerase complex proteins (PB1, PB2 and PA) which drive transcription and replication; and the NP, which forms homo-oligomers and wraps the genomic vRNA (minus strand). The RNP particles are located inside a layer of M1 protein that lines the viral lipid membrane. During virus replication, at least three other nonstructural proteins are expressed into the mature virion: (1) the non-structural protein 1 (NS1), a multi-functional protein that evades the host immune system; (2) NS2, also called nuclear export protein (NEP), which plays a crucial role in mediating the export of viral RNPs from the cell nucleus during replication as well as modulating transcription and replication; (3) and PB1-F2, a protein expressed by certain, but nor all, IAV strains that exhibits pleiotropic effects in the process of virus-host interaction, including induction of host-cell apoptosis, modulation of polymerase activity, viral virulence and innate immune responses (Krumbholz, Philipps et al. 2011; Rossman and Lamb 2011). In 2009, N40, an N-terminally truncated version of PB1 lacking transcriptase function was described for the first time, but its function remains elusive (Wise, Foeglein et al. 2009).

The genome of influenza A virus is composed of eight vRNA segments with negative polarity. For each genomic viral RNA segment, the coding region is flanked by non-coding sequences at both ends. The 5' and 3'terminal sequences of the vRNA molecules together constitute the promoter structure, in a stepwise interaction with viral RNA polymerase complex (Robertson 1979). Each viral RNA segment exists as an RNP complex in which the RNA is coated with NP and forms a helical hairpin that is bound on one end by the heterotrimeric polymerase complex formed by PB2, PB1, and PA. Although the first 13 and 12 nucleotides at 5' and 3' end, respectively, are highly conserved among the vRNA segments of all influenza A viruses, the length and nucleotide composition of additional noncoding regions varies significantly among the eight segments (Desselberger, Racaniello et al. 1980).

### 2.1.4. Viral RNA segments, gene products, and pathogenesis determinants

The eight influenza A viral RNA segments encodes 12 recognized gene products. These proteins are PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NEP/NS2 proteins. Besides PB1, segment 2 has also been show to encode PB1-F2 and N40 gene products. Proteins M2 and NEP/NS2 are derived from mRNA splicing of the M and NS genes, respectively. The major function of each viral protein is summarized in Table 2.1 Influenza virus virulence is considered a polygenic trait, in which disease outcome is determined by the gene constellation of a particular influenza virus strain within a specific host (Hatta, Gao et al. 2001; Chen, Bright et al. 2007; Basler and Aguilar 2008).

Table 2.1. Influenza A virus genes and their function

| RNA Segment | Size (nucleotides) | Encoded proteins | Function |
|---|---|---|---|
| 1 | 2341 | PB2 | RNA-dependent RNA polymerase: binds to Cap<br>Inhibits type I interferon |
| 2 | 2341 | PB1 | RNA-dependent RNA polymerase: catalysis of viral RNA polymerization |
| | | PB1-F2 | Strain-dependent pleiotropic effects: modulates host innate immune response, apoptosis, polymerase activity, enhances primary and secondary viral pneumonia, antibacterial effects |
| | | N40 | Unknown |
| 3 | 2231 | PA | RNA-dependent RNA polymerase: cap-snatching endonuclease activity |
| 4 | 1742 to 1778 | HA | Receptor binding, viral fusion, and major viral antigen |
| 5 | 1565 | NP | Viral encapsidation, transport of RNPs from cytoplasm to nucleus, type-specific antigen |
| 6 | 1467 | NA | Receptor destroying activity: viral release, limits virus superinfection, antigenic site |
| 7 | 1027 | M1 | Assembly, type-specific antigen |
| | | M2 | Ion channel |
| 8 | 890 | NS1 | Multifunctional protein: blocks host antiviral immunity, regulates transcription and replication, modulates RNA splicing, enhancement of viral mRNA translation, regulation of virus particle morphogenesis |
| | | NS2/NEP | Assembly: nuclear export of progeny vRNPs<br>Regulation of influenza virus transcription and replication |

**Segment 1: PB2**

The RNA segment 1 (2,341 nucleotides long) encodes a single protein: the polymerase basic protein PB2, which is a 759-amino acid long protein. The PB2 subunit plays an essential role in the initiation of transcription for viral mRNA by binding to the 5' methylated cap of host pre-mRNAs, which are subsequently cleaved after 10-13 nucleotides by the endonuclease activity of PA (Dias, Bouvier et al. 2009). The PB2 has been shown to be an important determinant of pathogenicity of some influenza A viruses. PB2 accomplishes this task by several mechanisms, including enhancement of polymerase activity and modulation of host antiviral innate immune pathways upon its interaction with the mitochondrial antiviral signaling protein, MAVS (Hatta, Gao et al. 2001; Salomon, Franks et al. 2006; de Wit, Munster et al. 2010; Graef, Vreede et al. 2010). In particular, presence of a lysine (K) at position 627 is a strong determinant of pathogenicity to mammals, whereas glutamic acid (E) is the virulence marker in avian isolates (Subbarao, London et al. 1993; Baigent and McCauley 2003). However, it must be noted that avian influenza viruses with a typical avian PB2 at position 627 can also display high virulence in mammals further supporting the notion that influenza virulence is a multigenic phenomenon (Chen, Bright et al. 2007).

**Segment 2: PB1, PB1-F2, and N40**

The RNA segment 2 (2,341 nucleotides long) encodes up to three proteins: the polymerase basic protein PB1, the most important protein encoded by this segment; PB1-F2; and N40. PB1 is a 757-amino acid long protein that serves as the backbone of the viral polymerase complex. PB1 is responsible for the catalysis of viral RNA polymerization and plays a prominent role in the assembly of the polymerase complex since it contains binding sites for PB2 and PA subunits. PB1 is a major determinant of pathogenicity of the influenza A viruses (Salomon, Franks et al. 2006).

PB1-F2 is an 87-90 amino-acid long protein encoded by an alternate (+1) open reading frame (ORF) within the PB1 gene. The protein was originally discovered in 2001 during a search for $CD8^{+}$T-cell epitopes in the influenza genome (Chen, Calvo et al. 2001). Translation of PB1-F2 mRNA is likely mediated by ribosomal scanning and the protein is expressed early and transiently in infected cells (Chen, Calvo et al. 2001). Full-length PB1-F2 is expressed by most IAV of avian origin, but the protein is truncated in the classical swine H1N1 (cH1N1) and human H1N1 viruses isolated after 1950. Truncated versions of PB1-F2

have been reported also in some human H3N2 isolates and North American swine triple reassortant (TR) viruses, which have a human-like PB1 gene (Zell, Krumbholz et al. 2007). Using *in vitro* and mouse models, several groups have shown that PB1-F2 has pleiotropic effects. This protein, while dispensable for viral replication in embryonated eggs, tissue culture, or *in vivo* (Conenello and Palese 2007), it enhances the replication kinetics of the laboratory virus, A/Puerto Rico/8/34 (H1N1; hereafter referred to as PR8), and the 1918 H1N1 virus, but has no effects in the growth kinetics of the H5N1 or human H3N2 viruses (McAuley, Zhang et al. 2010; Smith, Adler et al. 2011). By interacting with the PB1 subunit, PB1-F2 was shown to up-regulate the PR8 polymerase activity (Mazur, Anhlan et al. 2008) and, more recently, the highly pathogenic H5N1 (McAuley, Zhang et al. 2010) and pH1N1 (Chen, Chen et al. 2010). Importantly, PB1-F2 expression results in enhanced apoptotic-cell death in immune cells, viral pathogenicity in mice, and immunopathology (Chen, Calvo et al. 2001; Zamarin, Ortigoza et al. 2006; Conenello, Zamarin et al. 2007; McAuley, Hornung et al. 2007; McAuley, Chipuk et al. 2010). Intranasal administration of PB1-F2 peptides derived from the 20$^{th}$ century pandemic strains (1918 H1N1, 1957 H2N2, 1968 H3N2) and H5N1 led to acute lung injury, elevation of pro-inflammatory cytokines, and the influx of inflammatory cells to the lungs of mice (McAuley, Chipuk et al. 2010). However, this effect seems to be lost in recently circulating human H3N2 strains through accumulations of point mutations in the PB1-F2 protein (Alymova, Green et al. 2011). A rare polymorphism (N66S) in PB1-F2 has been associated with high lethality of the 1918 IAV and certain highly pathogenic H5N1 strains (Conenello, Zamarin et al. 2007; Schmolke, Manicassamy et al. 2011). Surprisingly, two recent studies investigating the role of PB1-F2 in the pathogenicity of clinically relevant human viruses and the pH1N1 concluded that the expression of PB1-F2 has minimal effects on the virulence of these viruses in murine and ferret models (Hai, Schmolke et al. ; McAuley, Zhang et al. 2010). Recent publications have shown that PB1-F2 has profound effects in genes involving the host innate immunity responses, although these reports disagreed on the role of PB1-F2 in modulating type I interferons, suggesting that this function of PB1-F2 remains unresolved and needs further investigation (Le Goffic, Bouguyon et al. 2010; Dudek, Wixler et al. 2011; Le Goffic, Leymarie et al. 2011; Varga, Ramos et al. 2011). Overall, the effects of PB1-F2 in relation to influenza-host interaction are not completely understood and seem to be cell-type, virus strain, and even host-specific.

Besides PB1 and PB1-F2, RNA segment 2 also encodes N40, an N-terminally truncated version of PB1 that was first described in 2009. N40 interacts with PB2 and the polymerase complex but lacks transcriptase activity. Similar to PB1-F2, N40 is not essential

for virus rescue but is detrimental for virus replication in certain viral genetic backgrounds. (Wise, Foeglein et al. 2009). Recent work has shown that N40 does not alter the host innate immune response (Le Goffic, Leymarie et al. 2011). The functional role of N40 in viral virulence is yet to be elucidated.

**Segment 3: PA**

The RNA segment 3 (2,231 nucleotides long) encodes a single protein: the polymerase acid protein PA, which is a 716-amino acid long protein. The N-terminus of PA directly interacts with the C-terminus of PB1, which is essential for viral transcription and replication (Perez and Donis 2001). Although initially attributed to PB2 and PB1, Dias *el al.* recently demonstrated that the cap-snatching endonuclease activity of influenza virus resides in the PA subunit. The endonuclease active site binds two manganese ions and comprises a histidine and a cluster of three acidic residues, which are conserved in all influenza viruses (Dias, Bouvier et al. 2009). Importantly, the PA subunit modulates polymerase activity, virus replication and the virulence of H5N1 avian influenza viruses ducks, a natural host for influenza virus (Song, Feng et al. 2011).

**Segment 4: HA**

The RNA segment 4 (1742 to 1778 nucleotides long) encodes a single protein: the hemagglutinin (HA), which is a 562 to 566-amino acid long glycoprotein. The HA protein is multifunctional, being responsible for binding of the virus to receptors on host cells, membrane fusion, and assembly (see "Influenza virus life cycle" below). Binding to host receptors is the first step in the viral life cycle and this property makes the HA a major contributor to the host range of influenza viruses (Suzuki, Ito et al. 2000). HA also plays a central role in the adaptive host immune system since it contains important antigenic sites against which neutralizing antibodies are directed. The variability of NA and HA proteins is responsible for the ability of this virus persists in the population despite the immune response mounted by their hosts. So far, 16 different HA subtypes and 9 different NA subtypes have been identified. This allows the formation of hundreds of possible subtypes HA and NA combinations. However, only a few subtypes have established permanent lineages in mammalian species. For example, only three of these HA subtypes (H1, H2, and H3) have established stable lineages in humans. In contrast, aquatic birds harbor a large repertoire of influenza subtypes, including viruses with the H5, H7, and H9 subtypes, which poses

pandemic threat (Webster, Bean et al. 1992; Sorrell, Ramirez-Nieto et al. 2007; Kimble, Sorrell et al. 2011).

The HA is synthesized in the rough endoplasmic reticulum (RER) as a single polypeptide HA0. Newly synthesized HA is cleaved to remove the amino-terminal hydrophobic sequence of 14 to 18 amino acids, which correspond to the signal sequence for transport to the cell membrane. To fold properly, HA must be glycosylated and acquire its six intramolecular disulphide bonds (Cross, Burleigh et al. 2001). The structure of the membrane anchored HA molecules form homotrimers and each molecule consists of a globular head on a stalk. The cleavage of HA0 is accomplished by host-produced trypsin-like proteases, which are serine proteases secreted from a restricted number of cell types of the respiratory and digestive tracts. In the case of the HA-containing polybasic cleavage site of the highly pathogenic avian influenza viruses, processing of HA is carried out by furin, an ubiquitous membrane-bound protease member of the subtilisin family (Rott, Klenk et al. 1995). Cleavage of HA0 into HA1 and HA2 is a prerequisite for virus infectivity (Lazarowitz and Choppin 1975) and a major determinant of virulence and systemic dissemination of the highly pathogenic avian influenza strains (Horimoto and Kawaoka 1994). These two HA subunits are linked by a single disulphide bond (Copeland, Doms et al. 1986).

The globular head is made up solely of HA1 and contains the receptor-binding domain as well as antigenic sites against which neutralizing antibodies responses are directed to. The stalk is composed of HA2 and the N-terminal portion of HA1. HA2 contains the transmembrane hydrophobic sequence which is highly conserved among different HA subtypes, and play an essential role in HA fusion upon virus entry (Cross, Burleigh et al. 2001). The HA, along with the NA, undergoes "antigenic drift" due to accumulation of amino acid substitutions in epitopes recognized by antibodies that neutralize viral infectivity. The rapid antigenic drift displayed by influenza viruses has precluded the development of broadly protective vaccines. It seems that the virus evolve by adjusting receptor binding avidity with steady alterations in antigenicity (Hensley, Das et al. 2009). Since the discovery that potentially more broadly neutralizing antibodies are directed against the conserved HA2 stalk domain, ongoing research efforts from different groups are trying to re-focus influenza immune responses in order to develop universal influenza vaccines (Steel, Lowen et al. 2010; Ekiert, Friesen et al. 2011; Wrammert, Koutsonanos et al. 2011). The HA is a major virulence determinant of the influenza A virus, an aspect that has been heavily studied (Kawaoka,

Naeve et al. 1984; Kobasa, Takada et al. 2004; Pappas, Aguilar et al. 2008; de Wit, Munster et al. 2010; Ping, Dankar et al. 2010).

**Segment 5: NP**

The RNA segment 5 (1565 nucleotides long) encodes a single protein: the nucleoprotein (NP), which is 498 amino acids in length and plays a pivotal role in viral encapsidation. NP is an arginine-rich protein that acts primarily as a single-strand RNA binding protein and serves as the structural protein in viral ribonucleoprotein particles (vRNPs). NP coats the RNA and is the major viral protein in the vRNPs. NP also plays a role in the transport of vRNPs from cytoplasm to nucleus by interacting with cellular cargo protein karyopherin α (Melen, Fagerlund et al. 2003) NP is also one of the type-specific antigens and a major target of the host cell-mediated immune responses (Yewdell, Bennink et al. 1985) (Yewdell, Bennink et al. 1985). Numerous studies suggest that NP plays a role in host range (Scholtissek, Koennecke et al. 1978; Scholtissek, Burger et al. 1985; Naffakh, Tomoiu et al. 2008) and virulence (Wasilenko, Lee et al. 2008; Tada, Suzuki et al. 2011).

**Segment 6: NA**

The RNA segment 6 (1413 nucleotides long, for A/Puerto Rico/8/34 (H1N1) strain) encodes a single protein: the neuraminidase (NA), which is a 454 amino acid long type II membrane glycoprotein. The NA's sialidase activity plays a role in virion release, facilitating budding of progeny virions from infected cells and contributing to the spread of progeny virions on the airway epithelium. Recently, it was shown that NA also plays an important role early during viral infection, presumably by removal of mucus covering the airways epithelium (Matrosovich, Matrosovich et al. 2004). By removing surface sialic acid receptors from the virion-producing cell, the NA also limits influenza A virus superinfection (Huang, Li et al. 2008). The NA monomer consists of four domains: a globular head, a stalk, a transmembrane and cytoplasmic domains. The NA catalytic and major antigenic sites are located in the globular head. Similarly to the HA, NA contains highly variable regions considered as antigenic sites targeted by the host immune system that contributes to antigenic drift. Even though NA-specific antibodies are not neutralizing, they have protective effects since they interfere with virus replication by preventing the release of new virus particles. As mentioned earlier, antigenic drift in NA can contributes for influenza evasion from neutralizing antibody response generated as a result of previous natural infection or

vaccination (Subbarao and Joseph 2007; Nayak, Kumar et al. 2010). In addition, the NA has also been associated with the virulence of several influenza viruses of avian and mammalian origin (Schulman and Palese 1977; Li, Schulman et al. 1993; Pappas, Aguilar et al. 2008; Matsuoka, Swayne et al. 2009; Sorrell, Song et al. 2010).

**Segment 7: M1 and M2**

The RNA segment 7 (1027 nucleotides long) is bicistronic and encodes the matrix protein M1 and the ion channel M2 protein. The M1 is a 252-amino acid long protein that is expressed late in the viral life cycle and is the most abundant polypeptide in the influenza virion. The M1 is located on the inner surface of the lipid bilayer. By coating the inner face of the envelope, M1 gives rigidity to the virion. M1 is crucial for the assembly of progeny virions and it has been shown that M1 is the only viral component which is absolutely essential for the formation of virus-like particles (VLPs) (Gomez-Puertas, Albo et al. 2000). M1 also interacts with both vRNPs and NEP/NS2 mediating the export of newly synthesized vRNPs from the nucleus to the cytoplasm (Ma, Roy et al. 2001).

M2 is 97-amino acid long protein that is expressed from splicing of the M mRNA. M2 mRNA splicing is controlled by both the viral polymerase and cellular splicing factors, such as the serine/arginine-rich splicing factor SF2/ASF (Shih, Nemeroff et al. 1995; Shih and Krug 1996). The M2 is a tetrameric type III integral membrane protein whose ion channel activity is essential for the uncoating process M2 also plays a role in assembly and budding of virus particles (Rossman and Lamb 2011). In addition, M2 has also been considered a vaccine candidate or as an adjuvant for inactivated vaccines because antibodies specific for M2 have been shown to be protective in vivo in the mouse model (Schotsaert, De Filette et al. 2009; Guo, Yao et al. 2010; Song, Van Rooijen et al. 2011).

The M segment has been recently identified as a major determinant for aerosol transmissibility of the 2009 pandemic H1N1 virus (Chou, Albrecht et al. 2011), although it is not clear whether M1 or M2 or both contribute to this phenotype.

**Segment 8: NS1 and NEP/NS2**

The RNA segment 8 (890 nucleotides long) is bicistronic and encodes the nonstructural protein 1 (NS1) and the nuclear export protein (NEP/NS2). NS1 is a ~230-amino long protein that is expressed early during the infection and exerts multiple functions

during the viral life cycle. NS1 blocks the host's antiviral response by inhibiting the IFN production as well as the antiviral effects of IFN-induced proteins, such as dsRNA-dependent protein kinase R (PKR) and 2'5'-oligoadenylate synthetase (OAS)/RNase L. By interacting with cellular factors, NS1 also modulates a variety of cellular processes during viral infection, including inhibition of host mRNA polyadenylation, inhibition of nuclear export of polyadenylated host mRNA, regulation of viral RNA transcription and replication, modulation of viral RNA splicing, enhancement of viral mRNA translation, regulation of virus particle morphogenesis, and activation of phosphoinositide 3-kinase (PI3K) (Hale, Randall et al. 2008). In addition, a large body of work has shown that NS1 is an important virulence factor (Lipatov, Andreansky et al. 2005; Fernandez-Sesma, Marukian et al. 2006; Li, Jiang et al. 2006; Jackson, Hossain et al. 2008; Jiao, Tian et al. 2008).

NEP/NS2 is a 121-amino acid long protein that interacts with both M1 protein and the cellular export machinery to participate in the assembly of virus particles (Yasuda, Nakada et al. 1993). This interaction is essential in the virus life cycle for the export of the new vRNP complex from the nucleus (O'Neill, Talon et al. 1998). Recent mapping of NEP/NS2 domains showed that its C-terminal domain (residues 81–100) is essential for M1 binding and the nuclear export of progeny vRNPs (Shimizu, Takizawa et al. 2011). In addition, NEP/NS2 has also been implicated in playing a role in the regulation of influenza virus transcription and replication (Robb, Smith et al. 2009).

### 2.1.5. Influenza virus life cycle

The replication cycle of influenza virus is initiated when a viral particle comes into contact with the target cell. The association between the virus and the cell is possible due to the affinity of the activated HA to sialic-acid-containing receptors present on cell surface glycoproteins or glycolipids. N-acetyl neuraminic acid (sialic acid) is bound to galactose (Gal) through α2-3, α2-6, or α2-8 glycosidic linkage by sialyltransferases, which are expressed in a cell type and species-specific fashion (Angata and Varki 2002; Gagneux, Cheriyan et al. 2003). The ability of different HA proteins to bind to SA receptors with either a2-3 or a2-6 linkages is a major determinant of the host range and tissue tropism of the influenza A viruses (Bean, Schell et al. 1992; Matrosovich, Tuzikov et al. 2000; Ibricevic, Pekosz et al. 2006). For example, avian and equine influenza strains preferentially bind cell surface oligosaccharides that contain the SAa2-3Gal receptor, whereas isolates of human and

swine origin binds preferably to SAa2-6Gal receptors (Ito and Kawaoka 2000; Gambaryan, Karasin et al. 2005).

The binding of the membrane-distal region of influenza HA to SA receptors induces the entry of the virus in the cell by receptor-mediated endocytosis. The cleavage of the HA0 molecule into HA1 and HA2 is a prerequisite for the membrane fusion and therefore a condition for viral infectivity. Virus entry is mediated by invagination of clathrin coated-pits on the host plasma membrane. As the endosomal vesicles containing influenza virus move towards the nucleus, their pH is acidified. When the endosome reaches pH 5.0, the HA undergoes a conformational change that exposes the fusion peptide located on HA2. The fusion peptide is a short hydrophobic sequence that inserts itself into the endosomal membrane and triggers the fusion with the viral envelope resulting in the release of vRNPs into the cytoplasm. Acidification of the endosome also activates the M2 protein, which pumps $H^+$ protons into the viral particle, thus destabilizing the M1 interaction with RNP complex and freeing it from the viral envelope (Cross, Burleigh et al. 2001).

The viral RNPs are then transported into the cell nucleus where transcription and replication occurs (Cross, Burleigh et al. 2001). Nuclear localization signals (NLS) located on NP, the major component of the RNPs, binds to NLS receptors (e.g., karyopherin family members), which are responsible for the transport of vRNP to the nucleus (Wang, Palese et al. 1997). A unique feature of influenza viruses among other RNA viruses is that all vRNA synthesis takes place in the nucleus by use of the nuclear machinery of the host cells. As mentioned earlier, the genome of influenza virus consists of an eight-segmented, single-stranded, negative-sense RNA. Similar to all viruses with a negative-sense genome, the influenza vRNA serves both as a template for mRNA synthesis and as a template for the synthesis of positive-sense (cRNA) replicated intermediates, which in turn serve as a template for the synthesis of progeny genomic vRNA. The heterotrimeric polymerase complex composed of PB2, PB1, and PA protein subunits carries out the transcription and replication of the eight separate segments of influenza vRNA. To initiate synthesis of its mRNA, influenza virus depends on cellular RNA polymerase II activity because the influenza RNA polymerase complex is a primer-dependent enzyme (Palese and Shaw 2007). Thus, the virus cannibalizes 5'-capped primer (5'-methyguanosine cap plus 10-13 nucleotides) from heterogeneous host pre-mRNA transcripts to initiate viral mRNA synthesis ('cap-snatching' mechanism). To accomplish this, the virus uses the cap-binding function of PB2 and the endonuclease activity assigned to PA (Dias, Bouvier et al. 2009). This mechanism requires a

continuous supply of cellular RNA polymerase II and justifies fully functional inhibition of mRNA synthesis by actinomycin D. Polyadenylation of viral mRNA is catalyzed by the polymerase complex via a sttutering mechanism when it reaches a polyuridine strech approximately 17 to 22 nucleotides from the 5' end of each vRNA segment (Robertson, Schubert et al. 1981). Mature mRNAs are then transported to the cytoplasm, where they direct the synthesis of viral proteins using the cellular machinery. Unlike mRNA synthesis, cRNA (+ sense) synthesis occurs in a primer-independent manner. The cRNAs lack 5'cap and poly-A tail. The cRNAs are not transported to the cytoplasm during viral infection and serve as templates for the synthesis of negative-sense genomic vRNA. Synthesis of all three RNA species (mRNA, cRNA, and vRNA) takes place in the nucleus. It is not completely understood how the switch between transcription and replication occurs, although it has been reported that the NP protein has an important function in this switching (Portela and Digard 2002). Recently, Perez and co-workers reported that influenza A virus generates small RNAs (22–27 nucleotide oligomers corresponding to the 5′ end of each vRNA) that regulates the switch from transcription to replication (Perez, Varble et al. 2010).

Progeny vRNPs are exported out of the nucleus into the cytoplasm associated with the M1 and NEP/NS2 proteins. These complexes are transported to the assembly site on the plasma membrane either via cytoskeleton elements or by piggybacking on the cytoplasmic tail of HA and NA through interactions with the M1 protein. Newly synthesized HA and NA glycoproteins are targeted to lipid rafts domains on the apical membrane of polarized cells, where budding of progeny viral particles takes place. Lipid raft domains are regions of the plasma membrane rich in cholesterol and sphingolipids. Expression of lipid raft-associated proteins, such as the influenza virus HA and NA, causes the coalescence and enlargement of the raft domains. This clustering of HA and NA may cause a deformation of the membrane and the initiation of the virus budding event. M2, which is not a raft-associated protein, is recruited to the periphery of the budding virus though interactions with M1 (Rossman and Lamb 2011). M2 initially stabilizes the site of budding, possibly enabling the polymerization of the matrix protein and the formation of filamentous virions. Subsequently, M2 is able to alter membrane curvature at the neck of the budding virus, causing membrane scission ("pinching off"") and the release of the budding virus (Leser and Lamb 2005; Schmitt and Lamb 2005; Chen, Leser et al. 2008; Rossman and Lamb 2011).

At the final stage of viral assembly, vRNAs are likely selectively (rather than randomly) incorporated into the virion as it buds from the cell's apical plasma. The packaging

of vRNPs into virions typically involves the recognition of packaging signals in the vRNA. These packaging signals are cis-acting sequences that span both the untranslated regions (UTR) and regions of the ORF at both ends of the vRNA segments. The UTR's and packaging signals are unique to each gene segment but conserved amongst gene segments across influenza A viruses (Watanabe, Watanabe et al. 2003; Fujii, Fujii et al. 2005; Liang, Hong et al. 2005; Hutchinson, von Kirchbach et al. 2010). In addition to the 8 RNA segments and nine viral structural proteins, influenza virus has been shown to package 36 host derived proteins in the virion (Shaw, Stone et al. 2008). The implications of these host-derived proteins for the interactions of influenza virus with its host are yet to be deciphered.

### 2.1.6. Advancements in influenza virus reverse genetics and its use as a vector

Because influenza viruses contain a negative strand RNA, transfection of the naked vRNA, without the replication complex, cannot initiate transcription or translation. Rather, the vRNA must be transcribed into positive-sense mRNA and cRNA by the viral RNA polymerase complex in order to initiate an infection. This limitation of negative-sense RNA viruses compared with positive-sense RNA viruses hampered the progress in developing reverse genetics systems to manipulate the IAV genome. For example, the first reverse genetic system for a virus was developed in 1981 by Racaniello and Baltimore, who were able to rescue poliovirus entirely from cloned cDNAs (Racaniello and Baltimore 1981).

The first reverse genetics system for negative-strand RNA viruses was described in 1989 by Luytjes and co-workers, who engineered an IAV vector expressing foreign sequences (Luytjes, Krystal et al. 1989). The authors engineered a plasmid expressing the chloramphenicol-acetyltransferase (CAT) gene in negative polarity flanked by the NS UTR under the transcriptional control of a T7 promoter. The influenza-like CAT reporter gene was then transfected into cells along with purified vRNP protein components (PB2, PB1, PA, and NP) to transcribe the added RNA. The transfected cells were previously infected with an influenza helper virus, which was able to replicate, transcribe, and package the foreign CAT gene. Although this allowed the production of influenza viruses with transgenic RNA, the purification of the RNPs was troublesome and required the use of a helper influenza virus, which required specialized methods to select the virus with the foreign gene. Furthermore, expression of the foreign CAT transgene was significantly reduced after a few passages suggesting that the segment encoding CAT was not stably preserved. To distinguish the wild type helper virus from the desired recombinant virus, several selection methods were

developed including host-range growth restrictions (Enami, Luytjes et al. 1990; Subbarao, Kawaoka et al. 1993), temperature sensitive mutations (Enami, Sharma et al. 1991), or plaque purification in the presence of antibodies (Enami and Palese 1991; Barclay and Palese 1995) or amantadine (Castrucci and Kawaoka 1995).

Subsequent work demonstrated successful vRNP formation of viral model RNAs in influenza virus-infected cells using a influenza-reporter replicon under the control of the RNA polymerase I (Pol-I) promoter. The Pol-I complex is involved in transcription of ribosomal RNA that lacks both a 5′ cap and a 3′ poly (A) tail. In addition, the hepatitis delta virus ribozyme was incorporated downstream of the vRNA in order to ensure adequate processing of the 3'end of the vRNA (Neumann, Zobel et al. 1994; Neumann and Hobom 1995; Pleschka, Jaskunas et al. 1996). Although this system eliminated the need to purify viral NP and polymerase proteins for RNP reconstitution *in vitro*, it still relied on the co-infection with a helper virus for generation of recombinant influenza viruses (Pleschka, Jaskunas et al. 1996). In 1999, two independent groups succeeded, for the first time, in the generation of influenza A viruses entirely from cloned cDNAs (Fodor, Devenish et al. 1999; Neumann, Watanabe et al. 1999). This technique was a major breakthrough in influenza virus research since it eliminated the need for purified RNPs and helper viruses. In this 12-plasmid rescue system, virus rescue was accomplished by transfecting cells with eight plasmids encoding authentic sequences of each vRNA and four expression plasmids encoding the NP and polymerase proteins needed for transcription and replication. To generate vRNA-expressing plasmids, vRNA extracted from the virus is cloned in a negative sense, flanked by a truncated RNA polymerase I promoter and the hepatitis delta virus ribozyme, or a RNA polymerase I terminator. The use of these flanking sequences assures the intracellular expression of viral RNAs with correct start and end sequences as described above. In contrast to the RNAs generated by cellular RNA polymerase II, these vRNAs lack the 5'cap. Upon transfection, authentic vRNAs are transcribed by the cellular RNA polymerase I and then recognized and amplified by viral polymerase (PB1, PB2 and PA) expressed from the cellular RNA polymerase II coding plasmids. Because NP is required for both replication and encapsidation of the viral RNA, an NP expression plasmid is also included. This method reliably produces recombinant influenza viruses without the need of further selection methods.

This initial plasmid-based reverse genetics was further improved to an eight-plasmid based reverse genetics system, which dispensed the need for RNP expression plasmids

(Hoffmann, Neumann et al. 2000). Using this approach, the cDNA encoding each vRNA was cloned in the negative–sense orientation between human RNA polymerase I promoter at the 5' end and the mouse RNA polymerase I terminator at the 3'end. This entire gene cassette was then cloned in the positive-sense orientation between an RNA polymerase II promoter and polyadenilation site. The orientation of the two transcription units allows the synthesis of negative sense vRNA by RNA polymerase I and positive-sense mRNA by RNA polymerase II from one cDNA. This system is commonly referred to as the bidirectional (dual promoters: Pol-I and Pol-II) reverse genetic system to differentiate form the unidirectional (single promoter: Pol-I) system described above.

In 2005, a one-plasmid-based rescue system was first developed with the goal of improving transfection efficiency by reducing the number of plasmids required for viruses rescue. In this system, the eight RNA polymerase I transcription cassettes for viral RNA synthesis were combined on one plasmid. The authors suggested that protein synthesis from a cryptic RNA polymerase II promoter, present in the vector or in the RNA polymerase I promoter or terminator region, was responsible for the generation of recombinant virus from one-plasmid-transfected cells (Neumann, Fujii et al. 2005).

A limitation of all the previously described reverse genetics systems is the species specificity of the RNA polymerase I promoter systems, which impede virus recovery from a heterologous host either in vitro or in vivo and can lead to host adaptation when generating recombinant viruses from cloned cDNAs. These limitations were partly circumvented by the use of a species-independent T7 promoter rescue system (de Wit, Spronken et al. 2007) or by the development of the chicken (Zhang, Kong et al. 2009) and the canine Pol-I reverse genetics systems (Wang and Duke 2007; Murakami, Horimoto et al. 2008). Although the T7 RNA polymerase-based reverse-genetics can be use for virus rescue in cells from different species, rescue efficiency with this system is inferior to the bidirectional eight-plasmid rescue system and therefore have not gained much popularity (de Wit, Spronken et al. 2007). Even though the draft swine genome sequence has been released, the porcine Pol-I reverse genetics system is yet to be developed.

The use of viral vectors for the delivery of traceable reporter genes and bioactive molecules has broad applications in the fields of gene therapy and infectious disease. A number of live recombinant viral vectored vaccines have been licensed for veterinary use and many are in clinical development for humans. These vaccines combine some of the positive

features of DNA vaccines with those of live attenuated vaccines. Similarly to DNA vaccines, viral vectors deliver the DNA encoding protein immunogens into target host cells, but with the added advantage of inducing more robust immune by the replicating viral entity (Draper and Heeney 2010). Since the development of reverse genetic methods for segmented negative sense RNA viruses, influenza A viruses (IAV) have also been considered as potential vaccine vectors (Hoffmann, Neumann et al. 2000). Martínez-Sobrido and García-Sastre have reviewed in details the use of IAV as a vector (Martínez-Sobrido and García-Sastre 2007) . The engineering of IAV as expression and delivery vectors offers several advantage: (i) induce strong cellular and humoral immune responses both systemically and at the mucosal surfaces (Garulli, Kawaoka et al. 2004), (ii) the virus does not have a DNA phase during replication, eliminating thus safety concerns regarding integration of viral DNA into the hosts genome, (iii) IAV have 16 different HA and 9 different NA surface protein genes, which readily permits reconstitution of the vector surface proteins, making boost vaccinations feasible(Fouchier, Munster et al. 2005), and (iv) IAV are well characterized with attenuated strains already used as vaccines for humans and livestock (Belshe, Edwards et al. 2007). However, most of the AIV vectors developed to date have either been unstable or tolerated only short gene inserts. Thus, both the instability of the transgene and the insert size limitation has prevented IAV vectors from being used as vaccines (Martínez-Sobrido and García-Sastre 2007).

Among all the IAV proteins, the surface glycoproteins HA and NA have been successfully engineered for expression of small protein epitopes (Li, Mueller et al. 2005; Martínez-Sobrido and García-Sastre 2007; Martina, van den Doel et al. 2011). Nevertheless, neither HA nor NA glycoproteins are optimal targets for the insertion of foreign sequences because of insert size constraints and the fact that booster immunizations could be affected by pre-existing immunity to the vector or the transgene itself. Another approach that has been attempted is the incorporation of the foreign gene as an additional gene segment. Several groups have successfully expressed transgenes as additional influenza segments, including the CAT reporter gene (Luytjes, Krystal et al. 1989), the ectodomain of the classical swine fever virus E2 protein (Zhou, Konig et al. 1998) , GFP or the tumor-associated antigen MAGE-3 (Strobel, Krumbholz et al. 2000), or even an additional HA protein to generate an influenza virus expressing two HA proteins from the H1 and H3 (Gao, Lowen et al. 2010) or H9 and H1 subtypes (Wu, Guan et al. 2010).

Additionally, expression of foreign genes has also been achieved by fusion or co-expression of foreign proteins downstream of the NS1 protein, but expression of NEP is still dependent of splicing of segment 8 RNA (Ferko, Stasakova et al. 2001; Kittel, Ferko et al. 2005; Sereinig, Stukova et al. 2006; Wolschek, Samm et al. 2011). Recently, Manicassamy and collaborators adapted a previously developed strategy to express NEP as a single ORF (Basler, Reid et al. 2001) and constructed IAV expressing GFP reporter gene fused to viral NS1 protein via a linker sequence. Nevertheless, the stability of the GFP-expressing virus was compromised during multicycle replication (Manicassamy, Manicassamy et al. 2010).

Alternatively, IAV expressing foreign genes have been constructed by replacing the NA (Shinya, Fujii et al. 2004) or the PB2 (Ozawa, Victor et al. 2011) ORF with the gene of interest. Although this approach is interesting, it requires the use of specialized cell lines that express reduced levels of sialic acid or provides the essential PB2 protein in *trans*.

Bicistronic influenza virus RNA segments have been constructed with two open reading frames as fusion proteins (Kittel, Ferko et al. 2005) or as discrete gene products separated by either an internal ribosomal entry site (IRES) (Garcia-Sastre, Muster et al. 1994); by use of an internal influenza promoter (Fujii, Ozawa et al. 2009); by a putative caspase cleavage site (Kittel, Sereinig et al. 2004), by a linker sequence (Manicassamy, Manicassamy et al. 2010) or connected to each other via a cleavage recognition signal, such as the foot-and-mouth disease virus 2A cis-acting hydrolase element (CHYSEL) (Percy, Barclay et al. 1994).

Although these strategies have contributed to advances in the field, further developments in influenza virus vector design are needed to improve foreign gene stability as well as the packaging capacity of these viruses. This knowledge will permit the development of alternative safe and effective influenza-based vectors against influenza as well as other diseases. Furthermore, the generation of non-attenuated influenza viruses stably carrying traceable reporter genes will serve as an important tool to more effective tracking of the manipulated virus during its interaction with the host and will provide important insights into influenza pathogenesis.

## *2.2. Influenza virus infection of domestic swine*

### 2.2.1. Overview

Swine influenza is an acute respiratory disease of pigs. The disease has a high morbidity rate, but mortality is generally low. However, severe outbreaks can be seen depending on many factors, including animal's age, virus strain, and secondary bacterial infections. Swine influenza not only affects the swine industry, but also represents a constant threat to the turkey industry and is of particular concern to public health (OIE 2008). The emergence of the 2009 pH1N1 containing all eight RNA segments derived from swine viruses provided further evidence of the pivotal role of domestic pigs in the ecology of influenza A virus infections.

The causative agent of swine influenza is the type A influenza virus of the *Orthomyxoviridae* family. The genetic, structural and biologic characteristics of SIVs are identical to other type A influenza viruses characterized by negative-sense, segmented RNA genomes and were described in detail above.

### 2.2.2. Epidemiology

Influenza A viruses (IAV) display a remarkable ability to infect a variety of animal species, including humans, pigs, wild and domestic birds, horses, cats, dogs, ferrets, and sea mammals (Palese and Shaw 2007). Among these animals, wild aquatic birds are thought to be the primordial reservoir from which all IAV have emerged (Webster, Bean et al. 1992).

IAV of three different subtypes: H1N1, H1N2 and H3N2 are endemic in swine populations worldwide. Other subtypes that have been identified in pigs include H1N7, H3N1, H2N3, H4N6, H3N3, and H9N2 (OIE 2008). Equine-origin H3N8 influenza viruses have also been isolated from pigs in central China (Tu, Zhou et al. 2009). In addition, wholly H5N1 avian influenza viruses have been isolated from asymptomatic pigs in Korea and Indonesia (Lee, Pascua et al. 2009; Nidom, Takano et al. 2010). Moreover, an H7N2 influenza virus reassortant between avian H7N2 and H5N3 influenza viruses has been isolated from pigs in Korea (Kwon, Lee et al. 2011).

Despite the same subtype classification, swine influenza viruses in Europe and North America are antigenically and genetically distinct (Vincent, Ma et al. 2008). In Europe, an avian-like H1N1 virus and human-like H3N2 swine influenza viruses have been considered widespread among pigs since the 1980s. Furthermore, a novel H1N2 reassortant emerged in the mid 1990s and is also endemic in European swine populations (Van Reeth, Brown et al. 2008). This virus has a human-like H1 HA, a human-like swine H3N2 viruses NA and its internal genes are most closely related to avian H1N1 viruses (Brown, Harris et al. 1998).

In North America, classical H1N1 viruses (cH1N1), descendents of the 1918 "Spanish flu" pandemic, were the predominant subtype circulating in swine populations since their first isolation in 1930. The classical H1N1 swine influenza viruses in the United States remained antigenically and genetically highly conserved for almost 70 years (Sheerar, Easterday et al. 1989; Luoh, McGregor et al. 1992; Noble, McGregor et al. 1993). In 1998, however, the epidemiology of swine influenza infection in North American pigs changed dramatically. A new genotype of H3N2 triple reassortant IAV (TR) emerged in the U.S. swine population and established permanent lineages. TR swine IAV have demonstrated exceptional ability to further reassort with either classical swine H1N1 and human H1N1 and H3N2 viruses, generating at least seven different reassortant lineages in the last decade (Karasin, Olsen et al. 2000; Karasin, Schutten et al. 2000; Webby, Rossow et al. 2004; Karasin, Carman et al. 2006; Lekcharoensuk, Lager et al. 2006; Ma, Gramer et al. 2006; Olsen, Karasin et al. 2006; Ma, Vincent et al. 2007). Currently, TR strains of the H3N2, H1N2, H1N1, and the pH1N1 subtypes predominate in the US swine population (Vincent, Ma et al. 2008). Interestingly, some of these viruses have expanded their host range and cause outbreaks in turkeys in North Carolina, Minnesota, and Ohio in 2003-2004 (Choi, Lee et al. 2004). A unique feature shared by all of these endemic reassortants is the maintenance of the triple reassortant internal gene (TRIG) cassette, which consists of the avian-like (PA and PB2), swine-like (M, NP, and NS), and human-like (PB1) gene segments. The TRIG cassette appears to accept multiple HA and NA types, which could provide a selective advantage to swine viruses possessing this internal gene constellation (Vincent, Ma et al. 2008; Ma, Lager et al. 2009).

In addition, the 2009 pandemic H1N1 virus (pH1N1), which is also a TR, have been commonly isolated from commercial swine operations throughout the world. In all incidents, epidemiological investigations have linked humans as the possible source of the infection to the pigs (Hofshagen, Gjerset et al. 2009; Howden, Brockhoff et al. 2009). Since then, several

reassortants between pH1N1 and circulating influenza A viruses have been isolated from pigs in several countries (Vijaykrishna, Poon et al. 2010; Howard, Essen et al. 2011; Moreno, Di Trani et al. 2011; Pereda, Rimondi et al. 2011; Starick, Lange et al. 2011). In the US alone, pH1N1 have reassorted promiscuously with endemic TR strains given rise to at least 7 distinct and novel viral genotypes in this animal species (Ducatez, Hause et al. 2011).

### 2.2.3. The pig as an intermediate host and sialic acid receptor distribution

For many years, pigs have been considered a "mixing vessel" for the generation of novel influenza viruses (Scholtissek, Burger et al. 1983; Scholtissek, Burger et al. 1985; Scholtissek 1995). Pigs are susceptible to human influenza viruses, and to a wide range of avian influenza viruses. Experimental infection studies showed that pigs were susceptible to 13 different influenza virus subtypes (H1-H3 and non-human-type HA types H4-H13). From these studies, a reassortant virus was generated in pigs upon co-infection with a parental SIV strain and an avian strain; the latter naturally unable to replicate by itself in pigs (Kida, Ito et al. 1994). The mixing vessel attribute is consistent with the presence in the respiratory tract of pigs of prototypical human-like and avian-like influenza receptors, N-acetylneuraminic acid-α2,6-galactose (SAα2,6Gal) and N-acetylneuraminic acid-α2,3-galactose (SAα2,3Gal), respectively (Ito, Couceiro et al. 1998). However, several recent studies have questioned the "mixing vessel" hypothesis. First, comprehensive mapping of the sialic acid distribution showed that despite SAα2,3Gal and SAα2,6Gal receptors in pig respiratory tracts, SAα2,3Gal is found only in the smaller airways (bronchiole and alveoli) and not in the trachea or nose (Nelli, Kuchipudi et al. 2010; Van Poucke, Nicholls et al. 2010; Trebbien, Larsen et al. 2011), which is in disagreement with a previous report by Ito et al that found both type of receptors in pig trachea (Ito, Couceiro et al. 1998). Experimentally, pigs display low susceptibility to infection with low and highly pathogenic H5N1 avian influenza viruses (Choi, Nguyen et al. 2005; Lipatov, Kwon et al. 2008; De Vleeschauwer, Atanasova et al. 2009). Moreover, the prevalence of H5N1 avian influenza virus infection in Asia appears to very low relative to prevalence in bird populations (Choi, Nguyen et al. 2005) and swine H5N1 isolates are less virulent to mice than avian isolates, suggesting that these viruses may become less virulent during their replication in pigs (Takano, Nidom et al. 2009).

The receptor specificity theory alone does not explain the apparently increased susceptibility of this species since both types of receptors are also found in the respiratory tract of quail, pheasant, turkey, guinea fowl (Wan and Perez 2006; Kimble, Nieto et al. 2010)

and humans (Shinya, Ebina et al. 2006), implying that these species can also serve as intermediate hosts for influenza reassortment. Nevertheless, the hypothesis that the pig acts as a mixing vessel between human and avian influenza viruses cannot be rejected since pigs have been commonly associated with two-way transmission of influenza viruses to and from humans (Myers, Olsen et al. 2007; Robinson, Lee et al. 2007; Newman, Reisdorf et al. 2008; Vincent, Swenson et al. 2009). In addition, pigs were involved in the genesis of the 2009 pH1N1 (Garten, Davis et al. 2009) and a greater number of reassortants between pH1N1 and circulating IAV have been isolated from pigs than from any other species that have been infected by pH1N1(Vijaykrishna, Poon et al. 2010; Howard, Essen et al. 2011; Moreno, Di Trani et al. 2011; Pereda, Rimondi et al. 2011; Starick, Lange et al. 2011, Gramer, Lee et al. 2007; Kumar, Deflube et al. 2011; Nfon, Berhane et al. 2011).

In addition, there have been at least 50 cases described in the literature of zoonotic swine influenza virus infection with a case-fatality rate of 14% (Myers, Olsen et al. 2007). In fact, an epidemiological study conducted in swine workers from Iowa, the US largest pork producing state, concluded that occupational exposure to pigs greatly increases workers' risk of swine influenza virus infection (Myers, Olsen et al. 2006). A 2-year prospective study of zoonotic influenza transmission in Iowa concluded that swine workers and their nonswine-exposed relatives are at increased risk of zoonotic influenza virus infections (Gray, McCarthy et al. 2007). Thus, these studies highlight the importance of swine in the ecology and evolution of the influenza A viruses and suggest that systematic swine surveillance may allow the early detection of potentially pandemic strains in this reservoir.

### 2.2.4. Pathogenesis, pathology, and clinical features

The pathogenesis of swine influenza is well known and bears remarkable resemblance to human influenza with respect to the clinical presentations and pathologic features. Swine influenza infection follows a typical pattern of respiratory viral infections without systemic spread. Viral replication takes place in cells of the nasal mucosa, tonsils, trachea, lungs and tracheobronchial lymph nodes (Brown, Done et al. 1993). Consistent with the sialic acid receptor distribution, immunohistochemistry studies in influenza virus infected pigs have revealed that swine influenza antigens are widespread in the respiratory tract (alveoli, bronchioles, bronchi as well as nasal and tracheal epithelium) of pigs, whereas avian influenza antigens are located only in the lower respiratory tract (alveoli and bronchioles) (Trebbien, Larsen et al. 2011). Under experimental conditions, reproduction of typical swine influenza clinical signs and pathology is most reliably accomplished by intratracheal

inoculation of high viral doses, as opposed to less invasive methods such as intranasal inoculation, which usually result in mild or subclinical infection (Maes 1984).

In uncomplicated infections, the gross lesions are mainly those of a primary viral pneumonia. Animals develop bronchointerstitical pneumonia that is characterized by depressed dark red to purple-red and sharply demarcated areas in the lungs. Lesions may be found throughout the lungs but they are usually more extensive in the cranio-ventral portions of the lung (Fig. 2.2A). Other parts of the lungs may be pale and emphysematous. The airways are often dilated and filled with mucopurulent exudate. The bronchial and mediastinal lymph nodes are typically edematous but not congested (Brown, Done et al. 1993; Van Reeth, Van Gucht et al. 2002; Vincent, Ma et al. 2008). Microscopically, the hallmark of swine influenza infection is necrosis and desquamation of epithelial cells lining the airways. In addition, epithelium attenuation in the bronchioles and interstitial pneumonia may be present (Fig. 2.2B). Mild to moderate peribronchiolar and perivascular lymphocytic infiltration occurs at nearly all levels of the airways. The influx of inflammatory cells in the airways causes obstruction of the bronchi, bronchiole and significant lung damage due to release of enzymes (Van Reeth 2000; Barbe, Atanasova et al. 2011). As mentioned earlier, viral antigen can be detected throughout the respiratory tree, but is predominantly detected in the luminal, rather than the basal epithelial cells (Fig. 2.2C). Infection with influenza viruses induces the production of pro-inflammatory cytokines mediators in pigs that are thought to be key players in developing disease. Specifically, experimental infection with representative swine influenza virus strains demonstrated highly significant correlations between clinical signs and the levels of IL-1, IL-6, IL-12, IFN-α, IFN-γ, TNF-α, and acute phase protein in bronchoalveolar lavage fluid (Van Reeth, Van Gucht et al. 2002; Barbe, Atanasova et al. 2011).

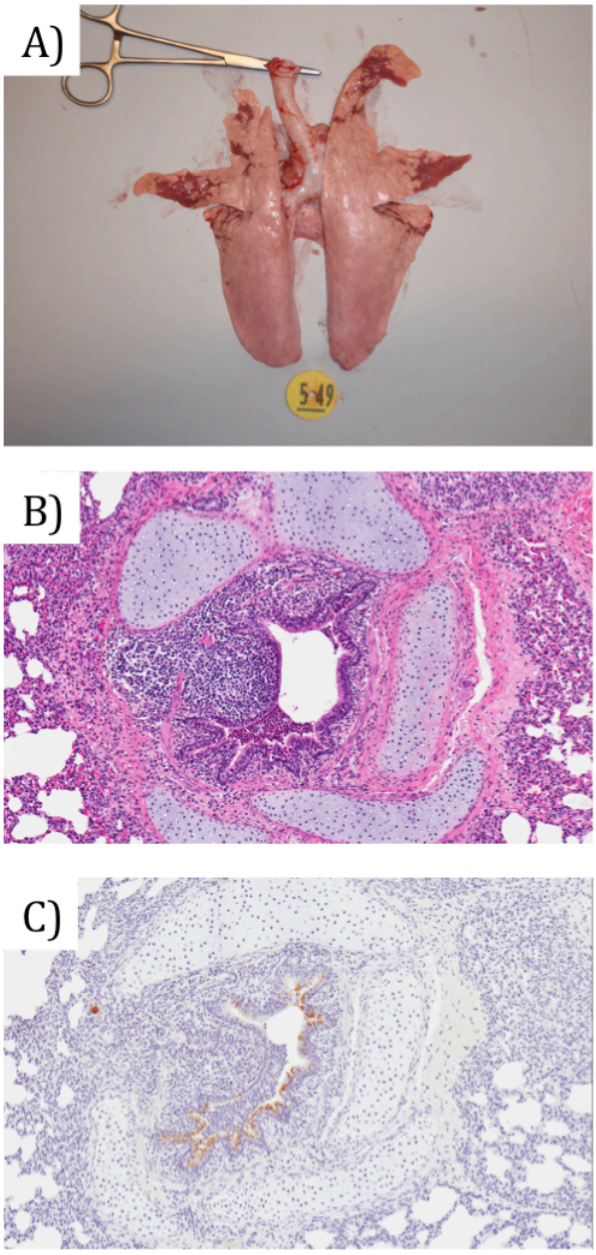



**Figure 2.2. Lung pathology induced by influenza in pigs.** Pigs were infected with A/turkey/Ohio/313053/04 (H3N2) and pneumonia was assessed at 3 dpi. (A) Gross lung pathology. There are multifocal dark red areas of consolidation, which are consistent with bronchointerstitial pneumonia. (B) Microscopic pneumonia. The bronchus contains neutrophils. The epithelium is infiltrated by neutrophils and there is a loss of cilia and goblet cells. The adjacent BALT is present. (C) Immunohistochemistry. The brown spots indicate the presence of the virus (NP antigen) in the bronchial epithelium. The bronchus is also infiltrated by neutrophils and lymphocytes and plasma cells.

Typical signs of influenza in pigs include abrupt onset fever, anorexia, inactivity and respiratory signs (sneezing, coughing, irregular abdominal breathing and ocular and nasal discharges). Although mortality is relatively low (1-3%), it can be higher in piglets and in breeding females, where the abortion rate can reach up to 7% (Easterday 1999; Zhou, Senne et al. 1999). Morbidity can be as high as 100% and can precipitate outbreaks of more serious respiratory diseases causing increased mortality. Outbreaks of swine influenza can have serious economic consequences because of the increased time needed for convalescent animals to attain slaughter weight (Kay, Done et al. 1994). Swine influenza virus is spread via aerosolized respiratory secretions and fomites, including contaminated inanimate objects and people moving between infected and uninfected swine. Outbreaks of swine influenza are most commonly associated with the introduction of new animals into a herd. The incubation period is usually one to three days. Pigs begin excreting the virus within 24 hours of infection, and may shed the virus for seven to ten days (OIE 2008). With respect to the 2009 pH1N1, pigs inoculated with this strain virus show clinical disease signs and pathology similar to infection with other swine influenza viruses (Brookes, Irvine et al. 2009; Itoh, Shinya et al. 2009; Lange, Kalthoff et al. 2009; Weingartl, Albrecht et al. 2009; Vincent, Lager et al. 2010).

### 2.2.5. Diagnosis

According to clinical and pathologic findings only a presumptive diagnosis can be made since there are no pathognomonic signs of the disease and the clinical signs may be confused with other respiratory diseases of pigs including porcine reproductive and respiratory syndrome virus (PRRSV), Aujeszky's disease (pseudorabies) virus, porcine respiratory coronavirus, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, and other bacterial agents. Moreover, in outbreaks of primary influenza virus infection, clinical signs many be mild or absent. Virus isolation is carried out on embryonated chicken eggs and/or in MDCK cell lines using nasal or pharyngeal swabs collected of live animals in the febrile phase. Tissue samples from trachea and lungs can be used to isolate viruses from animals that were euthanized or died during the acute stage of the disease. For routine diagnosis, however, RT-PCR and quantitative real time RT-PCR (qPCR) has replaced virus isolation, thanks to their speed, sensitivity, automation, and for enabling rapid diagnosis on samples where the virus is no longer viable. Nevertheless, virus isolation is still used to provide enough material for genome sequencing (Richt, Lager et al. 2004; OIE 2008; Lorusso, Faaberg et al. 2010; Nagarajan, Simard et al. 2010).

Serology on paired samples can diagnose swine influenza, retrospectively. However, the presence of maternal antibodies may limit the applicability of antibody-based assays. The presence of swine influenza specific antibodies can be determined by serological assays such as the hemagglutination inhibition test (HI), which is subtype specific. Additionally, serum neutralization and ELISA can also be used. Virus present in fixed tissue samples can be detected by immunofluorescence or immunohistochemistry methods (Vincent, Janke et al. 1997; OIE 2008). Recent developments in metagenomics-based strategies, such as pan-viral microarrays and deep sequencing, may potentially transform the way clinical microbiology laboratories approach the diagnosis and genetic characterization swine influenza as well as other respiratory diseases of swine (Greninger, Chen et al. 2010; Nicholson, Kukielka et al. 2011).

### 2.2.6. Prevention and control

Vaccine and strict biosecurity practices are the primary means of preventing and controlling swine influenza in pig populations. Because influenza can be introduced into a facility in an infected animal, strict quarantine of newly acquired pigs can decrease the risk of transmission to the rest of the herd. Segregation and partial depopulation of early-weaned piglets, "all-in/all-out" system and rigorous hygiene, are considered essential steps to control the spread of the swine influenza within the farm and minimize the economical effects of the disease. Infected swine herds can be cleared of influenza viruses by depopulation. Where exclusion is not feasible, vaccines can be used to control the disease (Kothalawala, Toussaint et al. 2006; Vincent, Ma et al. 2008). Currently, commercially available swine influenza vaccines are based on inactivated whole virus preparations of the H1N1 and H3N2 subtypes. The use of vaccination has become a common practice to control swine influenza in the US. Nonetheless, these commercially available vaccines provide limited protection against the antigenically diverse influenza viruses that circulate in North American pigs, and consequently, forced swine producers to use autogenous inactivated vaccines with the hope of achieving better antigenic matching (Vincent, Lager et al. 2007). These vaccines do not always prevent infection or virus shedding, but the disease is usually milder if it occurs. However, it is important to note that the use of inactivated vaccines has been associated with enhanced pneumonia when immunized pigs were challenged with divergent viruses (Vincent, Lager et al. 2007).

Experimental live attenuated influenza vaccines (LAIV) have been shown to induce immune responses in pigs that provide protection to both homologous and heterologous

challenge (Vincent, Ma et al. 2007; Masic, Booth et al. 2009). However, the attenuation mechanism used in these platforms was not temperature-sensitive (ts), the attenuation mechanism used in vaccines currently available for humans (Belshe 2004) and horses (Townsend, Penner et al. 2001). Recently, a LAIV using ts genetic markers for attenuation was developed using a contemporary IAV strain with the triple reassortant backbone and shown to be efficacious in swine. Importantly, these vaccines provided sterilizing immunity in pigs (Pena, Vincent et al. 2011). LAIV closely mimic natural infection and have several advantages over inactivated vaccines. LAIVs trigger cell-mediated immunity and mucosal immune responses, thereby providing longer-lasting immunity and broader antigenic coverage than conventional inactivated vaccines (Cox, Brokstad et al. 2004; He, Holmes et al. 2006; Horimoto and Kawaoka 2009). Additionally, LAIV are more cost effective to produce than inactivated vaccines since there are inherently less processing steps during production.

In general, the antigenic evolution (antigenic drift) of swine viruses occurs at a rate approximately six times slower than the rate in human viruses, suggesting that swine influenza vaccines may be changed less often than their human counterparts (de Jong, Smith et al. 2007). Nevertheless, the high genetic and antigenic diversity of influenza viruses circulating in swine populations underscores the need for effective vaccines to mitigate the impact of swine influenza in pigs as well as in populations at risk such as workers with occupational exposure to swine.

# Chapter 3: Role of Surface Glycoproteins in the Virulence of Triple Reassortant H3N2 Influenza Viruses in Swine

## *3.1. Abstract*

H3N2 triple reassortant (TR) swine influenza viruses first emerged in the United States in 1998 and have since become endemic in swine populations. Little is known about the molecular markers that modulate the pathogenesis of influenza viruses in swine. Therefore, there is a need to understand the molecular basis of pathogenesis in this important natural host. Here we focused on the role of surface glycoproteins in the virulence of TR H3N2 influenza virus in pigs. Two closely related H3N2 TR influenza viruses were chosen for these studies. These strains are influenza A/swine/WI/14094/99 (H3N2) and A/turkey/OH/313053/04 (H3N2), referred herein as sw/99 and ty/04, respectively. By using reverse genetics, we exchanged the HA and NA genes between the low virulent (sw/99) and the highly virulent (ty/04) viruses to prepare 6:2 reassortant and determine whether the surface proteins are responsible for the differential virulence seen in pigs. The clinical signs when present were milder than with infection ty/04 WT virus and none of the animals had respiratory signs of disease, suggesting that the surface genes are necessary but not sufficient for the clinical disease. Interestingly, the 6 ty/04:2 sw/99 reassortant had higher body temperature than the 6 sw/99:2 ty/04, suggesting that the internal genes from the ty/04 are the major contributors for the febrile response. Remarkably, swapping the surface genes was sufficient for reverting the lung pathology phenotype. Taken together, these studies suggest that surface glycoproteins are responsible for the macroscopic pneumonia induced by TR H3N2 influenza viruses in pigs.

## *3.2. Introduction*

Influenza A viruses (IAV) are single-stranded RNA viruses with negative polarity that belong to the family *Orthomyxoviridae*. Its genome contains eight RNA segments, which encode 10-12 viral proteins. Two major viral glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA), are present in the viral envelope. To date, 16 HA and 9 NA subtypes have been described (Fouchier, Munster et al. 2005). These surface glycoproteins are the main antigenic determinants of the virus. The HA is the viral ligand that recognizes sialic acid receptors present on glycoproteins and glycolipids on host cells. Binding to host receptors is the first step in the viral life cycle and this property makes the HA a major contributor to the host range of influenza viruses (Suzuki, Ito et al. 2000). The NA glycoprotein virus promotes the release of virus particles from infected cells contributing to the spread of progeny virions at the final stage of infection. Recently, it was formally proven that NA also plays an important role early during the viral infection, presumably by removal of mucus covering the airways epithelium (Matrosovich, Matrosovich et al. 2004).

IAV display a remarkable ability to infect a variety of animal species, including humans, pigs, wild and domestic birds, horses, cats, dogs, ferrets, and sea mammals (Palese and Shaw 2007). Among these animals, wild aquatic birds are thought to be the primordial reservoir from which all IAV have emerged (Webster, Bean et al. 1992). Little is known about the molecular markers that lead to the emergence of novel viruses subtypes with the ability to cross the species barrier and spread efficiently among humans. However, genetic reassortment played a role in the genesis of the last three influenza pandemics. In both the 1957 Asian (H2N2) and 1968 Hong Kong (H3N2) pandemics, reassortment occurred between human and avian viruses. The 2009 (H1N1) pandemic (pH1N1), besides having gene segments derived from avian and human IAV, also acquired gene segments from the swine IAV.

Pigs have been suggested as intermediate hosts for the generation of novel influenza viruses because they are susceptible to infection with avian and human influenza viruses (Scholtissek, Burger et al. 1983; Scholtissek, Burger et al. 1985; Scholtissek 1995) and have both the human and avian type sialic acid receptors (Ito, Couceiro et al. 1998). Since both types of receptors are also found in the respiratory tract of quail, pheasant, turkey, guinea fowl (Wan and Perez 2006; Kimble, Nieto et al. 2010) and humans (Shinya, Ebina et al. 2006), the receptor specificity does not explain the increased susceptibility of this species. However, pigs have been commonly associated with two-way transmission of influenza

viruses to and from humans (Myers, Olsen et al. 2007; Robinson, Lee et al. 2007; Newman, Reisdorf et al. 2008; Vincent, Swenson et al. 2009) and a greater number of reassortants between pH1N1 and circulating IAV have been isolated from pigs than from any other species (Vijaykrishna, Poon et al. 2010; Howard, Essen et al. 2011; Moreno, Di Trani et al. 2011; Pereda, Rimondi et al. 2011; Starick, Lange et al. 2011; Zhu, Zhou et al. 2011).

Three influenza subtypes circulate in the swine populations of the world: H1N1, H3N2, and H1N2. In North America, classical H1N1 viruses (cH1N1), descendents of the 1918 "Spanish flu" pandemic, were the predominant subtype circulating in swine populations since their first isolation in 1930. In 1998, however, the epidemiology of swine influenza infection in North American pigs changed dramatically. A new genotype of H3N2 triple reassortant IAV (TR) emerged in the U.S. swine population and established permanent lineages. TR swine IAV have demonstrated an exceptional ability to reassort with classical swine H1N1, human H1N1, and H3N2 viruses, generating at least seven different reassortant lineages in the last decade (Karasin, Olsen et al. 2000; Karasin, Schutten et al. 2000; Webby, Rossow et al. 2004; Karasin, Carman et al. 2006; Lekcharoensuk, Lager et al. 2006; Ma, Gramer et al. 2006; Olsen, Karasin et al. 2006; Ma, Vincent et al. 2007). Currently, TR strains of the H3N2, H1N2, H1N1, and the pH1N1 subtypes are predominant in the US swine population (Vincent, Ma et al. 2008). These viruses are not just limited to swine hosts: between 2003-2004, turkeys experienced outbreaks in North Carolina, Minnesota, and Ohio (Choi, Lee et al. 2004). A unique feature shared by all of these endemic reassortants is the maintenance of the triple reassortant internal gene (TRIG) cassette, which consists of the avian-like (PA and PB2), swine-like (M, NP, and NS), and human-like (PB1) gene segments. The TRIG cassette appears to accept multiple HA and NA types, which could provide a selective advantage to swine viruses possessing this internal gene constellation (Vincent, Ma et al. 2008; Ma, Lager et al. 2009) .

Typical signs of influenza in pigs include abrupt onset fever, anorexia, inactivity and respiratory signs (sneezing, coughing, irregular abdominal breathing, and ocular and nasal discharges). Although mortality is relatively low (1-3%), it can be higher in piglets and in breeding females, where the abortion rate can reach up to 7% (Easterday 1999; Zhou, Senne et al. 1999). Morbidity can be as high as 100% and can precipitate outbreaks of more serious respiratory diseases causing increased mortality. Outbreaks of swine influenza can have serious economic consequences because of the increased time needed for convalescent animals to attain slaughter weight (Kay, Done et al. 1994).

The genetic, antigenic, and pathobiological attributes of TR H3N2 swine influenza virus have been evaluated. At the genetic level, at least three introductions of human H3-subtype viruses, leading to phylogenetic clusters I, II, and III, have been documented (Richt, Lager et al. 2003; Webby, Rossow et al. 2004). The cluster III has evolved into cluster III variants, also known as cluster IV, which are now the predominant H3N2 viruses circulating in swine populations in North America (Gramer, Lee et al. 2007; Kumar, Deflube et al. 2011; Nfon, Berhane et al. 2011). Experimental infection of swine with viruses belonging to clusters I to III were able to induce clinical disease and pneumonia, whose severity varied between individual strains even within one antigenic cluster (Richt, Lager et al. 2003). However, to our knowledge there have been no studies investigating the molecular basis for strain-dependent virulence of the TR H3N2 swine influenza virus.

Virulence of IAV is considered a polygenic trait, in which disease outcome is determined by the gene constellation of a particular influenza virus strain within a specific host (Hatta, Gao et al. 2001; Chen, Bright et al. 2007; Basler and Aguilar 2008). Several virulence determinants in the genome of IAV have been described, including the HA (Kawaoka, Naeve et al. 1984; Kobasa, Takada et al. 2004; Pappas, Aguilar et al. 2008; de Wit, Munster et al. 2010; Ping, Dankar et al. 2010), NA (Pappas, Aguilar et al. 2008; Matsuoka, Swayne et al. 2009) , NS1 (Lipatov, Andreansky et al. 2005; Fernandez-Sesma, Marukian et al. 2006; Li, Jiang et al. 2006; Jiao, Tian et al. 2008), NP and the polymerase complex formed by PB2, PB1, and PA proteins (Snyder, Buckler-White et al. 1987; Hatta, Gao et al. 2001; Fouchier, Schneeberger et al. 2004; de Jong, Simmons et al. 2006; Salomon, Franks et al. 2006; de Wit, Munster et al. 2010; Ping, Dankar et al. 2010; Song, Feng et al. 2011). The balance between the HA and NA activities are crucial for influenza virus replication, virulence, and transmission (Mitnaul, Matrosovich et al. 2000; Hulse, Webster et al. 2004; Lu, Zhou et al. 2005; Yen, Liang et al. 2011).

To understand the molecular mechanism underlying the strain-specific virulence of the TR H3N2 swine influenza virus in pigs, two genetically related H3N2 TR IAV were selected for these studies. These strains are A/swine/Wisconsin/14094/99(H3N2) [sw/99] and A/turkey/OH/313053/04(H3N2) [ty/04], a relative of sw/99 virus that caused an outbreak on a turkey farm. Because these viruses displayed different pathotypes in pigs, we used reverse genetics to decipher the role of their surface glycoproteins in virulence for swine. We found that although the HA and NA genes were not sufficient for reverting the clinical disease phenotype, swapping these surface genes was necessary and sufficient for the development of

lung pathology. These results demonstrate that surface glycoproteins play a major role in the pneumonia induced by the TR H3N2 swine influenza virus in pigs.

## *3.3. Material and Methods*

### 3.3. 1. Ethics Statement

Swine studies were conducted under BSL-2 conditions approved by USDA and performed according to protocols R-08-16 “Transmissibility of Influenza A Viruses in Swine", approved by the Institutional Animal Care and Use Committee of the University of Maryland. Animal studies adhere strictly to the US Animal Welfare Act (AWA) laws and regulations.

### 3.3. 2. Cells and viruses

Human embryonic kidney cells (293-T) were cultured in OptiMEM I (GIBCO, Grand Island, NY) containing 10% FBS and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), L-glutamine and antibiotics.

A/swine/Wisconsin/14094/99 (H3N2) (Genbank taxonomy 136472) was a kind gift from Dr. Sagar Goyal, University of Minnesota. A/turkey/Ohio/313053/04 (Genbank taxonomy ID 533026) was kindly provided by Dr. Yehia Saif, Ohio State University. Both viruses were successfully rescued from cloned cDNAs as described below and amplified in either MDCK cells (sw/99) or embryonated eggs (ty/04) following the same substrates used for isolation of the WT viruses. Surface reassortants between sw/99 and ty/04 were generated by reverse genetics and amplified in MDCK cells.

### 3.3. 3. Reverse genetics

The eight-plasmid-based reverse genetics (RG) system (Hoffmann, Neumann et al. 2000) was used to rescue WT A/swine/Wisconsin/14094/99 (H3N2) (sw/99), A/turkey/Ohio/313053/04 (H3N2) (ty/04) and their 2:6 surface reassortant. Briefly, viral RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and reverse-transcribed to cDNA using the uni-12 primer (5′-AGCAAAAGCAAAGG-3′) and the AMV reverse transcriptase (Promega, Madison, WI, USA). Viral RNA segments were PCR amplified using a set of universal primers (Hoffmann,

Stech et al. 2001). Purified amplicons were digested with either BsaI or BsmBI (NEB, Ipswich, MA) and cloned into the pDP2002 vector (Song, Nieto et al. 2007). The resulting clones were fully sequenced and compared to the WT viral consensus. Mutations found in plasmid clones were corrected by site-directed mutagenesis using a commercially available kit (Stratagene, La Jolla, CA).

The recombinant WT viruses as well as the 6:2 surface gene reassortants were generated by transfecting the RG plasmids into co-cultured 293-T and MDCK cells as previously reported (Hoffmann, Neumann et al. 2000). 6 ty/04:2sw/99 is a 6:2 reassortant with the 6 internal genes from ty/04 and 2 surface genes from the sw/99, whereas the 6 sw/99:2 ty/04 has the 6 internal genes from sw/99 and 2 surface genes from the ty/04.

3.3. 4. Swine studies

To evaluate the pathogenicity of the sw/99 and ty/04 strains in swine, two independent experiments were carried out. A total of 14 female Yorkshire swine (5 weeks old and weighing approximately 40 lbs each) were obtained from a commercial farm and shown to be negative for antibodies against influenza A viruses by a commercially available NP ELISA (Synbiotics/Pfizer, Kansas City, MO). Pigs were sedated by intramuscular injection of 6 mg/kg of tiletamine-zolazepam (Telazol®, Pfizer Animal Health, NY) and 2.2 mg/kg of xylazine. In each experiment, groups of 3 pigs each were infected intratracheally with $1x10^8$ $TCID_{50}$ with either sw/99 or ty/04 virus diluted in 4 mL of sterile PBS. Two pigs served as an uninfected control and were inoculated through the same route with 4 mL of PBS. For five days post inoculation, pigs were monitored daily for hyperthermia and clinical signs of disease and virus shedding in nasal secretions were measured. At the end of the experiment, the animals were euthanized and necropsied. Nasal swabs were collected daily in 1 mL of Brain-Heart Infusion (BHI) media containing antibiotics and stored at -80°C until further analysis. During necropsy, lungs were removed in toto and a score was given based on the percentage of gross lesions of lobes showing the purple-red consolidation. Bronchoalveolar lavage fluid (BALF) and sections of the pulmonary lobes were also obtained for virus isolation.

To evaluate the virulence of the surface reassortants between sw/99 and ty/04, two groups of pigs (n=3) were infected with each of the reassortants following the same experimental design for the infection with WT viruses.

3.3. 5. Virus titration

Viral stocks and viruses present in biological samples (clarified lung homogenates, nasal swabs or BALF) were titrated on MDCK cells and the $TCID_{50}$/ml was determined by the method of Reed and Muench (Reed and Muench 1938). Briefly, samples were serially diluted 10-fold in OptiMEM I media (GIBCO, Grand Island, NY) containing antibiotics and 1 µg/ml TPCK-trypsin (SIGMA, Saint Louis, MO). Next, 200 µL of the inoculum were overlaid onto confluent monolayers of MDCK cells seeded in 96-well plates. Plates were incubated for 3 days and the endpoint viral titer was determined by an HA assay readout using 0.5% turkey red blood cells.

3.3. 6. Cytokine levels in porcine BALF

The levels of nine porcine cytokines (IFNγ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and TNFα) in BALF samples were determined by multiplex sandwich ELISA following the manufacter's protocol (Aushon Biosystems, MA).

3.3. 7. Alignment and phylogenetic analysis

The surface glycoproteins of the sw/99 and ty/04 viruses were aligned with other North American H3N2 swine IAV using ClustalW method in MegAlign (Lasergene v.8.1.5., DNAStar, Madison, WI). The phylogenetic tree was generated by Neighboor-Joining method using the using PAUP 4.0b10 program (Sinauer Associates, Inc., Sunderland, MA). Robustness of phylogenetic trees was tested by performing 1000 bootstrap replicates with 111 random seeds.

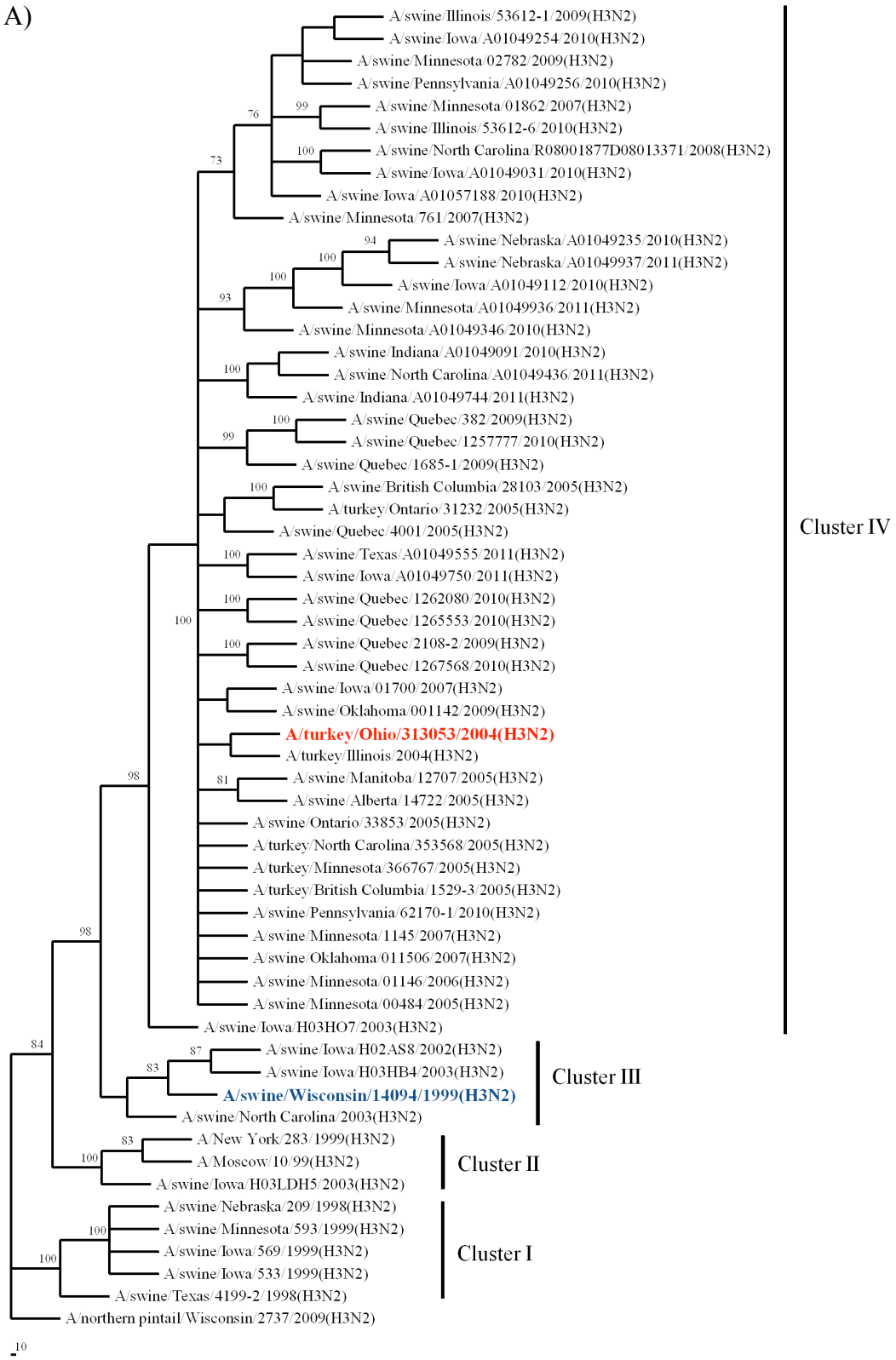
3.3. 8. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student t-test, whereas multiple comparisons were carried out by analysis of variance (ANOVA) using Tukey's post hoc test, unless otherwise specified. The differences were considered statistically significant at $p< 0.05$.

## *3.4. Results*

### 3.4.1. Evolutionary relationships and sequence alignment of the sw/99 and ty/04 surface glycoproteins

The A/swine/Wisconsin/14094/99 (H3N2) belongs to cluster III of the North America TR H3N2 swine IAV based on the nucleotide sequence of its HA gene. Initially, phylogenetic analysis of the HA gene of A/turkey/Ohio/313053/04 (H3N2) suggested that the virus also belonged to cluster III and was closely related to A/Swine/WI/14094/99 (H3N2) (Tang, Lee et al. 2005). However, the cluster III viruses have continued to evolve into cluster III variants, also known as cluster IV. With the inclusion of more recent TR H3N2 strains isolated from different hosts, it has become clear that the ty/04 strain is a variant belonging to cluster IV of TR H3N2 IAV, not to cluster III (Fig. 3.1A). Deduced amino acid sequences of the HA protein of both the sw/99 and ty/04 viruses showed that these viruses differ by 26 amino acids (95.4% similarity), whereas their NA differ by 27 amino acids (94.2% similarity). Major amino acid changes were shown at the antigenic sites of sw/99 and ty/04 HA1 molecule. Furthermore, there are drastic changes in receptor binding and glycosylation sites between the two strains (Fig. 3.1B).


A)
A/swine/Illinois/53612-1/2009(H3N2)
A/swine/Iowa/A01049254/2010(H3N2)
A/swine/Minnesota/02782/2009(H3N2)
A/swine/Pennsylvania/A01049256/2010(H3N2)
A/swine/Minnesota/01862/2007(H3N2)
A/swine/Illinois/53612-6/2010(H3N2)
A/swine/North Carolina/R08001877D08013371/2008(H3N2)
A/swine/Iowa/A01049031/2010(H3N2)
A/swine/Iowa/A01057188/2010(H3N2)
A/swine/Minnesota/761/2007(H3N2)
A/swine/Nebraska/A01049235/2010(H3N2)
A/swine/Nebraska/A01049937/2011(H3N2)
A/swine/Iowa/A01049112/2010(H3N2)
A/swine/Minnesota/A01049936/2011(H3N2)
A/swine/Minnesota/A01049346/2010(H3N2)
A/swine/Indiana/A01049091/2010(H3N2)
A/swine/North Carolina/A01049436/2011(H3N2)
A/swine/Indiana/A01049744/2011(H3N2)
A/swine/Quebec/382/2009(H3N2)
A/swine/Quebec/1257777/2010(H3N2)
A/swine/Quebec/1685-1/2009(H3N2)
A/swine/British Columbia/28103/2005(H3N2)
A/turkey/Ontario/31232/2005(H3N2)
A/swine/Quebec/4001/2005(H3N2)
A/swine/Texas/A01049555/2011(H3N2)
A/swine/Iowa/A01049750/2011(H3N2)
A/swine/Quebec/1262080/2010(H3N2)
A/swine/Quebec/1265553/2010(H3N2)
A/swine/Quebec/2108-2/2009(H3N2)
A/swine/Quebec/1267568/2010(H3N2)
A/swine/Iowa/01700/2007(H3N2)
A/swine/Oklahoma/001142/2009(H3N2)
A/turkey/Ohio/313053/2004(H3N2)
A/turkey/Illinois/2004(H3N2)
A/swine/Manitoba/12707/2005(H3N2)
A/swine/Alberta/14722/2005(H3N2)
A/swine/Ontario/33853/2005(H3N2)
A/turkey/North Carolina/353568/2005(H3N2)
A/turkey/Minnesota/366767/2005(H3N2)
A/turkey/British Columbia/1529-3/2005(H3N2)
A/swine/Pennsylvania/62170-1/2010(H3N2)
A/swine/Minnesota/1145/2007(H3N2)
A/swine/Oklahoma/011506/2007(H3N2)
A/swine/Minnesota/01146/2006(H3N2)
A/swine/Minnesota/00484/2005(H3N2)
A/swine/Iowa/H03HO7/2003(H3N2)
A/swine/Iowa/H02AS8/2002(H3N2)
A/swine/Iowa/H03HB4/2003(H3N2)
A/swine/Wisconsin/14094/1999(H3N2)
A/swine/North Carolina/2003(H3N2)
A/New York/283/1999(H3N2)
A/Moscow/10/99(H3N2)
A/swine/Iowa/H03LDH5/2003(H3N2)
A/swine/Nebraska/209/1998(H3N2)
A/swine/Minnesota/593/1999(H3N2)
A/swine/Iowa/569/1999(H3N2)
A/swine/Iowa/533/1999(H3N2)
A/swine/Texas/4199-2/1998(H3N2)
A/northern pintail/Wisconsin/2737/2009(H3N2)
Cluster IV
Cluster III
Cluster II
Cluster I

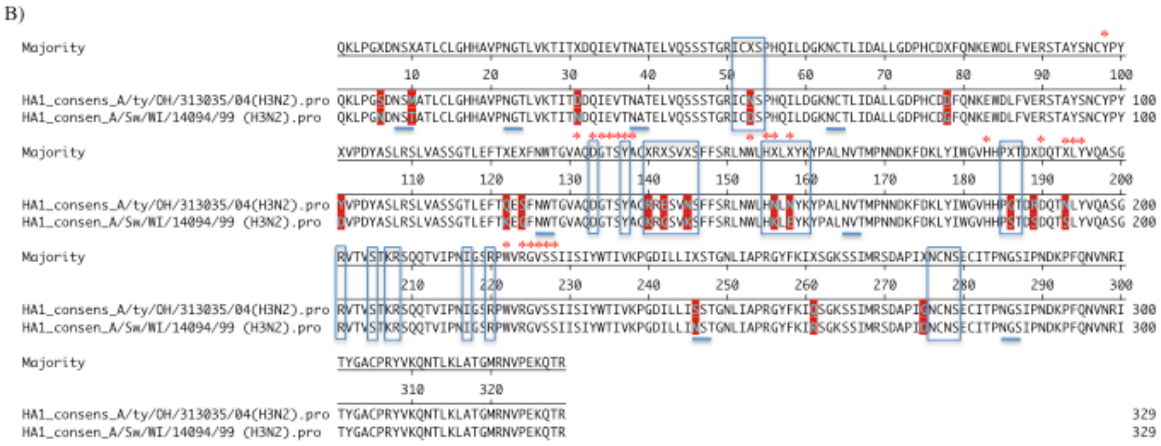



**Figure 3.1. Phylogenetic analysis and alignment of of the HA derived from the TR H3N2 viruses used in this study.** (A) Phylogenetic relationships of sw/99 and ty/04 with other H3N2 IAV. Analysis was based on the nucleotide sequences of the HA genes. The phylogenetic tree was generated by Neighboor-Joining method using the PAUP 4.0 beta 10 program. (B) Alignment of the HA1 protein. Shaded residues indicate amino acids that differ from the consensus. Boxed residues represent antigenic sites in the H3 molecule. Underlined residues are possible N-glycosylation sites. Residues with asterisks represent receptor binding sites.

3.4.2. Genetically related triple reassortant H3N2 influenza viruses display distinct pathotypes in swine

To determine the pathotypes of the two parental WT TR H3N2 viruses used in this study, we initially inoculated groups of pigs with $1x10^8$ $TCID_{50}$ with either sw/99 or ty/04 virus through the intratracheal route because experimental reproduction of typical signs of influenza in pigs is most consistently accomplished by intratracheal inoculation with high doses of virus (Maes 1984). After inoculation with sw/99, none of the pigs had fever or other clinical signs, with exception of a single pig that had a transient cough and a two-day decrease in food consumption. This suggests that this virus is of low virulence for pigs under experimental conditions. In contrast, pigs inoculated with ty/04-developed signs of severe clinical disease, which were characterized by high fever, hyporexia, inactivity, huddling, nasal and ocular discharge, coughing, and dyspnea. Fifty percent (3 out of 6) of pigs inoculated with ty/04 developed conjunctivitis between 1-5 dpi (Fig. 3.2 Table 3.1).

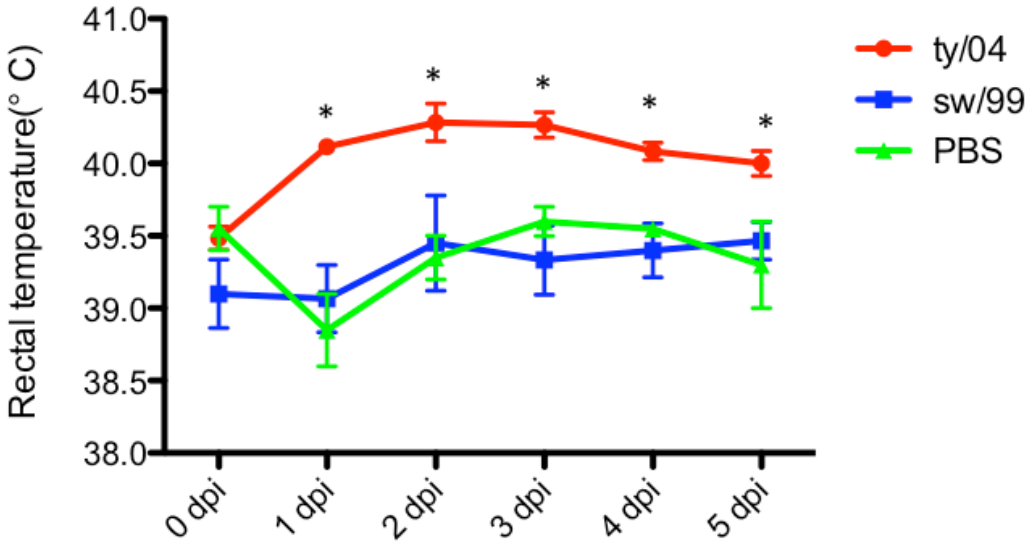



**Figure 3.2. Rectal temperature of pigs intratracheally infected with H3N2 triple reassortant Influenza A viruses.** Daily mean rectal temperatures of pigs following inoculation with either sw/99, ty/04, or PBS control (Values are shown as the mean ± SEM. * $p<0.05$ (Two-way ANOVA).

At necropsy, pigs infected with sw/99 had discrete lung lesions, which were characterized by small areas and scarce with purple-red consolidation. In sharp contrast, pigs infected with ty/04 virus had pneumonia, which was characterized by multiple areas of purple-red lung consolidation (Fig.3.3), airways filled with blood-tinged fibrinous exudates, and enlargement of the tracheobronchial lymph nodes (data not shown). The severity of macroscopic lung lesions correlated with the clinical outcome of the infection by these two viruses. As expected, mock-infected animals had neither clinical disease nor macroscopic lung lesions (data not shown).

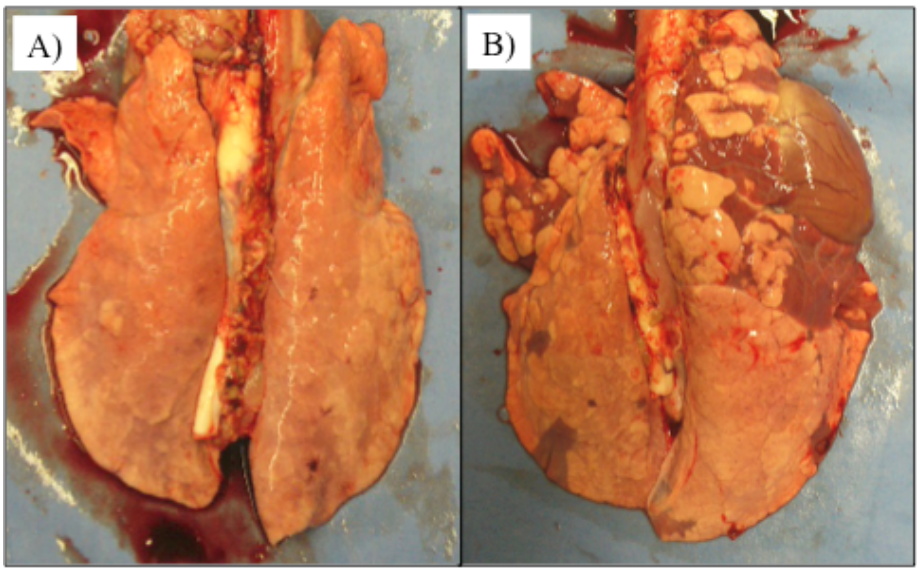



**Figure 3.3. Gross pathology of pigs infected with H3N2 triple reassortant Influenza A viruses.** On day 5 post-infection, pigs were euthanized and the gross pathology was evaluated. Representative lungs from pigs infected with either sw/99 (A) or ty/04 (B) are shown. Lungs from pigs infected with sw/99 display minimum pathology, whereas lungs from the ty/04 group have multifocal areas of dark red consolidation that typical of swine influenza pneumonia.

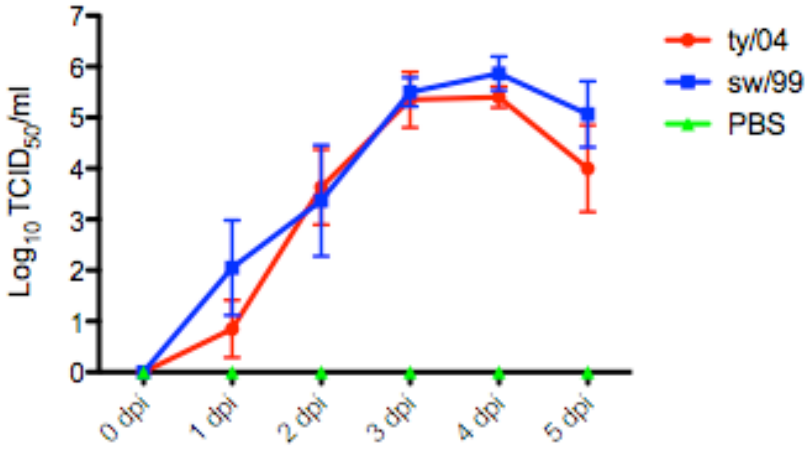
The degrees of macroscopic lung pathology were mirrored by the changes found at the microscopic levels. Pigs infected with the sw/99 virus generally showed only mild histopathological lesions as opposed to the ty/04-inoculated pigs that had severe bronchopneumonia. In general, pulmonary lesions were typical of influenza virus infection in pigs and were characterized by multifocal to widespread necrotizing bronchitis and bronchiolitis, light peribronchiolar lymphocytic cuffing, mild multifocal interstitial pneumonia and varying degrees of alveolar involvement (data not shown). These results suggest that in spite of the genetic similarity between ty/04 and sw/99, the two viruses display different signs of clinical disease and lung pathology in swine.

### 3.4.3. Pneumonia caused by ty/04 does not correlate with levels of viral replication in the respiratory tract

We then compared the levels of virus shedding in the upper respiratory tract and the viral lung load in both the sw/99 and the ty/04-infected pigs. There was no statistical significant difference in the kinetics and absolute levels of virus shedding between the two viruses during the first five days of infection. Nasal shedding was first detected at 1 dpi and peaked at 4 dpi, reaching 5-6 $\log_{10}$ $TCID_{50}$/mL (Fig. 3.4A).

Virus titers in BALF and lung homogenate ranged from $10^4$ to $10^5$ $TCID_{50}$ /ml of BALF or gram of tissue and the differences were not statistically significant between two viruses (Fig. 3.4B). In conclusion, these results suggest that both viruses replicate similarly in the respiratory tract of pigs and the levels of replication do not correlate with the severity of clinical disease or pneumonia.

**A)**




**B)**

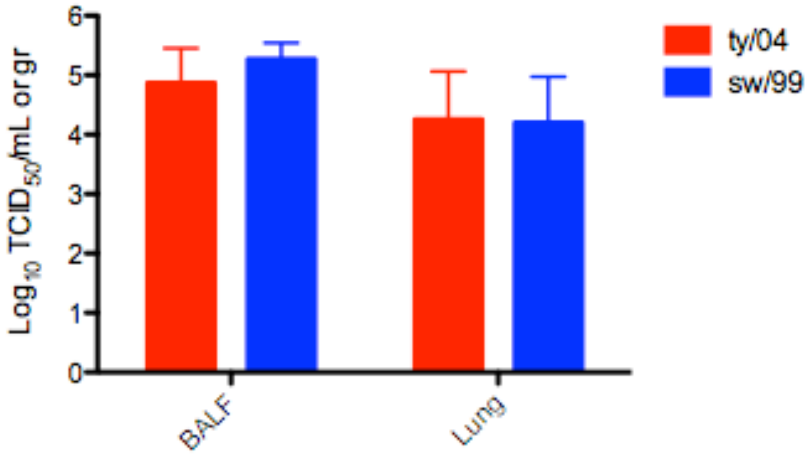



**Figure 3.4. Shedding and viral lung load following inoculation of pigs with H3N2 triple reassortant Influenza A viruses.** Groups of pigs (n=6) were inoculated intratracheally with $10^8$ $TCID_{50}$/animal of either sw/99 or ty/04 diluted in 4 ml of PBS. A third group (n=3) was mock vaccinated with PBS alone. (A) Viral shedding in nasal secretions of pigs infected with either WT sw/99 or ty/04 (B) Viral lung load. Viral titers in bronchoalveolar lavage fluid (BALF) and in the right cranial lobe collected at 5 dpi. Viral titers were determined by $TCID_{50}$ on MDCK cells. Values are shown as the mean ± SEM. The differences are not statistically significant.

3.4.4. Febrile response induced by ty/04 correlates with pulmonary levels of IL-1β

Cytokine production after influenza infection has been implicated in the pathogenesis of influenza in mammals, including pigs (Van Reeth 2000). To further understand the host response following infection with sw/99 or ty/04 in swine, we measured the protein levels of nine porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-γ) in BALF collected at 5 dpi necropsies. The concentrations of IL-2 were elevated in infected pigs relative to mock controls, but there was no statistical significant difference between the two viruses. Interestingly, ty/04-infecteded pigs exhibited statistically significant higher levels of the proinflammatory cytokine IL1-β as compared to sw/99 group (Fig.3.5). The high levels of IL1-β in the ty/04 group correlated with the fever induced by the infection with this virus strain. These results indicate that ty/04 infection induces higher expression levels of IL1-β relative to sw/99 and this heightened production of pro-inflammatory mediator may have contributed to the sustained febrile response developed by ty/04-infected animals.

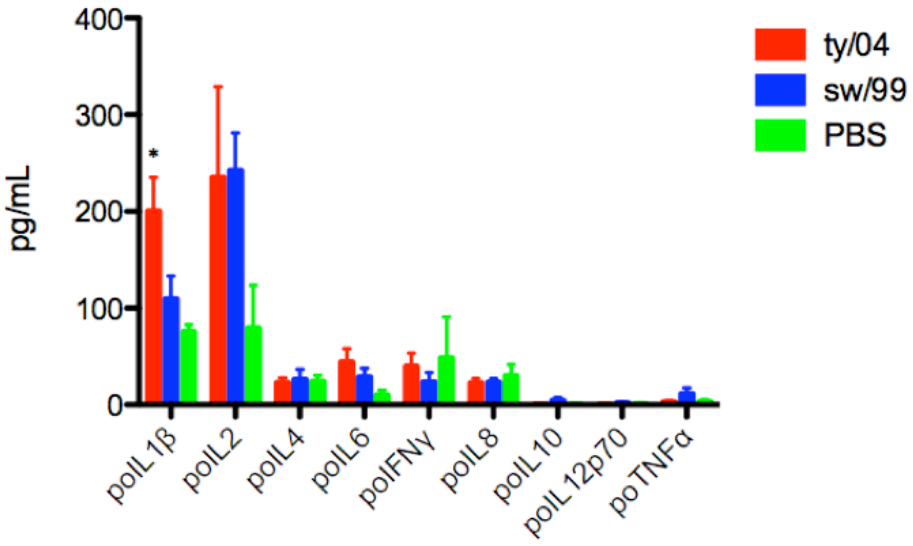



**Figure 3.5. Cytokine profile in the lungs of pigs infected with WT H3N2 TR viruses**. The levels of nine porcine cytokines in BALF samples were determined by multiplex sandwich ELISA. * Indicates a statistically significant difference between ty/04 and sw/99 ($p < 0.05$, Student's t-test).

### 3.4.5. Surface glycoproteins modulate the lung pathology of TR H3N2 influenza viruses in swine

We next attempted to determine the molecular attributes that led to enhanced virulence of the ty/04 strain compared to sw/99. By using reverse genetics, we exchanged the HA and NA genes between the less virulent (sw/99) and the more virulent (ty/04) viruses to determine whether the surface proteins are responsible for the differential virulence seen in pigs. We focused on HA and NA surface proteins because of their pivotal role in influenza virulence for other species and the fact that they co-evolve (Mitnaul, Matrosovich et al. 2000; Pappas, Aguilar et al. 2008).

Six female Yorkshire swine were divided in two groups of three pigs each and infected with the 6:2 surface reassortants between sw/99 and ty/04 using the same dose and route as described above for infection with the parental WT viruses. The clinical signs, when present, were milder than with infection WT viruses and none of the animals had respiratory signs of disease, suggesting that the surface genes are necessary but not sufficient for the clinical disease induced by ty/04 (Table 3.1).

Table 3.1. Clinical signs of pigs infected with H3N2 triple reassortant Influenza A viruses and their 6:2 surface reassortants.

| | **Viruses** | | | |
|---|---|---|---|---|
| **Clinical signs** | **sw/99** | **ty/04** | **6 ty/04:2 sw/99** | **6 sw/99:2 ty/04** |
| hyporexia | **6/6** | **6/6** | **3/3** | **3/3** |
| inactivity | 0/6 | **6/6** | **3/3** | **3/3** |
| huddling | 0/6 | **6/6** | 0/3 | 0/3 |
| nasal discharge | 0/6 | **6/6** | 0/3 | 0/3 |
| conjunctivitis | 0/6 | **3/6** | 0/3 | **3/3** |
| coughing | **1/6** | **6/6** | 0/3 | 0/3 |
| dyspnea | 0/6 | **6/6** | 0/3 | 0/3 |

Strikingly, swapping the surface genes was sufficient for reverting the lung pathology phenotype (compare Fig. 3.6 with 3.2). Pigs infected 6sw/99:2ty/04 had the same percentage of lung involvement than pigs infected with the WT ty/04, suggesting that the surface genes from H3N2 TR influenza viruses are necessary and sufficient for induction of macroscopic pneumonia in pigs. The severity of lung pathology did not correlate with viral burden in the lungs since there was no statistically significant difference in lung and BALF viral titers among the groups (Fig. 3.7). In addition, the levels of viral shedding in nasal secretions were similar between the two viruses. Taken together, these studies suggest that the surface genes are involved in the macroscopic pneumonia in pigs.

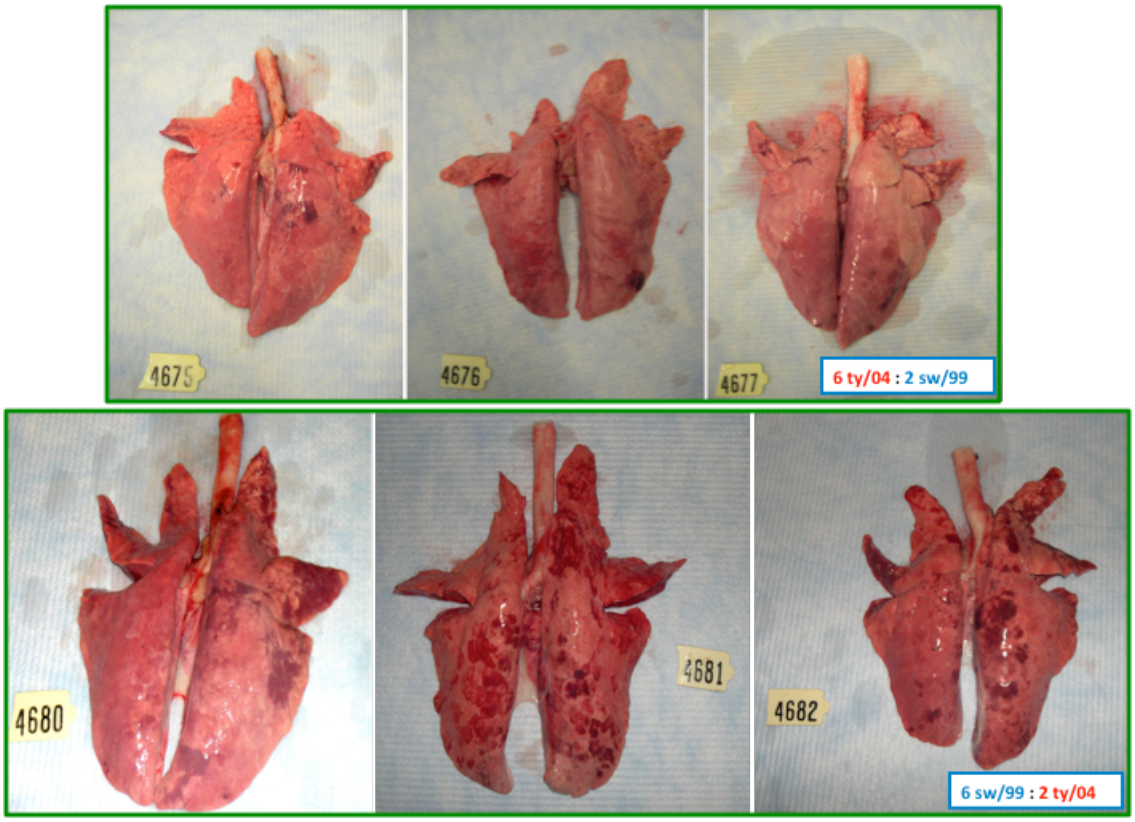



**Figure 3.6. Gross pathology of pigs infected with 6:2 surface reassortants between ty/04 and sw/99.** Groups of pigs (n=3) were inoculated intratracheally with $10^8$ $TCID_{50}$/animal of with 6:2 surface reassortants between sw/99 and ty/04viruses. At 5dpi, pigs were euthanized and their lungs were removed to evaluate gross pathology. Lungs from pigs infected with 6ty/04:2sw/99 (top panels) display minimum pathology, whereas lungs from 6sw/99:2ty/04-infected pigs (bottom panels) have multifocal areas of dark red consolidation that are undistinguishable from the ones induced by ty/04 WT.

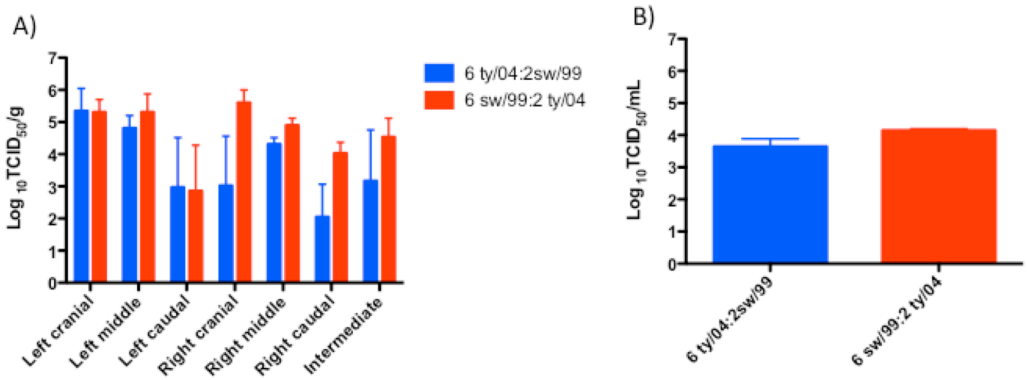



**Figure 3.7. Virus lung load after infection with 6:2 surface reassortants between sw/99 and ty/04 viruses.** Groups of pigs (n=3) were inoculated intratracheally with $10^8$ $TCID_{50}$/animal of with 6:2 surface reassortants between sw/99 and ty/04 viruses. At 5 dpi, pigs were euthanized and viral load in all lung lobes (A) and BALF (B) were determined by $TCID_{50}$ on MDCK cells. Values are shown as the mean ± SEM. The differences are not statistically significant.

3.4.6. The internal genes from ty/04 play a major role in the febrile response

Because infection with WT ty/04 caused sustained high body temperatures during the acute phase of the infection, we sought to study the role of surface and internal genes in this parameter of disease. Replacing both the HA and NA genes in ty/04 with those of sw/99 did not affect body temperature because animals infected with 6ty/04:2sw/99 reassortant had a higher body temperature ($p<0.05$, two-way ANOVA) than those animals infected with the 6 sw/99:2 ty/04 (Fig. 3.8). This suggests that ty/04 internal genes are the major contributors for the febrile response. The levels of IL1-β in 6ty/04:2sw/99 were not statistically different from the levels of IL1-β in 6 sw/99:2 ty/04 (data not shown), which may have been caused by the small number of animals used and the variability among them. Taken together, these experiments suggest that although surface genes are critical for the lung pathology, the internal genes also play a role in the febrile response and the clinical presentation of the disease.

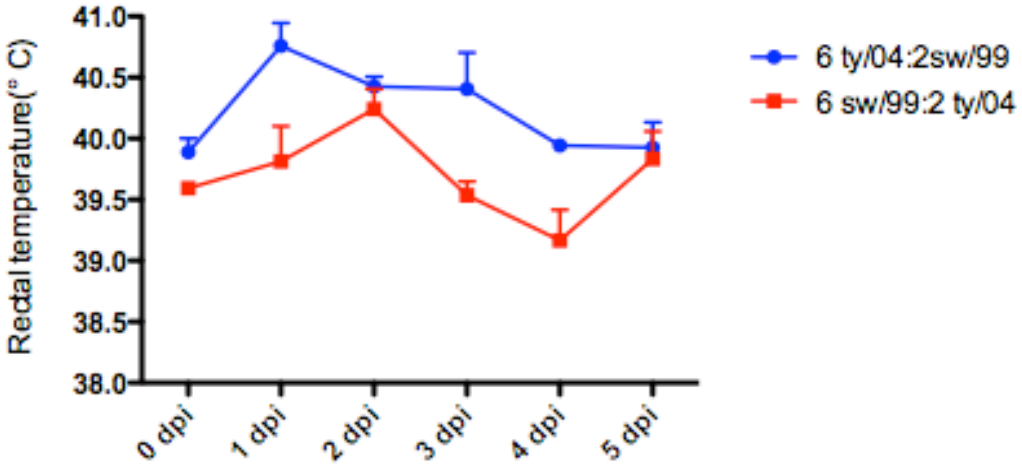



**Figure 3.8. Rectal temperature of pigs infected with 6:2 surface reassortants**. Groups of pigs (n=3) were inoculated intratracheally with $10^8$ $TCID_{50}$/animal of 6:2 surface reassortants between sw/99 and ty/04 viruses diluted in 4 ml of PBS. Values are shown as the mean ± SEM of the daily mean rectal temperatures of pigs following infection. The differences between the two groups are statistically significant as determined by Two-way ANOVA ($p< 0.05$).

## *3.5. Discussion*

The emergence of TR H3N2 influenza A viruses (IAV) in swine populations in 1998 has significantly affected the epidemiology of animal influenza in North America. Importantly, TR H3N2 IAV have expanded their host range and caused outbreaks in turkeys in North Carolina and Minnesota in 2003 (Choi, Lee et al. 2004), and Ohio in 2004 (Tang, Lee et al. 2005). Usually, infection of turkey flocks with swine influenza viruses is associated with close proximity to swine herds (Tang, Lee et al. 2005). In 2007, swine-like TR H3N2 viruses were isolated, for the first time, from migratory waterfowl in South Dakota, highlighting their potential for interspecies transmission (Ramakrishnan, Wang et al. 2010). Currently, TR H3N2 IAV, belonging to phylogenetic cluster IV, is the most prevalent TR H3N2 genotype circulating in North America animal populations, including swine, turkey, quails, and waterfowl (Ramakrishnan, Wang et al. 2010; Kumar, Deflube et al. 2011; Nfon, Berhane et al. 2011). TR swine IAV have demonstrated the ability to reassort with either classical swine H1N1 or human H1N1 and H3N2 viruses, generating at least seven different reassortant lineages in the last decade. Remarkably, the 2009 pandemic H1N1 resulted from genetic reassortment between TR swine IAV circulating in North America and Eurasia swine H1N1 IAV (Garten, Davis et al. 2009).

In our current study, we used reverse genetics with the goal of identifying molecular attributes responsible for the enhanced virulence of certain TR H3N2 IAV. We chose two representative strains, sw/99 and ty/04, because the ty/04 is a derivative of the sw/99 strain, a virus that had been circulating among pigs in the United States since 1998 (Tang, Lee et al. 2005). The evolutionary pathways and transmission events that led to the emergence of ty/04 had been hindered by a lack of systematic surveillance in turkey and swine populations. It has been shown that the ty/04 isolate is fully adapted to replication and transmission in both turkeys and swine (Yassine, Al-Natour et al. 2007). With respect to the sw/99 virus, to our knowledge, no studies have characterized this strain in pigs. Although both viruses were initially classified into genetic cluster III, it has become evident that the ty/04 virus is a cluster III variant, which is now denominated cluster IV (Fig. 3.1A). Richt and collaborators studied the pathogenesis of TR H3N2 swine IAV belonging from clusters I to III with respect to their ability to replicate and induce clinical signs and influenza-specific lesions in pigs. Pigs infected with H3N2 cluster I, II, or III virus demonstrated macroscopic pneumonia and there were differences in the severity of lesions between individual strains within genetic

cluster III (Richt, Lager et al. 2003). Although these studies have been important for the characterization of field isolates *in vivo*, the genetic basis of virulence of TR H3N2 IAV is yet to be elucidated.

To further characterize the pathotype of WT viruses in swine, we used an aggressive intratracheal challenge model. Only mild changes in the clinical appearances and lung pathology were observed in pigs infected with sw/99 strain. Fever was not observed in these animals. In sharp contrast, infection with ty/04 was associated with severe clinical disease, extensive gross pathological and histological pulmonary lesions, and sustained fever throughout the experimental period. Thus, our challenge protocol using high doses of virus ($1x10^8$ $TCID_{50}$) successfully reproduced disease manifestations under laboratory conditions. We have previously shown that intranasal infection with low dose ($1x10^5$ $TCID_{50}$) of ty/04 only caused mild clinical signs of disease (Pena, Vincent et al. 2011).

Surprisingly, the enhanced pneumonia caused by viruses containing the ty/04 surface glycoproteins (ty/04 WT and 6sw/99:2ty/04) did not correlate with either levels of viral shedding in nasal secretions or the viral lung load. This lack of correlation between viral burden and pneumonia may be due to the high infectious dose used in these experiments. It is possible that the use of a high infectious dose led to the synchronous infection of a large number of respiratory cells, which did not allow for the detection of modest differences in the levels of viral replication. Interestingly, when we used lower viral dose for infection ($10^5$ $TCID_{50}$), ty/04 replicated to higher titers than sw/99 (Pena et al, manuscript in preparation). Furthermore, Yassine et al. reported that ty/04 replicated more efficiently than a TR H3N2 swine IAV (A/swine/North Carolina/03 (H3N2) *in vitro* and *in vivo* and displayed better binding affinity to plasma membrane preparations from pig tracheas (Yassine, Khatri et al. 2011).

The infectivity of IAV is mainly determined by the presence of sialic acid receptors on susceptible host cells to which the HA is able to bind and internalize. Recently, a study investigating the distribution of human-like (α2,6Gal) and avian-like (α2,3Gal) influenza receptors (Nelli, Kuchipudi et al. 2010; Van Poucke, Nicholls et al. 2010; Trebbien, Larsen et al. 2011) challenged an earlier work that suggested that the presence of both types of receptors in the porcine trachea made pigs more susceptible to IAV and allowed them to serve as "mixing vessels" for human-avian influenza A virus reassortants (Ito, Couceiro et al. 1998). The current model suggests that the distribution of the two type sialic acid receptors in the pig respiratory tract closely resembles the published data of the human tract, in which

α2,6Gal is the predominant receptor species in all areas of the respiratory tract and the α2,3Gal receptor is found in small amounts exclusively in the lower respiratory tract (bronchioles and alveoli) (Nelli, Kuchipudi et al. 2010; Van Poucke, Nicholls et al. 2010; Trebbien, Larsen et al. 2011). Additionally, the avidity with which the ty/04 HA binds to each sialic acid species may determine its enhanced pneumonia *in* vivo. In agreement with this, titration of either ty/04 or sw/99 or their surface reassortants, using turkey red blood cells (RBC) yield the same titer, but when chicken RBC was used in these experiments, ty/04-containing viruses had a titer four-fold higher than sw/99 (data not shown). Since turkey RBC has predominantly α2,6Gal type receptor, whereas α2,3Gal is the preponderant sialic acid moiety in chicken RBC (Thompson, Barclay et al. 2004), it is conceivable that ty/04 may reach the deeper respiratory tract more effectively than sw/99 to cause bronchopneumonia.

Although introduction of ty/04 surface glycoproteins into the sw/99 backbone reproduced pneumonia caused by parental ty/04, the typical clinical disease induced by ty/04 could not be duplicated by this 6:2 reassortant. Here, we demonstrated that ty/04 internal genes, but not the surface genes, are responsible for the febrile response. Rescue and characterization of reassortant viruses containing the ty/04 surface genes and additional IAV virulence determinants located in the internal segments may shed light into the constellation of genes responsible for the clinical expression of disease. These studies are beyond the scope of this work and warrant further investigation.

In summary, we have shown that genetically related TR H3N2 IAV display distinct pathotypes in the swine host. Our current study indicates that the surface glycoproteins are necessary and sufficient for the induction of severe pneumonia, while their internal genes are responsible for the fever. The ability of pathogenic surface glycoproteins to induce pneumonia in the background of a low virulent virus has implications for the development of safer vaccines for this target species. Given the similarities between pigs and humans regarding the distribution of sialic acid receptors, cytokine production, and clinical-pathological outcomes after influenza infection (Van Reeth, Van Gucht et al. 2002; Barnard 2009; Barbe, Atanasova et al. 2010; Barbe, Saelens et al. 2010), our findings may be relevant for human medicine. Knowledge gained from understanding the molecular basis of virulence may aid in the selection of surface glycoproteins that are both innocuous and highly immunogenic.

# Chapter 4: Restoring PB1-F2 into the 2009 Pandemic H1N1 Influenza Virus Enhances Viral Replication, Histopathology, and Cytokine Responses in Swine.

## *4.1. Abstract*

PB1-F2 is an 87-90 amino acid long protein expressed by certain influenza A viruses. Previous studies have shown that PB1-F2 contributes to virulence in the mouse model; however, its role in natural hosts - pigs, humans, or birds - remains largely unknown. Outbreaks of domestic pigs infected with the 2009 pandemic H1N1 influenza virus (pH1N1) have been detected worldwide. Unlike previous pandemic strains, pH1N1 viruses do not encode a functional PB1-F2 due to the presence of three stop codons resulting in premature truncation after codon 11. However, pH1N1s have the potential to acquire the full-length form of PB1-F2 through mutation or reassortment. In this study, we assessed whether restoring the full-length PB1-F2 ORF in the pH1N1 background would have an effect in virus replication and virulence in pigs. Restoring the PB1-F2 ORF resulted in up-regulation of viral polymerase activity at early time points *in vitro* and enhanced virus yields in porcine respiratory explants and in the lungs of infected pigs. There was a significant increase in the severity of pneumonia in pigs infected with isogenic virus expressing PB1-F2 as compared to the WT pH1N1. The extent of microscopic pneumonia correlated with increased pulmonary levels of IFN-α and IL-1β in pigs infected with pH1N1 encoding a functional PB1-F2. Together, our results indicate that PB1-F2 in the context of pH1N1 modulates viral replication, lung histopathology, and local cytokine response in pigs.

## *4.2. Introduction*

Influenza A viruses (IAV) belong to the family *Orthomyxoviridae* and represent important pathogens of humans and animals. In the 20th century, humans experienced three IAV pandemics (1918, 1957, and 1968) that resulted in significant morbidity and mortality (Taubenberger and Kash 2010). These pandemic strains emerged through genetic reassortment between influenza viruses of avian origin and the circulating human strain. These events resulted in antigenic shift and the successful dissemination, in an immunologically naïve population, of a virus carrying a novel hemagglutinin (HA). In addition to the new HA, pandemic strains inherited the PB1 gene segment from the avian influenza donor virus (and the NA gene in 1957) (Smith, Vijaykrishna et al. 2009). Like most avian influenza viruses, these previous pandemic strains encoded a full-length PB1-F2 gene. However, human influenza strains have invariably evolved to introduce truncations in the PB1-F2 open reading frame (ORF). It is tempting to speculate that PB1-F2 function is necessary for virus survival in birds but unnecessary or detrimental in humans. Its significance in pandemic strains remains obscure.

In the spring of 2009, a novel H1N1 IAV emerged in North America causing acute respiratory disease in humans. The virus quickly spread throughout most regions of the world, prompting the World Health Organization (WHO) to declare an influenza pandemic on June 11th, 2009 (Dawood, Jain et al. 2009; Garten, Davis et al. 2009). The new virus (pH1N1) resulted from genetic reassortment between swine influenza viruses (SIVs) circulating in North America and Eurasia. Specifically, six of its RNA segments (PB2, PB1, PA, HA, NP, and NS) are closely related to North American triple-reassortant (TR) H1N1 SIVs, whereas the NA and M gene segments are related to Eurasian H1N1 SIVs (Garten, Davis et al. 2009). This unique gene constellation has never been described among influenza isolates from anywhere in the world and the precise evolutionary history of the 2009 pH1N1 is unknown (Dawood, Jain et al. 2009; Gibbs, Armstrong et al. 2009; Smith, Vijaykrishna et al. 2009; Al Hajjar and McIntosh 2010). Several outbreaks of pH1N1 virus infections in animals have been reported worldwide. These outbreaks were predominantly documented in pigs, but incidental infection in turkeys, cats, ferrets, dogs and wild animals has been described (Howden, Brockhoff et al. 2009; Nofs, Abd-Eldaim et al. 2009; Britton, Sojonky et al. 2010; Dundon, De Benedictis et al. 2010; Mathieu, Moreno et al. 2010; Pereda, Cappuccio et al. 2010; Song, Lee et al. 2010; Sponseller, Strait et al. 2010; Swenson, Koster et al. 2010; Pereda, Rimondi et al. 2011). In pigs, field outbreaks of pH1N1 have been reported in more

than 20 countries and epidemiological investigations have linked humans as the probable source (Hofshagen, Gjerset et al. 2009; Howden, Brockhoff et al. 2009). Experimentally, pigs are susceptible to human pH1N1 viruses and the virus is highly transmissible in swine (Brookes, Irvine et al. 2009; Itoh, Shinya et al. 2009; Lange, Kalthoff et al. 2009; Weingartl, Albrecht et al. 2009; Vincent, Lager et al. 2010). Pigs inoculated with the pH1N1 virus show clinical disease signs and pathology similar to infection with other SIVs (Brookes, Irvine et al. 2009; Itoh, Shinya et al. 2009; Lange, Kalthoff et al. 2009; Weingartl, Albrecht et al. 2009; Vincent, Lager et al. 2010).

Pigs have been historically considered a "mixing vessel" for the generation of novel influenza viruses (Scholtissek, Burger et al. 1983; Scholtissek, Burger et al. 1985; Scholtissek 1995). Pigs are susceptible to human influenza viruses; however, and perhaps unlike humans, they appear susceptible to a wide range of avian influenza viruses. Experimental infection studies showed that pigs were susceptible to 13 different influenza virus subtypes (H1-H3 and non-human-type HA types H4-H13). From these studies, a reassortant virus was generated in pigs upon co-infection with a parental SIV strain and an avian strain; the latter naturally unable to replicate by itself in pigs (Kida, Ito et al. 1994). The mixing vessel attribute is consistent with the presence in the respiratory tract of pigs of prototypical human-like and avian-like influenza receptors, N-acetylneuraminic acid-α2,6-galactose (α2,6Gal) and N-acetylneuraminic acid-α2,3-galactose (α2,3Gal), respectively (Ito, Couceiro et al. 1998). Although subsequent studies have shown both types of receptors are also found in the respiratory tract of quail, pheasant, turkey, guinea fowl (Wan and Perez 2006; Kimble, Nieto et al. 2010) and humans (Shinya, Ebina et al. 2006), pigs have been commonly associated with two-way transmission of influenza viruses to and from humans (Myers, Olsen et al. 2007; Robinson, Lee et al. 2007; Newman, Reisdorf et al. 2008; Vincent, Swenson et al. 2009). Pigs were undoubtedly involved in the genesis of the 2009 pH1N1 (Garten, Davis et al. 2009). Since then, several reassortants between pH1N1 and circulating influenza A viruses have been isolated from pigs in several countries (Vijaykrishna, Poon et al. 2010; Howard, Essen et al. 2011; Moreno, Di Trani et al. 2011; Pereda, Rimondi et al. 2011; Starick, Lange et al. 2011), raising great concerns about the potential acquisition of virulence markers by the pH1N1 virus upon reassortment with other strains in the swine host.

Among the virulence factors that could be acquired by pH1N1 is the non-structural protein PB1-F2. In contrast to the three previous pandemic influenza viruses, pH1N1 does not encode a functional PB1-F2 due to the presence of three stop codons that causes a premature truncation (Trifonov and Rabadan 2009). PB1-F2 is an 87-90 amino-acid long protein

encoded by an alternate (+1) open reading frame (ORF) within the PB1 gene. Translation of PB1-F2 mRNA is likely mediated by ribosomal scanning and the protein is expressed early and transiently in infected cells (Chen, Calvo et al. 2001). PB1-F2 is expressed mostly by IAVs of avian origin (Zell, Krumbholz et al. 2007) and its presence is not required for viral replication in embryonated eggs, tissue culture, or *in vivo* (Conenello and Palese 2007). However, PB1-F2 expression results in enhanced apoptotic-cell death in immune cells, viral pathogenicity in mice, and immunopathology (Chen, Calvo et al. 2001; Zamarin, Ortigoza et al. 2006; Conenello, Zamarin et al. 2007; McAuley, Hornung et al. 2007; McAuley, Chipuk et al. 2010). Additionally, the presence of PB1-F2 in both the 1918 and the mouse-adapted PR8 influenza A virus enhances secondary bacterial pneumonia in the mouse model (McAuley, Hornung et al. 2007). Surprisingly, two recent studies investigating the role of PB1-F2 in the pathogenicity of clinically relevant human viruses and the pH1N1 concluded that the expression of PB1-F2 has minimal effects on the virulence of these viruses in murine and ferret models (Hai, Schmolke et al. ; McAuley, Zhang et al. 2010). From these studies, it appears that the role of PB1-F2 in modulating influenza virus pathogenicity is cell-type and virus strain-specific and/or species-dependent. The contribution of PB1-F2 to the virulence of influenza viruses in swine has yet to be determined.

Here, we restored the PB1-F2 ORF in pH1N1 and studied its effects on viral pathogenicity and host responses in pigs. Restoring PB1-F2 in the pH1N1 virus resulted in increased virus replication in swine respiratory explants. More importantly, in pigs, a pH1N1 virus encoding a complete PB1-F2 ORF increased virus replication in the lung, enhanced lung histopathology, and induced higher pulmonary levels of IFN-α and IL-1β than the WT virus. These findings suggest that PB1-F2 modulates pH1N1 pathogenicity and cytokine responses in swine.

## *4.3. Material and Methods*

### 4.3.1. Ethics Statement

Pig explants were prepared according to protocol R-08-16 “Transmissibility of Influenza A Viruses in Swine" approved by the Institutional Animal Care and Use Committee, University of Maryland, College Park. Swine pathogenicity studies were conducted in the high containment facilities at the National Animal Disease Center in Ames, IA under protocol 3950 "Influenza A virus pathogenesis and host response in swine" approved by the USDA- ARS Animal Care and Use Committee. Animal studies adhered strictly to the US Animal Welfare Act (AWA) laws and regulations.

### 4.3.2. Cell lines and virus strains

Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), L-glutamine.and antibiotics. Human embryonic kidney cells (293-T) were cultured in OptiMEM I (GIBCO, Grand Island, NY) containing 5% FBS and antibiotics. A/California/04/09 (H1N1) (Ca/04) was kindly provided by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Ca/04 wild type (WT) and the recombinants thereof were propagated in MDCK cells for 3 days at 35°C to produce viral stocks. The recombinant Ca/04 viruses used in this study were generated from cloned cDNAs and are described below.

### 4.3.3. Mutagenesis and rescue of recombinant influenza viruses

The eight gene segments of Ca/04 were amplified by RT-PCR and cloned in the bidirectional reverse genetics (RG) plasmid derived from pHW2000 (Hoffmann, Neumann et al. 2000). The QuikChange II site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) was used according to manufacturer’s protocols to introduce changes in the PB1-F2 open reading frame (ORF). The PB1-F2 ORF in the PB1 segment was restored by mutating the stop codons at positions 12, to encode for serine, and at positions 58 and 88, to encode for tryptophan (Fig.1). The mutations did not change the PB1 ORF. The recombinant viruses were generated by transfecting co-cultured 293-T and MDCK cells as previously described (Hoffmann, Neumann et al. 2000). In order to improve virus rescue and growth in tissue

culture, the HA gene from the mouse-adapted Ca/04 (Ye, Sorrell et al. 2010) was used in these experiments. All RG plasmids and recovered recombinant viruses were fully sequenced to confirm their identity.

### 4.3.4. Alignment and phylogenetic analysis

The restored Ca/04 PB1-F2 was aligned with the 20$^{th}$ century pandemic strains (1918 H1N1, 1957 H2N2, and 1968 H3N2), some of the ancestors of the 2009 Influenza A H1N1 strain, and previously characterized PR8 and A/VietNam/1203/04 (H5N1) PB1-F2 protein using ClustalW method in MegAlign (Lasergene v.8.1.5., DNAStar, Madison, WI). The phylogenetic tree was generated by Neighboor-Joining method using the MegAlign program. Robustness of phylogenetic trees was tested by performing 1000 bootstrap replicates with 111 random seeds.

### 4.3.5. Minigenome assay to study polymerase activity

The minigenome assay was performed as described previously (Pena, Vincent et al.). Briefly, 1 μg of the plasmid encoding the influenza virus-like NS vRNA carrying the *Gaussia* Luciferase (GLuc) reporter gene was transfected in 293-T cells along with 1 μg of each of the RG plasmids encoding Ca/04 PB2, PB1, PA and NP plasmids using the TransIT-LT1 (Mirus, Madison, WI) reagent following the manufacturer's recommendations. The Ca/04 PB1 plasmid encoded either the WT PB1-F2 (truncated after codon position 11) or the artificially restored PB1-F2 ORF. The pCMV/SEAP (SEAP) plasmid, which expresses the secreted alkaline phosphatase, was co-transfected into cells to normalize the transfection efficiency. Alternatively, MDCK cells were transfected with GLuc reporter under the control of the canine Polymerase I promoter (PolI) for 12 hours followed by infection with Ca/04 (WT or KI) virus at an MOI of 1 or 0.01. At the indicated time points, supernatant from transfected cells was harvested and assayed for both luciferase and secreted alkaline phosphatase activities using the BioLux™ *Gaussia* Luciferase Assay Kit (NEB, Ipswich, MA) and the Phospha-Light™ Secreted Alkaline Phosphatase Reporter Gene Assay System (A&D, Foster City, CA) according to the manufacturers' instructions. Relative polymerase activity was calculated as the ratio of luciferase and SEAP luminescence from four independent experiments.

4.3.6. Isolation, culture, and infection of porcine respiratory explants

Porcine nasal turbinates (NT), tracheal, and lung explants were prepared as described previously (Van Poucke, Nicholls et al. 2010), with some modifications. All respiratory explants were cultivated at an air-liquid interface in an incubator at 37°C and 5% $CO_2$. NT and trachea explants were cultivated in 50% DMEM (Gibco)/50% RPMI (Gibco) media supplemented with 100 U/ml penicillin (Gibco), 100 µg/ml of streptomycin (Gibco), 0.1 mg/ml gentamycin (Gibco), 25 µg/ml Amphotericin B (Gibco), 0.3 mg/ml glutamine (BDH Biochemical) and nonessential amino acids (Sigma). Media for lung explants consisted of M199 (Sigma) containing the same antibiotics as above, nonessential amino acids (Sigma). Vitamin supplement (ATCC, Rockville, MD), 0.5 µg/ml hydrocortisone and ITS supplement (Insulin, Transferrin, Selenium) added at 10 ml/L medium. A five-week-old swine donor obtained from a high-health status farm whose animals are negative for IAV was used for this study. The animal was humanely euthanized with Beuthanasia®-D (Intervet/Schering-Plough, Summit, NJ) at a dosage of 1 ml/4.5 Kg of body weight and the respiratory tissues were collected. NT and trachea explants were dissected and washed 10 times with PBS containing antibiotics to remove bacterial contamination. Tissues were then cut into squares of 25 $mm^2$ each and placed with the epithelial surface upwards onto the filter membrane of the polyester tissue culture-treated inserts (Transwells®, Corning, Lowell, MA) at an air-liquid interface in 12-well plates. The lower compartment was filled with 1 ml of explant media. The right apical lung lobe was expanded with a 1% type VII-A low gelling temperature agarose solution (Sigma, St. Louis, MO) that had been dissolved in lung explants media and cooled down to 37°C. The expanded lung was placed at 4°C for 10 min in a sterile container until the agarose solidified. The embedded lung tissue was then cut into 1 mm thick slices using a microtome blade and hand microtome. Two sections of the lungs were obtained: the proximal lung (close to the start of bronchial tree) and distal lung (close to the lung alveoli). The procedure for cultivation of lung explants was similar to the procedure used for the NT and tracheal explants. After 24 h, lung explants were washed with warm PBS to remove most of the agarose before infection. At 24 h of culture, explants were washed with PBS and $10^6$ tissue culture infectious dose 50% ($TCID_{50}$) of the recombinant viruses diluted in 500 µl of explants media were deposited in the upper compartment of Transwells® for 1 h. After the 1 h adsorption at 37°C, explants were washed three times with PBS and the culture replenished with 500 µl of explants media. One hundred µl of upper compartment explant

bathing media was collected at 24, 48, and 72 h post inoculation to assess virus yields. Virus titers in respiratory explants were determined by standard $TCID_{50}$ in MDCK cells.

### 4.3.7. Virus titration

Viral stocks and virus present in biological samples were titrated on MDCK cells and the $TCID_{50}$/ml was determined by the Reed and Muench method (Reed and Muench 1938). Briefly, samples were serially diluted 10-fold in serum-free media containing antibiotics and 1 µg/ml TPCK-trypsin (SIGMA) and 100 or 200 µl of the inoculum were overlaid onto confluent monolayers of MDCK cells seeded in 96-well plates. The cells with the sample were incubated for 3 days and the endpoint viral titer was determined by an HA assay using 0.5% turkey red blood cells.

### 4.3.8. Swine pathogenicity experiment

Three-week-old cross-bred pigs were obtained from a high-health herd free of SIV and porcine reproductive and respiratory syndrome virus. All pigs were treated with ceftiofur crystalline free acid (Pfizer Animal Health, New York, NY) to reduce bacterial contaminants prior to the start of the experiment. Twenty pigs were randomly divided in two groups (n=10) and housed in separate isolation rooms. Pigs were infected intratracheally with $1\times10^5$ $TCID_{50}$ of either Ca/04 WT or an isogenic virus expressing PB1-F2 (Ca/04 KI) diluted in 2 ml of MEM using previously described protocols (Vincent, Lager et al. 2010). Following inoculation, pigs were monitored daily for clinical signs of disease and nasal swabs were collected to measure viral shedding. Five pigs from each group were euthanized on day 1 and the remaining five animals were euthanized at 3 dpi for evaluation of viral lung load, pathology, and host response to infection. Five additional pigs were inoculated with 2 ml MEM as above and served as mock-controls. Control pigs were euthanized at 3 days post infection (dpi).

### 4.3.9. Pathologic examination of swine lungs

At necropsy, lungs were removed *in toto* and evaluated to determine the percentage of the lung affected by purple-red, consolidated lesions that are typical of influenza virus infection in pigs. The percentage of the surface affected with pneumonia was visually estimated for each lobe, and a total percentage for the entire lung was calculated based on

weighted proportions of each lobe to the total lung volume as previously described (Halbur, Paul et al. 1995). Each lung was then lavaged with 50 ml MEM to obtain bronchoalveolar lavage fluid (BALF). A veterinary pathologist scored all lungs and was blinded to the treatment groups.

4.3.10. Histopathology and immunohistochemistry

Tissue samples from the trachea and right middle lung lobe were taken and fixed in 10% buffered formalin for histopathological examination. Tissues were routinely processed and stained with hematoxylin and eosin. A board-certified veterinary pathologist blinded to treatment groups evaluated microscopic lesions. Scoring of lesions was based on scales adapted from Gauger et al (Gauger, Vincent et al. 2011). Individual scores were assigned to four parameters: bronchial and bronchiolar epithelial changes, bronchitis/bronchiolitis, peribronchiolar lymphocytic cuffing and interstitial pneumonia. Scores were based on percentage of airways with lesions that included epithelial changes and inflammation on a 5-point scale: 0: No lesions, 1: 1-25%, 2: 26 to 50%, 3: 51-75%, 4: greater than 75% of airways affected with airway epithelial damage and inflammation. Peribronchiolar cuffing by lymphocytes was graded on a 4-point scale: 0: None, 1: Mild, loosely formed cuff, 2: Moderate, well formed cuffs, and 3: Prominent, thick well formed cuffs. Interstitial pneumonia was graded on 5-point scale based on severity and distribution: 0: No lesions, 1: Mild, focal to multifocal, 2: Moderate, locally extensive to multifocal, 3: Moderate, multifocal to coalescing, and 4: Severe, coalescing to diffuse.

Trachea sections were scored similar to the bronchi and bronchioles and were based on epithelial changes and degree of inflammation. Tracheal epithelial changes were graded on a 5-point scale: 0: No lesions, 1: Early epithelial changes characterized by focal to multifocal loss of cilia and epithelial degenerative changes, 2: Mild epithelial flattening with loss of cilia and goblet cells, 3: Moderate epithelial flattening with decreased thickness of respiratory epithelium, loss of cilia and goblet cells, 4: Flattened epithelium with areas of mucosa covered by a single layer of cuboidal epithelium and epithelial loss (necrosis). Degree of tracheitis was graded on a simple 4-point scale: 0: None, 1: Mild, 2: Moderate, and 3: Severe.

Influenza virus Type A-specific antigen was detected in lung tissues using a previously described immunohistochemical (IHC) method with minor modification (Vincent, Janke et al. 1997). Briefly, tissue sections were deparaffinized and hydrated in distilled water. Slides were quenched in 3% hydrogen peroxide for 10 min, rinsed three times in de-ionized

water and treated in 0.05% protease for 2 min. Slides were then rinsed three times in de-ionized water and once in Tris-buffered saline (TBS). Influenza A virus-specific monoclonal antibody (MAb) HB65 (ATCC, Manassas, VA), specific for the nucleoprotein (NP) of influenza A viruses, was applied at 1:100 dilution and slides were incubated at room temperature for 1 h. Bound MAbs were stained with peroxidase-labeled anti-mouse IgG followed by chromogen using the DAKO LSAB2-HRP Detection System (DAKO, Carpinteria, CA) according to the manufacturer's instructions. The slides were rinsed in deionized water and counterstained with Gill's hematoxylin. Antigen detection was given two scores: 1) airway epithelial labeling and 2) alveolar/interstitial labeling. In airway epithelium a 5-point scale was used: 0: None,1: Few cells with positive labeling, 2: Mild scattered labeling, 3: Moderate scattered labeling, 4: Abundant scattered labeling (greater than 50% epithelium positive in affected airways). In the interstitium/alveoli, a 4-point scale was used: 0: None, 1: Minimal focal signals, 2: Mild multifocal signals, 3: Abundant signals.

#### 4.3.11. Quantification of cytokine/chemokine protein levels in bronchoalveolar lavage fluid

Levels of nine porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-γ) in BALF were determined by multiplex ELISA following the manufacturer's recommendations (SearchLight, Aushon Biosystems). Levels of IFN-α protein were measured by ELISA using F17 monoclonal antibody, K9 MAb and recombinant porcine IFN-α (R&D Systems Inc., Minneapolis, MN) as previously described (Brockmeier, Lager et al. 2009).

#### 4.3.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student t-test, whereas multiple comparisons were carried out by analysis of variance (ANOVA). The differences were considered statistically significant at $p < 0.05$.

## *4.4. Results*

### 4.4.1. Restoring PB1-F2 in the Ca/04 pH1N1 background up-regulates early polymerase activity

It has been shown that PB1-F2 derived from the laboratory strain A/Puerto Rico/8/34 (H1N1) (PR8) interacts with the polymerase subunit PB1 and increases polymerase activity (Mazur, Anhlan et al. 2008). We wanted to determine if polymerase activity of a pH1N1 virus would be affected when the PB1-F2 ORF was restored. To this end, we first developed the entire reverse genetic system for the Ca/04 strain. The three stop codons present in the Ca/04 WT PB1-F2 were changed to encode for serine (codon 12) and tryptophan (codons 58 and 88) and thus allowing translation of the full-length PB1-F2 (Ca/04 KI), as previously described (Hai, Schmolke et al. ; Ozawa, Basnet et al. 2011) (Fig 4.1). These point mutations were silent in the PB1 ORF.

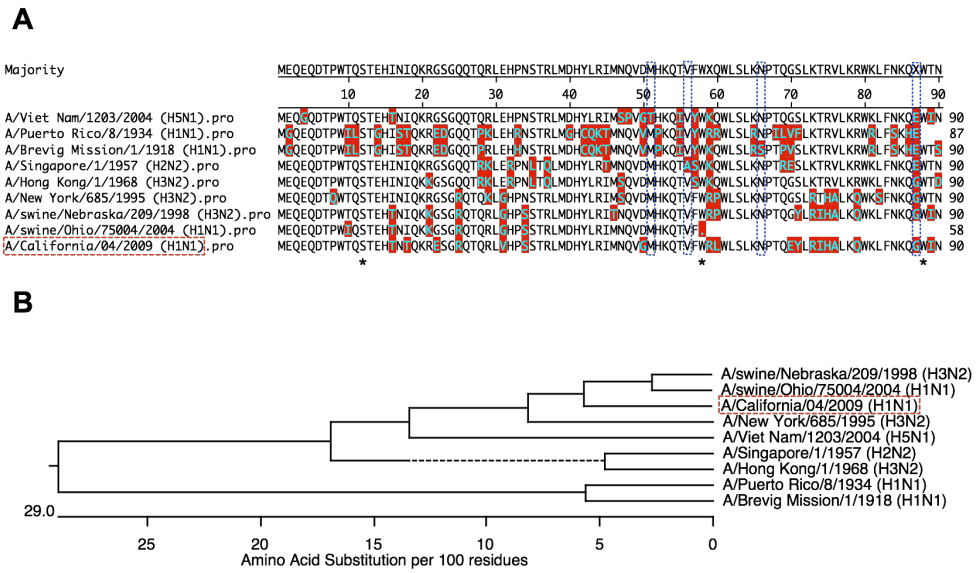



**Figure 4.1. Alignment and phylogenetic analysis of artificially restored PB1-F2 full-length ORF in Ca/04 pH1N1.** (A) Alignment of the PB1-F2 open reading frames. The three stop codons (asterisks) present in the Ca/04 PB1-F2 gene were replaced to serine (12), and tryptophan (58 and 88). Ca/04 PB1-F2 ORF was aligned to those of the 20$^{th}$ century pandemic strains (1918 H1N1, 1957 H2N2, and 1968 H3N2), the ancestors of the 2009 Influenza A H1N1 strain, and the previously characterized PR8 and A/VietNam/1203/04

(H5N1) strains. Boxed residue highlights amino acid residues that have been associated with increased virulence: 51(T), 56(V), 66(S), and 87(E) (Marjuki, Scholtissek et al. ; Conenello, Zamarin et al. 2007). (B) Phylogenetic relationships of Ca/04 PB1-F2 with other relevant PB1-F2 proteins. The phylogenetic tree was generated by Neighboor-Joining method using the MegAlign program.

To study viral polymerase activity in the absence or presence of PB1-F2, we used an influenza minigenome assay as previously described (Pena, Vincent et al.). Co-transfection of 293-T cells was performed with plasmids encoding the Ca/04 PB2, PB1 (with or without full-length PB1-F2), PA, and NP genes and the influenza replicon carrying GLuc. Transfection efficiency was normalized using a plasmid encoding the SEAP reporter gene under a RNA pol II promoter. Polymerase activity is monitored by the ratio of Gluc/SEAP. These experiments revealed that the presence of PB1-F2 led to significantly higher polymerase activities at 12 h post transfection than in its absence (Fig. 4.2A). At latter time points, no such difference was observed, suggesting that pH1N1 PB1-F2 modulates early polymerase activity in mammalian cells. Although the mingenome assay has become the method of choice for studying IAV polymerase activity, this method does not reflect all of the regulatory events that occur during the course of infection since it involves only the minimal components required for viral transcription and replication (Robb, Smith et al. 2009). To overcome this drawback and study the effect of PB1-F2 on polymerase activity in the context of the viral life cycle, we transfected MDCK cells with GLuc reporter under the control of the canine Pol-I followed by infection with Ca/04 (WT or KI) virus at an MOI of 1 or 0.01. Consistent with the results from the minigenome, the presence of PB1-F2 enhanced early viral polymerase activity. The enhancement in polymerase activity was statistically significant ($p<0.01$) between 6 and 12 hpi when MOI 0.01 was employed (Fig. 4.2B) and between 1 and 10 hpi when MOI 1 was used (Fig. 4.2C). Together, these results imply that PB1-F2 promotes temporal regulation of pH1N1 polymerase activity.

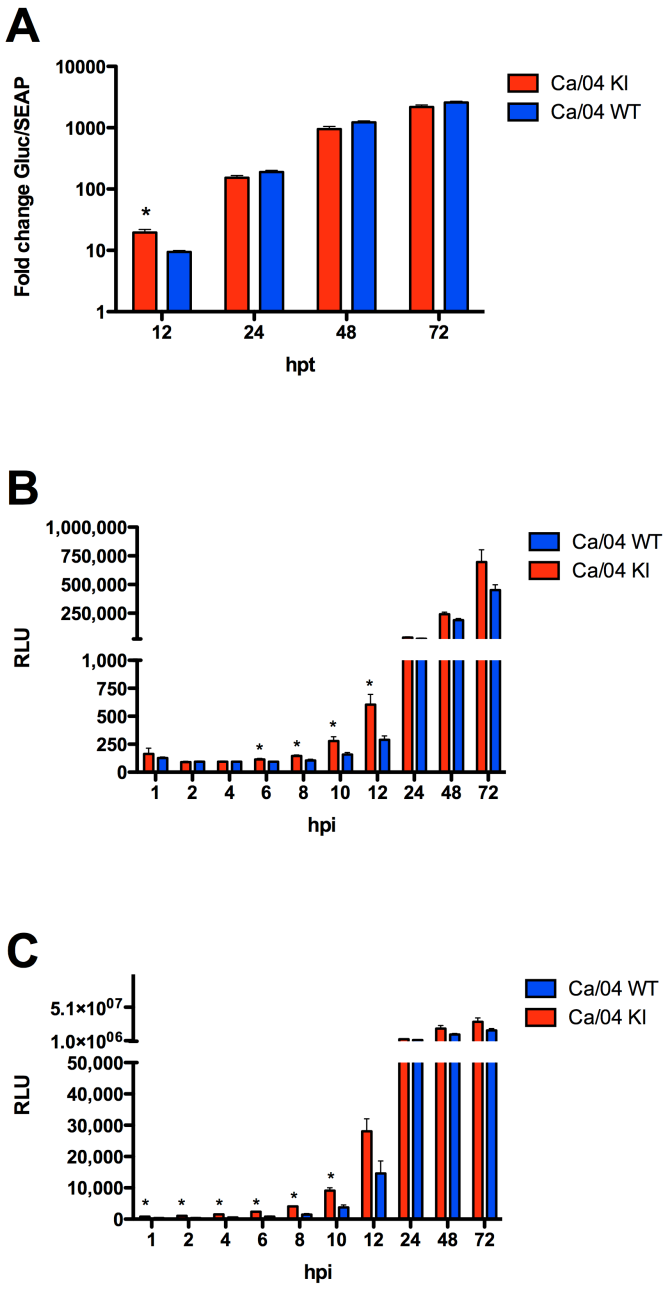



**Figure 4.2. PB1-F2 up-regulates early polymerase activity.** (A) Minigenome assay. 293-T cells were transfected with plasmids encoding the minimal components required for viral transcription and replication (PB2, PB1, and PA polymerase subunits, NP and a vRNA influenza driven-luciferase reporter replicon expressing GLuc) and pCMV/SEAP, plasmid to normalize transfection efficiency as previously described (Pena, Vincent et al.). At the indicated time points post transfection, the supernatant was harvested and assayed for both luciferase and phosphatase activities. (B) and (C) Kinetics of polymerase activity after virus infection. MDCK cells were transfected with 1 µg of GLuc reporter under the control of the canine PolI followed by infection with Ca/04 (WT or KI) virus at an MOI of 0.01 (B) or 1

(C). Data are expressed as polymerase activities (mean ± SE) determined from four independent experiments.

### 4.4.2. PB1-F2 enhances Ca/04 replication in porcine respiratory explants

It has been demonstrated that expression of full-length wild type PB1-F2 by the pH1N1 has no effects on viral replication in human A549 cells or MDCK cells (Hai, Schmolke et al. ; Ozawa, Basnet et al. 2011). However, cell lines do not exhibit the natural physiological conditions and cellular complexity present in the respiratory tract. To address this limitation and study the impact of PB1-F2 expression in a relevant biological system, we developed an *ex vivo* organ culture model of the pig respiratory tract maintained at an air–liquid interface. These tissue explants retained their cytoarchitecture (data not shown) and supported productive replication of the recombinant Ca/04 influenza viruses (Fig. 4.3). Restoring PB1-F2 in Ca/04 improved virus replication in explants of nasal turbinates (Fig. 4.3A), trachea (Fig. 4.3B), and proximal lung (Fig. 4.3C). No differences were observed in explants of the distal lung (Fig. 4.3D). This enhancing effect of PB1-F2 in virus production was mainly seen at early time points. These data indicate that PB1-F2 modulates early viral production in swine respiratory tissues infected *ex vivo*.

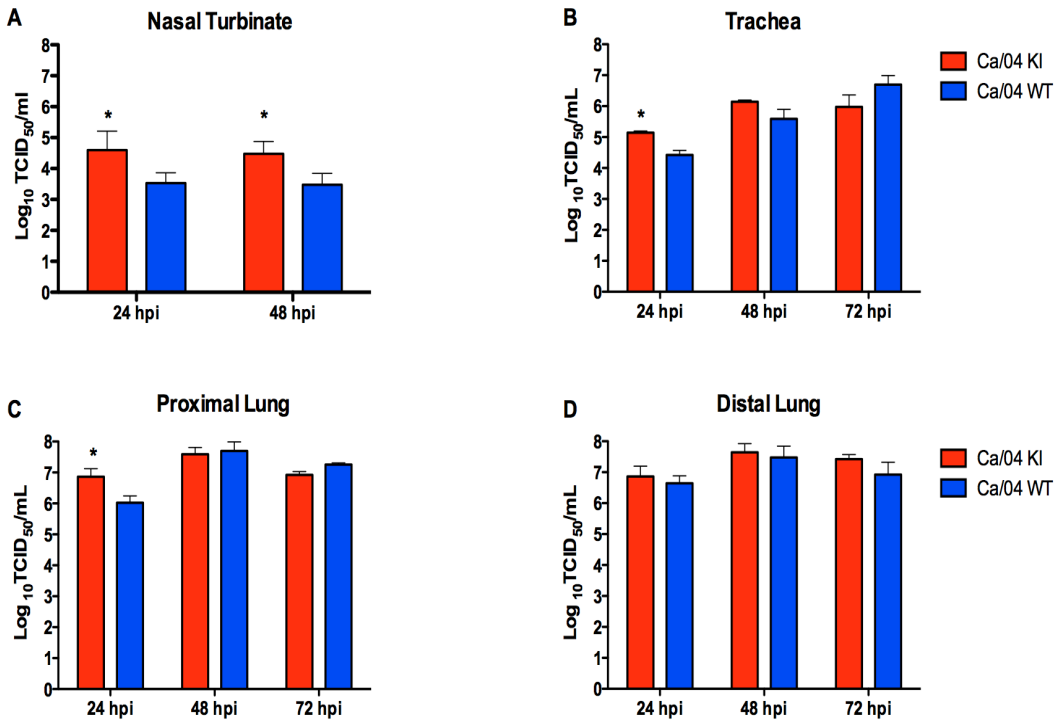



**Figure 4.3. Replication of PB1-F2 recombinant viruses in porcine respiratory explants.** Explants from nasal turbinates (A), trachea (B), and proximal (C), and distal lung (D) were infected with $10^6$ $TCID_{50}$ of either Ca/04 WT or KI. The bathing media was collected at the indicated time points and titrated by the $TCID_{50}$ method in MDCK cells. Values shown are the mean ± SEM of virus titer ($log_{10}$ $TCID_{50}$/ml) from four independent experiments. * $p < 0.05$.

### 4.4.3. Restored PB1-F2 ORF increases virus replication in swine lungs

To evaluate whether the enhanced viral yields in porcine respiratory explants displayed by Ca/04 KI would correlate with increased viral replication *in vivo*, we inoculated groups of pigs (n=10/group) with the Ca/04 WT or Ca/04 KI viruses. From each group, 5 pigs were euthanized at days 1 and 3 pi and viral titers in BALF were determined. Consistent with the explants data, restoring the PB1-F2 ORF resulted in statistically significant increases in viral loads in the lungs at either time point (Fig. 4.4A). Increased Ca/04 KI replication in the lungs was further corroborated by the more pronounced pulmonary expression of influenza NP antigen by IHC analysis (Fig. 4.4B-C). As expected, mock-inoculated pigs had neither detectable virus nor IHC influenza-positive cells in BALF or lung tissues (data not shown).

Collectively, these results suggest that restoring the PB1-F2 ORF in the Ca/04 virus leads to increased viral replication *in vivo*.

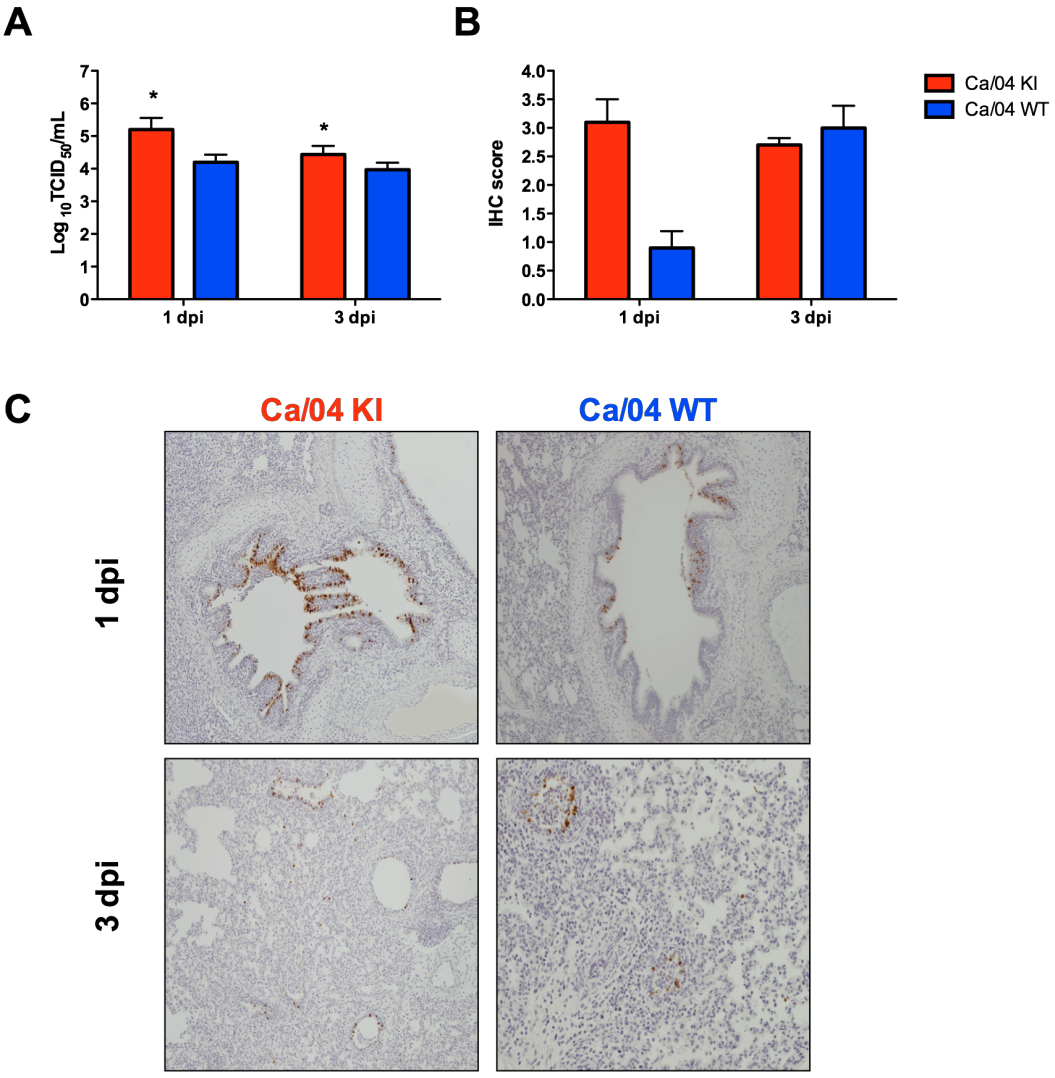



**Figure 4.4. Replication and immunohistochemical analysis of PB1-F2 recombinant viruses in swine lungs.** Groups of pigs (n=10) were infected with $10^5$ $TCID_{50}$ of PB1-F2 recombinant viruses and five animals from each group were euthanized either at 1 or 3 dpi. The lungs were collected and processed for virus titration and immunohistochemical analysis. (A) Pulmonary replication of PB1-F2 isogenic viruses in pigs. Values are mean ± SEM of virus titer ($log_{10}$ $TCID_{50}$/ml) in bronchoalveolar lavage fluid (BALF) collected at the indicated time points. (B) Immunohistochemical staining against

influenza A virus nucleoprotein (NP) in the lungs of infected pigs. Values given are the mean ± SEM IHC scores based on the percent of influenza-positive cells in the airway and lung interstitium. (C) Representative IHC slides depicting viral antigen primarily in airway epithelium at 1 and 3 dpi. Scattered labeling in the interstitium at 3 dpi is present.

### 4.4.4. PB1-F2 aggravates microscopic pneumonia despite no differences in nasal virus shedding or macroscopic lesions in pigs

To determine if PB1-F2 affects the kinetics of viral shedding in pigs, nasal swabs collected from each pig from 1 to 3 dpi were titrated in MDCK cells. Virus shedding in nasal secretions was only detected at 3 dpi and there was no difference between the Ca/04 WT and Ca/04 KI viruses (Fig. 4.5A). Infected pigs did not show any overt clinical signs of disease regardless of the virus used. At necropsy, pigs in both the Ca/04 WT and Ca/04 KI had cranioventral lung consolidation; with 5% to 10% of lung involvement at 3 dpi (Fig. 4.5B-C). There were no differences between the two virus groups, indicating that PB1-F2 has no effect on the macroscopic lung pathology caused by Ca/04 in swine.

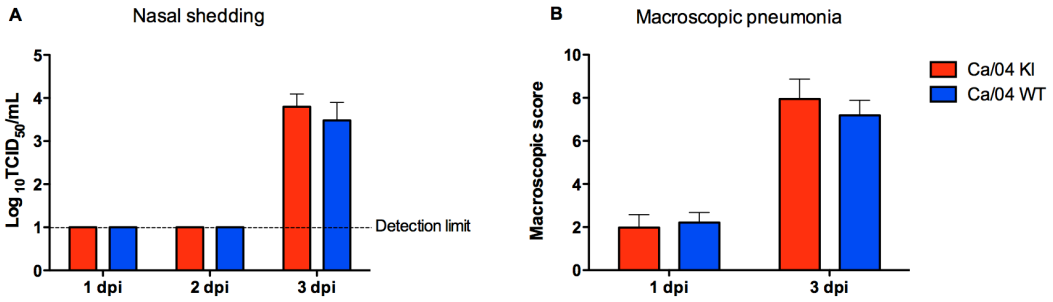



**Figure 4.5. Viral shedding and macroscopic pneumonia are unaltered by PB1-F2 expression in swine.** Groups of pigs (n=10) were infected with $10^5$ $TCID_{50}$ of PB1-F2 recombinant viruses and nasal swabs were collected from 1-3 dpi for measuring virus shedding. At 1 and 3 dpi, five animals from each group were euthanized and the lungs were scored for gross pneumonia. (A) Viral shedding in nasal secretions of pigs. Viral titers in nasal swabs were determined by $TCID_{50}$ on MDCK cells. (B) Percentage of macroscopic lung lesions. Values are shown as the mean ± SEM.

However, differences in lung inflammation were detected at the microscopic level. Quantitative histopathological analyses revealed that pulmonary inflammation was enhanced

in pigs in the Ca/04 KI group as compared to the Ca/04 WT group (Fig. 4.6A and 4.6C). Unlike the mock-inoculated animals that had normal histological features, the lesions in virus-infected pigs were typical of influenza virus pneumonia in pigs and were characterized by multifocal to widespread necrotizing bronchitis and bronchiolitis, light peribronchiolar lymphocytic cuffing, and multifocal interstitial pneumonia. Both viruses induced similar levels of tracheitis and cilia loss in the tracheal epithelium (Fig. 4.6B).

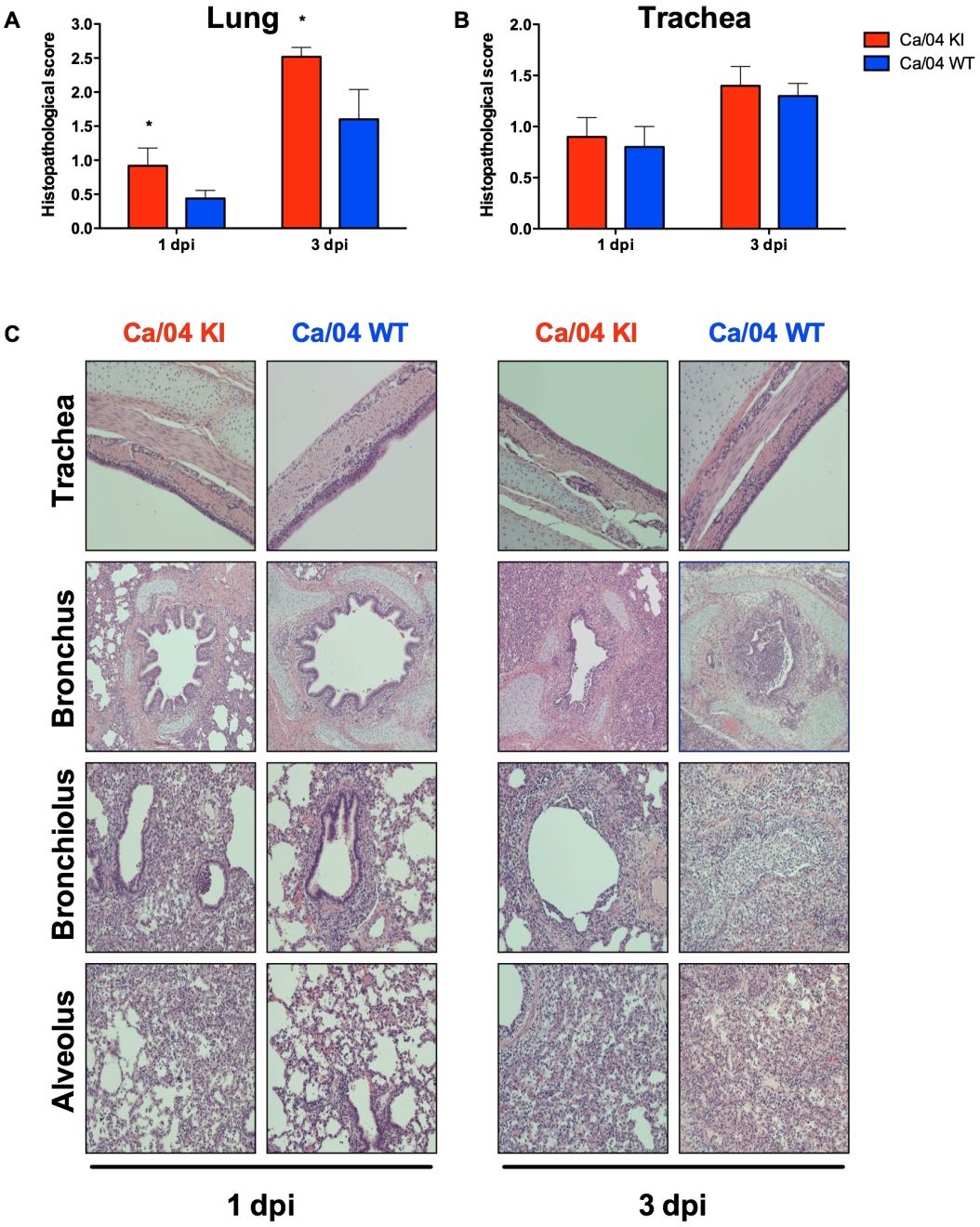


**Figure 4.6. PB1-F2 exacerbates microscopic pneumonia in swine.** Groups of pigs (n=10) were infected with $10^5$ $TCID_{50}$ of PB1-F2 recombinant viruses. At 1 and 3 dpi, five animals from each group were euthanized and the histopathological changes in the lower respiratory tract was evaluated. (A) Histopathologic scores in the lungs. (B) Histopathologic scores of trachea. (C) Photomicrographs representing microscopic pneumonia in Ca/04 KI and Ca/04 WT virus.

4.4.5. PB1-F2 exacerbates the pulmonary levels of IFN-α and IL-1β in swine

Cytokine production after influenza infection has been implicated in the pathogenesis of influenza in mammals, including pigs (Van Reeth 2000). To further characterize the effect of PB1-F2 in the pathological manifestations of pH1N in swine, we measured the protein levels of ten porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-α, and IFN-γ) in BALF collected at 1 and 3 dpi necropsies. The production of the cytokines/chemokines IL-6, IL-8, and IFN-γ was similar between the two groups infected with the PB1-F2 recombinants viruses (Fig. 4.7). However, pulmonary levels of IFN-α and IL-1β were significantly higher at 1 dpi in pigs infected with Ca/04 KI than with Ca/04 WT (Fig. 8). IL-2, IL-4, IL-10, IL-12p70, and TNF-α did not reach detectable levels in this experimental infection (data not shown). Collectively, these results indicate that PB1-F2 induces higher expression levels of IFN-α and IL-1β early during pH1N1 infection in pigs and this heightened production of pro-inflammatory mediators may have contributed to the more severe microscopic pneumonia developed in Ca/04 KI-infected animals (Fig. 4.6).

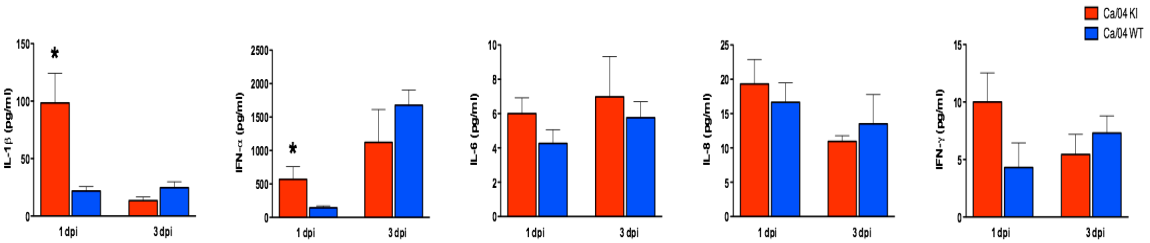



**Figure 4.7. PB1-F2 exacerbates the pulmonary levels of IFN-α and IL-1β in swine.** Groups of pigs (n=10) were infected intratracheally with $10^5$ $TCID_{50}$ of PB1-F2 recombinant viruses. On days 1 and 3 post-infection, animals from each group were euthanized and the cytokines/chemokines levels in BALF were determined by ELISA. Data are shown as mean ± SEM of four animals in each challenge group. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. * $p < 0.05$.

## *4.5. Discussion*

Outbreaks of influenza A viruses (IAV) are among the most significant causes of respiratory disease in both humans and animals. Occasionally, pandemic influenza viruses emerge when a virus acquires a new HA molecule to which the majority of the population is immunologically naïve, resulting in devastating levels of morbidity and mortality (Tscherne and Garcia-Sastre 2011). Virulence of influenza viruses is considered a polygenic trait. Molecular studies in different animal models have determined that the gene constellation of a particular influenza virus strain within a specific host plays a pivotal role in disease outcome (Hatta, Gao et al. 2001; Chen, Bright et al. 2007; Basler and Aguilar 2008). Several proteins in the viral genome have clearly identified roles in virulence: the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) that are responsible for viral binding, entry and release (Pappas, Aguilar et al. 2008); the polymerase protein complex formed by PB2, PB1, and PA proteins that drives genome transcription and translation (Watanabe, Watanabe et al. 2009; Graef, Vreede et al. 2010); and the nonstructural protein NS1 that disarms the interferon-based defense system of the host and also modulates other important aspects of the virus replication cycle (Hale, Randall et al. 2008).

A recently added member to the family of IAV proteins with a role in virulence is PB1-F2. Unlike other influenza viruses that have become adapted to humans, the 2009 pH1N1 virus lacks most of the known hallmarks of influenza virulence, including PB1-F2 (Trifonov and Rabadan 2009). However, with the continued circulation of pH1N1 in humans and animals, especially in swine, there are opportunities for acquisition of PB1-F2 through mutations or genetic reassortment with co-circulating animal influenza viruses. We examined the functional significance of PB1-F2 acquisition by pH1N1 on polymerase activity, viral replication, pathogenicity, and host response to infection in its natural host, the pig. The data demonstrated that the full-length PB1-F2 in a pH1N1 virus impacts polymerase activity, viral replication efficiency both *ex vivo* and *in vivo*, lung inflammation, and local cytokine responses to infection. Evolutionarily, the full-length PB1-F2 was lost in some humans and swine influenza viruses through incorporation of premature stop codons. This is in contrast to avian influenza viruses in which the entire PB1-F2 ORF is highly conserved (Zell, Krumbholz et al. 2007). Thus, PB1-F2 may have comparable functional roles in humans and pigs. Our pathogenicity studies in swine offers the possibility of translating the findings to

humans, given the outbred nature of this species, the comparable distribution of sialic acid receptors, and the similarities of influenza clinical signs, pathology, and cytokine response between the two species (Van Reeth, Van Gucht et al. 2002; Barnard 2009; Nelli, Kuchipudi et al. 2010).

In an attempt to characterize the molecular mechanisms by which PB1-F2 might affect viral replication, we studied the effects of Ca/04 PB1-F2 on polymerase activity using an IAV UTR-driven GLuc reporter gene. We found that PB1-F2 up-regulates early (12 hpt) viral polymerase activity, but not at later time points (24-72 hpt). These results contrast with those reported by Chen et al that indicated that Ca/04 PB1-F2 enhanced polymerase activity at 48 hpt (Chen, Chen et al. 2010). The discrepancy between this and the previous study with regard to polymerase function may be due to differences in the reporter gene used as polymerase activity readout and the length of the restored PB1-F2 ORF. Chen et al, used a chloramphenicol acetyltransferase (CAT) reporter system and mutated only 2 stop codons in PB1-F2 ORF generating an 87-aa product instead of a 90 peptide product (Hai, Schmolke et al.). It must be noted, however, that polymerase activity in our assay was evaluated in humans and canine cells; however, it appears to correlate well with the replication of these viruses in *ex vivo* swine respiratory explants and *in vivo* in swine lungs.

As the presence of PB1-F2 augmented replication of some viral strains in tissue culture (McAuley, Zhang et al. 2010; Smith, Adler et al. 2011), we studied the growth kinetics of PB1-F2 Ca/04 recombinant viruses in porcine respiratory explants. Respiratory explants have proved to be a valuable system to study influenza-host interaction in a scalable, well-defined and controlled experimental setting (Chan, Chan et al. 2010; Van Poucke, Nicholls et al. 2010). Our results are in harmony with the hypothesis that PB1-F2 modulates viral replication since higher viral yields were observed in explants derived from both the upper and lower respiratory tracts (Fig. 3). Interestingly, this effect of PB1-F2 in virus production (and polymerase activity, Fig 2) was mainly observed at early time points, consistent with the early and transient expression of PB1-F2 in the course of infection (Chen, Calvo et al. 2001; McAuley, Hornung et al. 2007; Le Goffic, Bouguyon et al. 2010). Furthermore, the increased *ex vivo* viral replication paralleled with lung viral load in pigs infected with Ca/04 KI (Fig. 5). In contrast, viral shedding in nasal secretions was not affected by PB1-F2. This may be explained by the route of inoculation used in our *in vivo* studies. Pigs were infected intratracheally, a route that deposits the virus in the lower respiratory tract and more consistently reproduces lung pathology (Maes 1984). Shedding

was detected only at 3 dpi, which could be a time point that makes it difficult to distinguish modest differences in shedding. However, we cannot rule out the possibility that PB1-F2 could affect viral shedding beyond 3 dpi and/or in intranasally infected animals. These studies are beyond the scope of this work and warrant further investigation.

Even though PB1-F2 had no effect on the gross lung pathology or clinical disease, Ca/04 KI-infected pigs developed more severe microscopic pneumonia as early as 1 dpi. This finding fit with previous reports that demonstrated that PB1-F2 increases the severity of pulmonary lesions in mouse models, either in the context of viral infection (McAuley, Hornung et al. 2007) or simply by administration of peptides (McAuley, Chipuk et al. 2010). Moreover, our results are in agreement with ferret studies by Hai et al that reported no changes in clinical outcome by a PB1-F2-expressing Ca/04 compared to WT controls, although the authors did not assess lung pathology (Hai, Schmolke et al.). Thus, it could be postulated that during primary viral infection the microscopic pulmonary changes induced by PB1-F2 are not sufficiently large enough to be manifested clinically or detected macroscopically. However, the effects of PB1-F2 enhancement of virulence might be seen under field conditions, where immunosuppressive conditions and secondary bacterial infections can aggravate the outcome of influenza infections.

The pro-inflammatory cytokine response is critical for recruiting effector cells to the site of an infection. However, elevated or prolonged cytokine production can also contribute to the pathological changes observed during infection. To better understand the mechanism of lesion severity in Ca/04 KI-infected pigs, we measured the pulmonary concentration of ten cytokines/chemokines during the acute stage of infection. Increased levels of IFN-α and IL-1β proteins were found in the lungs of pigs infected with Ca/04 KI compared to the Ca/04 WT (Fig. 7). This exacerbation of the host innate response was observed at 24 hpi, but not at 72 hpi, adding to the notion that changes mediated by PB1-F2 occur shortly after infection. To our knowledge, this is the first report that shows that PB1-F2 enhances type I IFN *in vivo*. Our results are consistent with a recent study that demonstrated that the WSN PB1-F2 exacerbates type I IFN expression in human respiratory epithelial cells (Le Goffic, Bouguyon et al. 2010). In both pigs and humans, there is a strong positive correlation between local IFN-α levels and viral titers in nasal secretions and clinical disease (Hayden, Fritz et al. 1998; Van Reeth, Van Gucht et al. 2002). In addition, increased levels of IL-1β have also been linked to neutrophil infiltration in the lungs and the severity of pneumonia in pigs (Van Reeth, Labarque et al. 1999; Loving, Brockmeier et al. 2010). The exacerbated inflammatory

response in pigs could be a direct effect of PB1-F2 early during infection. Thus, it is possible that enhanced expression of IFN-α and IL-1β are not the cause of respiratory disease, but markers of the extent of viral replication. The modulation of the porcine immune system by PB1-F2 and its interaction with host factors warrants further investigation and should shed light into the molecular mechanisms of PB1-F2 in strain and host-dependent manner. It remains to be determined whether the phenotypic differences observe with these two isogenic pH1N1 viruses are common features of other swine influenza viruses.

In summary, we have shown that PB1-F2 modulates several aspects of pH1N1 influenza virus interaction with swine, both *ex vivo* and *in vivo*. The present study fills a critical gap regarding the virulence determinants for influenza virus in swine. Our studies provide important insights into the impact that genetic changes may have on the virulence of pH1N1 for mammalian species, including humans.

# Chapter 5: Role of PB1-F2 in the Replication and Virulence of Triple Reassortant H3N2 Influenza Viruses in Swine

## *5.1. Abstract*

PB1-F2 acts as a virulence factor for the influenza A viruses (IAV) in the mouse model of infection. However, its contribution to the virulence of IAV in swine remains largely unexplored. In this study, we chose two genetically related H3N2 triple reassortant (TR) IAV to assess the impact of PB1-F2 in viral replication and virulence for pigs. Using reverse genetics, we disrupted the PB1-F2 open reading frame (ORF) of both the A/swine/WI/14094/99(H3N2)[Sw/99] and A/turkey/OH/313053/04(H3N2)[Ty/04]. In the Sw/99 strain we also introduced the N66S mutation, which has been shown to increase the virulence of H5N1 and 1918 influenza viruses. Knocking out PB1-F2 in the Ty/04 strain decreased its replication in swine respiratory explants, but did not affect its replication or viability in alveolar macrophage. In the context of the Sw/99 strain, PB1-F2 had no effect in virus yields in respiratory explants, though its presence increased viral yields and cell death in freshly isolated macrophages. *In vivo*, the expression PB1-F2 did not affect viral shedding or viral lung load of any of these strains. Upon necropsy, PB1-F2 had no effect on the lung pathology caused by Sw/99. Surprisingly, knocking out PB1-F2 from the Ty/04 strain significantly increased the lung pathology caused by this virus at 3 dpi. The polymorphism at position 66 had no effect in viral replication or virulence of the Sw/99 strain. The pulmonary levels of IL-6, IL-8, and IFN-γ were differentially regulated by the expression of PB1-F2. Together, our results indicate that PB1-F2 modulates viral replication, virulence, and innate immune response in pigs in a strain-dependent fashion.

## *5.2. Introduction*

Each year, infections with influenza A viruses (IAV) cause highly contagious respiratory diseases that are a major burden to human health, resulting in 250,000 to 500,000 deaths around the world (WHO 2009). Furthermore, IAV have caused three IAV pandemics (1918, 1957, and 1968) in the 20th century and, in 2009-2010, a worldwide epidemic caused by the swine-origin H1N1 virus resulted in major public health and economic losses. Importantly, IAV display a remarkable ability to infect a variety of animal species, including pigs, horses, cats, dogs, ferrets, sea mammals, and a wide variety of wild and domestic avian species (Palese and Shaw 2007). Among these species, swine are of particular importance, given the financial hardship resulting from natural IAV outbreaks in swine operations and the fact that pigs play important role in the generation of novel IAV with pandemic potential (Ma, Lager et al. 2009).

In pigs, AIV infection bears remarkable resemblance to human influenza with respect to the clinical presentations and pathologic features. In 1930, the first IAV to be isolated, A/swine/IA/15/30 (H1N1), was shown to be the etiologic agent of influenza in this species (Shope 1931). This virus, the so-called classical swine H1N1 viruses (cH1N1), is thought to be partially derived from the human 1918 pandemic H1N1 virus and evolved independently from other IAV in the swine population resulting in a distinct phylogenetic lineage (Smith, Bahl et al. 2009). cH1N1 was the only predominant IAV subtype circulating in North American swine populations for nearly 70 years (Vincent, Ma et al. 2008). In 1998, however, the epidemiology of swine influenza in North American pigs changed dramatically. H3N2 triple reassortant (TR) IAV, containing genes from human (HA, NA, and PB1), avian (PB2 and PA), and swine cH1N1 (NS, NP, and M) viral lineages emerged in the U.S. swine population and established permanent lineages. Over the next decade, these H3N2 TR IAV further reassorted with the co-circulating cH1N1 as well as with human H1N1 and H3N2 viruses, generating at least seven different reassortant lineages (Vincent, Ma et al. 2008). Interestingly, some of these viruses have expanded their host range and cause outbreaks in turkeys in North Carolina, Minnesota, and Ohio in 2003-2004 (Choi, Lee et al. 2004). A unique feature shared by all of these endemic reassortants is the maintenance of the triple reassortant internal gene (TRIG) constellation, which consists of the avian-like (PA and PB2), swine-like (M, NP, and NS), and human-like (PB1) gene segments. The TRIG cassette appears to accept multiple HA and NA types, which could provide a selective advantage to

swine viruses possessing this internal gene constellation (Vincent, Ma et al. 2008; Ma, Lager et al. 2009).

Currently, TR strains of the H3N2, H1N2, and H1N1 subtypes are endemic in the US swine population (Vincent, Ma et al. 2008). With respect to the H3N2 TR IAV, at least three introductions of human H3-subtype viruses, leading to phylogenetic clusters I, II, and III, have been documented (Richt, Lager et al. 2003; Webby, Rossow et al. 2004). The cluster III has evolved into cluster III variants, also known as cluster IV, which are now the predominant H3N2 viruses circulating in swine populations in North America (Gramer, Lee et al. 2007; Kumar, Deflube et al. 2011; Nfon, Berhane et al. 2011). In addition, the 2009 pandemic H1N1virus (pH1N1), which is also a TR, have been commonly isolated from US pigs and reassorted promiscuously with endemic TR strains given rise to at least 7 distinct and novel viral genotypes (Ducatez, Hause et al. 2011).

IAV belongs to the family *Orthomyxoviridae*, a family of enveloped viruses whose members contain a segmented, single-strand RNA genome with negative orientation. IAV genome contains eight RNA segments, which encode 10-12 viral proteins. Several virulence factors in the genome of IAV have been identified, including the surface glycoproteins HA and NA ; the multifunctional NS1 protein that inhibits the host immune responses; and the components of the ribonucleoprotein (RNP) complex (NP and the polymerase complex formed by PB2, PB1, and PA proteins), which drives genome transcription and translation. Virulence of IAV is considered a polygenic trait, in which disease outcome is determined by the gene constellation of a particular influenza virus strain within a specific host (Fukuyama and Kawaoka 2011).

Since the discovery of PB1-F2 in 2001 (Chen, Calvo et al. 2001), research has focused on using reverse genetics technology to elucidate the role of this small protein in the virulence of IAV. PB1-F2 is an 87-90 amino-acid long protein encoded by an alternate (+1) open reading frame (ORF) within the PB1 gene (RNA segment 2). Full-length PB1-F2 is expressed by most IAV of avian origin, but the protein is truncated in both cH1N1 and human H1N1 viruses isolated after 1950. Also, truncated versions of PB1-F2 have been reported in a some human H3N2 isolates and North American swine TR viruses, which have a human-like PB1 gene (Zell, Krumbholz et al. 2007). Using *in vitro* and mouse models, several groups have shown that PB1-F2 has pleiotropic effects. This protein, while dispensable for viral replication in embryonated eggs, tissue culture, or *in vivo* (Conenello and Palese 2007), it enhances the replication kinetics of the laboratory virus, A/Puerto Rico/8/34 (H1N1; hereafter referred to as PR8), and 1918 virus, but has no effects in the growth kinetics of the H5N1 or

human H3N2 viruses (McAuley, Zhang et al. 2010; Smith, Adler et al. 2011). By interacting with the PB1 subunit, PB1-F2 was shown to up-regulate the PR8 polymerase activity (Mazur, Anhlan et al. 2008) and, more recently, the highly pathogenic H5N1 (McAuley, Zhang et al. 2010) and pH1N1 (Chen, Chen et al. 2010). Importantly, PB1-F2 expression results in enhanced apoptotic-cell death in immune cells, viral pathogenicity in mice, and immunopathology (Chen, Calvo et al. 2001; Zamarin, Ortigoza et al. 2006; Conenello, Zamarin et al. 2007; McAuley, Hornung et al. 2007; McAuley, Chipuk et al. 2010). Intranasal administration of PB1-F2 peptides derived from the 20$^{th}$ century pandemic strains (1918 H1N1, 1957 H2N2, 1968 H3N2) and H5N1 led to acute lung injury, elevation of pro-inflammatory cytokines, and the influx of inflammatory cells to the lungs of mice (McAuley, Chipuk et al. 2010). However, this effect seems to be lost in recently circulating human H3N2 strains through accumulations of point mutations in the PB1-F2 protein (Alymova, Green et al. 2011). A rare polymorphism (N66S) in PB1-F2 has been associated with high lethality of the 1918 IAV and certain highly pathogenic H5N1strains (Conenello, Zamarin et al. 2007; Schmolke, Manicassamy et al. 2011). Overall, the effects of PB1-F2 in relation to influenza-host interaction are not completely understood and seem to be cell-type, virus strain, and even host-specific. Nevertheless, the contribution of PB1-F2 to the virulence of IAV to its natural hosts has been limited to avian species (Marjuki, Scholtissek et al. 2010; Schmolke, Manicassamy et al. 2011) and, to our knowledge, no studies have determined its role in the context of IAV infection of TR H3N2 in swine.

In this study, we compared the effects of PB1-F2 expression in the background of two TR H3N2 IAVs different pathotypes in pigs. We sought to delineate PB1-F2 contribution to viral polymerase activity measured *in vitro*, as well as its role in viral replication and cell death using *ex vivo* infected porcine tissues. Finally, we addressed whether the presence of PB1-F2 or the N66S polymorphism would translate into changes in pathogenicity, and host response to infection in an epidemiological relevant IAV host, the pig.

## *5.3. Material and Methods*

### 5.3.1. Ethics Statement

Swine pathogenicity studies were conducted in the high containment facilities at the National Animal Disease Center in Ames, IA under protocol 3950 "Influenza A virus pathogenesis and host response in swine" approved by the USDA- ARS Animal Care and Use Committee. Porcine explants were prepared according to protocol R-08-16 “Transmissibility of Influenza A Viruses in Swine" approved by the Institutional Animal Care and Use Committee, University of Maryland, College Park. Animal studies adhered strictly to the US Animal Welfare Act (AWA) laws and regulations.

### 5.3.2. Cell lines and virus strains

Human embryonic kidney cells (293-T) were cultured in OptiMEM I (GIBCO, Grand Island, NY) containing 10% FBS and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), L-glutamine.and antibiotics. A/swine/Wisconsin/14094/99 (H3N2) (Genbank taxonomy 136472) was a kind gift from Dr. Sagar Goyal, University of Minnesota. A/turkey/Ohio/313053/04 (Genbank taxonomy ID 533026) was kindly provided by Dr. Yehia Saif, Ohio State University. Both viruses were successfully rescued from cloned cDNAs as described below and amplified in either MDCK cells (Sw/99 ) or embryonated eggs (Ty/04) following the same substrates used for isolation of the WT viruses. Sw/99 and Ty/04 bearing mutations in PB1-F2 were generated by reverse genetics and are described below. All recombinant viruses were amplified in MDCK cells to produce viral stocks.

### 5.3.3. Reverse genetics and mutagenesis

The eight-plasmid-based reverse genetics (RG) system was used to rescue WT A/swine/Wisconsin/14094/99 (H3N2) (Sw/99), A/turkey/Ohio/313053/04 (H3N2) (Ty/04) and recombinants thereof. Each of the eight viral gene segments were amplified by RT-PCR and cloned in the bidirectional reverse genetics (RG) plasmid derived from pHW2000 (Hoffmann, Neumann et al. 2000). The QuikChange II site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) was used according to manufacturer’s protocols to disrupt the

PB1-F2 ORF of both Sw/99 and Ty/04, which display a low and high virulent phenotype for pigs, respectively (Pena *et al*., manuscript in preparation). To ensure complete abolition of PB1-F2 translation, we mutated the ATG start codon to ACG and introduced 2 stop codons at amino acid positions 12 (c153g) and 58 (g291a) as previously described by Zamarin *et al*. (Zamarin, Ortigoza et al. 2006). All the three above mutations were silent in the PB1 ORF. Since the Sw/99 virus is of low virulence to pigs, we introduced the virulence marker N66S to assess wheather this polymorphism is implicated in the high severity of TR H3N2-induced disease in pigs (Conenello, Zamarin et al. 2007). Viruses used in this study are summarized in table 1. All RG plasmids and recovered recombinant viruses were fully sequenced to confirm their identity. The recombinant viruses were generated by transfecting co-cultured 293-T and MDCK cells as previously described (Hoffmann, Neumann et al. 2000).

5.3.4. Sequence alignment

The Sw/99 and Ty/04 WT PB1-F2 gene products were aligned with the 20th century pandemic strains (1918 H1N1, 1957 H2N2, and 1968 H3N2), and some previously characterized PB1-F2 proteins. Alignment was carried out using ClustalW method in MegAlign (Lasergene v.8.1.5., DNAStar, Madison, WI).

5.3.5. Viral ribonucleoprotein (vRNP) reconstitution assay

In order to study the effects of PB1-F2 on polymerase activity, a previously developed viral ribonucleoprotein (vRNP) reconstitution assay was used (Pena, Vincent et al.). Briefly, 1 μg of the plasmid encoding the influenza virus-like NS vRNA carrying the *Gaussia* Luciferase (GLuc) reporter gene was transfected in 293-T cells along with 1 μg of each of the RG plasmids encoding PB2, PB1, PA and NP plasmids using the TransIT-LT1 (Mirus, Madison, WI) reagent following the manufacturer's recommendations. The PB1 plasmid encoded either the WT PB1-F2 or knockout (KO); or, in the case of Sw/99, the PB1-F2 gene with the N66S mutation. The pCMV/SEAP (SEAP) plasmid, which expresses the secreted alkaline phosphatase, was co-transfected into cells to normalize the transfection efficiency. At 24 hours post-transfection, supernatant from transfected cells was harvested and assayed for both luciferase and secreted alkaline phosphatase activities using the BioLux™ Gaussia Luciferase Assay Kit (NEB, Ipswich, MA) and the Phospha-Light™ Secreted Alkaline Phosphatase Reporter Gene Assay System (A&D, Foster City, CA) according to the manufacturers' instructions. Relative polymerase activity was calculated as the ratio of luciferase and SEAP luminescence from four independent experiments.

5.3.6. Isolation, infection, and viability of porcine alveolar macrophages

Porcine alveolar macrophages (PAMs) were isolated from five healthy pigs. At necropsy, the trachea was sectioned and 50 mL MEM were dispensed into the lungs using a 50 mL pipette. The fluid was withdrawn by aspiration and the procedure was repeated two times to maximize PAMs recovery. The retrieved bronchoalveolar lavage fluid (BALF) was placed on ice until centrifugation (500 g, 5 min, 4°C). The cell pellet was resuspended in 20 mL of PAMs complete media, which consisted of phenol red-free MEMα (Gibco, Grand Island, NY) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO) and antibiotics. PAMs were purified by adhesion. PAMs suspension derived from each donor was plated on 75 $cm^2$ tissue culture flasks (Corning, Lowell, MA) and incubated for 4 h at 37°C in 5% $CO_2$. The flasks were gently rocked at hourly intervals during incubation. Nonadherent cells were removed and adherents PAMs were harvested with a cell scraper. The cells were then collected, washed once, and counted using an automated cell counter PAMs were seeded at a density of $1x10^5$ cells per well in a 96-well flat-bottom plate with a final volume of 100 µl of PAMs complete media. At 24 h after plating, the media was removed and PAMs were infected with 100 µl of the recombinant viruses diluted in PAMs media at a multiplicity of infection (MOI) of 2 or 10. PAMs viability was measured by the XTT colorimetric method using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. At the indicated time points, 20 µl of XTT working solution (1 mg/mL), prepared immediately before use, were added to each well and the microplate was incubated for an additional 2 h at 37°C and read using an ELISA plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) at a wavelength of 650 nm. PAMs viability was determined as a relative percentage of the mock-treated non-infected control.

To determine the infectivity of PB1-F2 recombinant viruses in freshly isolated alveolar macrophages, PAMs were isolated from five healthy pigs as above, and seeded on a 6-well plate in a volume of 3 mL of MEMα supplemented with 10% fetal bovine serum and antibiotics. PAMs were incubated overnight and infected at MOI 2 with MEM without trypsin. Following 1 h adsorption at 37°C, PAMs were washed three times with PBS and the culture was replenished with 3 mL MEMα supplemented with 10% fetal bovine serum and antibiotics. At the indicated time points, 300 µl of supernatant was collected to assess virus replication. Virus yield in PAMs was determined by standard $TCID_{50}$ in MDCK cells.

### 5.3.7. TUNEL assay in *ex vivo* infected porcine alveolar macrophages

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used to quantify the levels of apoptotic-cell death induced by PB1-F2 recombinant viruses in *ex vivo* infected PAMs. Apoptotic cells were detected using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Briefly, PAMs were infected as described above at MOI of 2 and at 72 hpi cells were fixed with 4% paraformaldehyde and permeabilized in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate. Cells were then incubated in the TUNEL reaction mixture in the dark at 37°C for 60 min followed by detection of DNA strand breaks in apoptotic cells by fluorescence microscopy. A minimum of 600 cells was counted in a blinded-fashion from more than 10 random microscopic fields and the percentage of cell apoptosis was determined.

### 5.3.8. Isolation, culture, and infection of porcine respiratory explants

Porcine nasal turbinates, tracheal, and lung explants were prepared as described previously (Van Poucke, Nicholls et al. 2010), with some modifications. All respiratory explants were cultivated according to the air-liquid interface principle in an incubator at 37°C and 5% $CO_2$. NT and trachea explants were cultivated in 50% DMEM (Gibco)/50% RPMI (Gibco) media supplemented with 100 U/mL penicillin (Gibco), 100 µg/mL of streptomycin (Gibco), 0.1 mg/mL gentamycin (Gibco), 25 µg/mL Amphotericin B (Gibco), 0.3 mg/mL glutamine (BDH Biochemical) and nonessential amino acids (Sigma). Media for lung explants consisted of M199 (Sigma) containing the same antibiotics as above, nonessential amino acids (Sigma). Vitamin supplement (ATCC, Rockville, MD), 0.5 µg/mL hydrocortisone and ITS supplement (Insulin, Transferrin, Selenium) added at 10 mL/L medium. A five-week-old swine donor obtained from a high-health status farm whose animals are negative for IAV was used for this study. The animal was humanely euthanized with Beuthanasia®-D (Intervet/Schering-Plough, Summit, NJ) at a dosage of 1 mL/4.5 Kg of body weight and the respiratory tissues were collected. NT and trachea explants were dissected and washed 10 times with PBS containing antibiotics to remove bacterial contamination. Tissues were then cut into squares of 25 $mm^2$ each and placed with the epithelial surface upwards onto the filter membrane of the polyester tissue culture-treated inserts (Transwells®, Corning, Lowell, MA) at an air-liquid interface in 12-well plates. The

lower compartment was filled with 1 mL of explant media. The right apical lung lobe was expanded with a 1% type VII-A low gelling temperature agarose solution (Sigma, St. Louis, MO) that had been dissolved in lung explants media and cooled down to 37°C. The expanded lung was placed at 4°C for 10 min in a sterile container until the agarose solidified. The embedded lung tissue was then cut into 1 mm thick slices using a microtome blade and hand microtome. Two sections of the lungs were obtained: the proximal lung (close to the start of bronchial tree) and distal lung (close to the lung alveoli). The procedure for cultivation of lung explants was similar to the procedure used for the NT and tracheal explants. After 24 h, lung explants were washed with warm PBS to remove most of the agarose before infection. At 24 h of culture, explants were washed with PBS and $10^6$ tissue culture infectious dose 50% ($TCID_{50}$) of the recombinant viruses diluted in 500 µl of explants media were deposited in the upper compartment of Transwells® for 1 h. After the 1 h adsorption at 37°C, explants were washed three times with PBS and the culture replenished with 500 µl of explants media. One hundred µl of upper compartment explant bathing media was collected at 24, 48, and 72 h post inoculation to assess virus yields. Virus titers in respiratory explants were determined by standard $TCID_{50}$ in MDCK cells.

### 5.3.9. Virus titration

Viral stocks and virus present in biological samples were titrated on MDCK cells and the $TCID_{50}$/mL was determined by the Reed and Muench method (Reed and Muench 1938). Briefly, samples were serially diluted 10-fold in serum-free media containing antibiotics and 1 µg/mL TPCK-trypsin (SIGMA) and 100 or 200 µl of the inoculum were overlaid onto confluent monolayers of MDCK cells seeded in 96-well plates. The cells with the sample were incubated for 3 days and the endpoint viral titer was determined by an HA assay using 0.5% turkey red blood cells.

### 5.3.10. Swine pathogenicity experiment

Three-week-old cross-bred pigs were obtained from a high-health herd free of SIV and porcine reproductive and respiratory syndrome virus. All pigs were treated with ceftiofur crystalline free acid (Pfizer Animal Health, New York, NY) to reduce bacterial contaminants prior to the start of the experiment. Fifty pigs were randomly divided in five groups (n=10) and housed in separate isolation rooms. Pigs were infected intratracheally with $1\times10^5$ $TCID_{50}$

of each of the PB1-F2 recombinant viruses diluted in 2 mL of MEM using previously described protocols (Vincent, Lager et al. 2010). Following inoculation, pigs were monitored daily for clinical signs of disease and nasal swabs were collected to measure viral shedding. Five pigs from each group were euthanized on day 1 and the remaining five animals were euthanized at 3 dpi for evaluation of viral lung load, pathology, and host response to infection. Five additional pigs were inoculated with 2 mL MEM as above and served as mock-controls. Control pigs were euthanized at 3 days post infection (dpi).

5.3.11. Pathologic examination of swine lungs

At necropsy, lungs were removed *in toto* and evaluated to determine the percentage of the lung affected by purple-red, consolidated lesions that are typical of influenza virus infection in pigs. The percentage of the surface affected with pneumonia was visually estimated for each lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume as previously described (Halbur, Paul et al. 1995). Each lung was then lavaged with 50 mL MEM to obtain bronchoalveolar lavage fluid (BALF). A single veterinary pathologist scored all lungs and was blinded to the treatment groups.

5.3.12. Histopathology and immunohistochemistry

Tissue samples from the trachea and right middle lung lobe were taken and fixed in 10% buffered formalin for histopathological examination. Tissues were routinely processed and stained with hematoxylin and eosin. A board-certified veterinary pathologist blinded to treatment groups evaluated microscopic lesions. Scoring of lesions was based on scales adapted from Gauger et al (Gauger, Vincent et al. 2011). Individual scores were assigned to four parameters: bronchial and bronchiolar epithelial changes, bronchitis/bronchiolitis, peribronchiolar lymphocytic cuffing and interstitial pneumonia. Scores were based on percentage of airways with lesions that included epithelial changes and inflammation on a 5-point scale: 0: No lesions, 1: 1-25%, 2: 26 to 50%, 3: 51-75%, 4: greater than 75% of airways affected with airway epithelial damage and inflammation. Peribronchiolar cuffing by lymphocytes was graded on a 4-point scale: 0: None, 1: Mild, loosely formed cuff, 2: Moderate, well formed cuffs,, and 3: Prominent, thick well formed cuffs. Interstitial pneumonia was graded on 5-point scale based on severity and distribution: 0: No lesions, 1: Mild, focal to multifocal, 2: Moderate, locally extensive to multifocal, 3: Moderate, multifocal to coalescing, and 4: Severe, coalescing to diffuse.

Trachea sections were scored similar to the bronchi and bronchioles and were based on epithelial changes and degree of inflammation. Tracheal epithelial changes were graded on a 5-point scale: 0: No lesions, 1: Early epithelial changes characterized by focal to multifocal loss of cilia and epithelial degenerative changes, 2: Mild epithelial flattening with loss of cilia and goblet cells, 3: Moderate epithelial flattening with decreased thickness of respiratory epithelium, loss of cilia and goblet cells, 4: Flattened epithelium with areas of mucosa covered by a single layer of cuboidal epithelium and epithelial loss (necrosis). Degree of tracheitis was graded on a simple 4-point scale: 0: None, 1: Mild, 2: Moderate, and 3: Severe.

Influenza virus Type A-specific antigen was detected in lung tissues using a previously described immunohistochemical (IHC) method with minor modification (Vincent, Janke et al. 1997). Briefly, tissue sections were deparaffinized and hydrated in distilled water. Slides were quenched in 3% hydrogen peroxide for 10 min, rinsed three times in de-ionized water and treated in 0.05% protease for 2 min. Slides were then rinsed three times in de-ionized water and once in Tris-buffered saline (TBS). Influenza A virus-specific monoclonal antibody (MAb) HB65 (ATCC, Manassas, VA), specific for the nucleoprotein (NP) of influenza A viruses, was applied at 1:100 dilution and slides were incubated at room temperature for 1 h. Bound MAbs were stained with peroxidase-labeled anti-mouse IgG followed by chromogen using the DAKO LSAB2-HRP Detection System (DAKO, Carpinteria, CA) according to the manufacturer's instructions. The slides were rinsed in deionized water and counterstained with Gill's hematoxylin. Antigen detection was given two scores: 1) airway epithelial labeling and 2) alveolar/interstitial labeling. In airway epithelium a 5-point scale was used: 0: None, 1: Few cells with positive labeling, 2: Mild scattered labeling, 3: Moderate scattered labeling, 4: Abundant scattered labeling (greater than 50% epithelium positive in affected airways). In the interstitium/alveoli, a 4-point scale was used: 0: None, 1: Minimal focal signals, 2: Mild multifocal signals, 3: Abundant signals.

### 5.3.13. Quantification of cytokine/chemokine protein levels in bronchoalveolar lavage fluid

Levels of nine porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-γ) in BALF were determined by multiplex ELISA following the

manufacturer's recommendations (SearchLight, Aushon Biosystems). Levels of IFN-α protein were measured by ELISA using F17 monoclonal antibody, K9 MAb and recombinant porcine IFN-α (R&D Systems Inc., Minneapolis, MN) as previously described (Brockmeier, Lager et al. 2009).

5.3.14. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student t-test, whereas multiple comparisons were carried out by analysis of variance (ANOVA) using Dunnett's post hoc test, unless otherwise specified. The differences were considered statistically significant at $p < 0.05$.

## *5.4. Results*

### 5.4.1. Generation of PB1-F2 recombinant influenza viruses

In this study, we sought to understand how PB1-F2, the 11th defined IAV gene product, modulates the replication and virulence of triple reassortant influenza viruses in pigs. The sequences of the Sw/99 and Ty/04 PB1-F2 proteins are 90 amino acid (aa) long and differ only at four amino acid positions from each other. The Ty/04 contains the following mutations compared to sw/99: S23N, R44K F57S, and F83S. The functional roles of these polymorphisms have not been established. The Ty/04 PB1-F2 is 54.4 % divergent from the 1918 H1N1 PB1-F2 at the amino acid level. Compared with previously characterized human H3N2 IAV PB1-F2 proteins, the Ty/04 presents 74.4 % and 86.6 % of aa identity with 1968 pandemic strain A/Hong Kong/1/68 (H3N2) and with the seasonal human H3N2 A/Wuhan/359/95 (H3N2) isolate, respectively (Fig. 5.1).

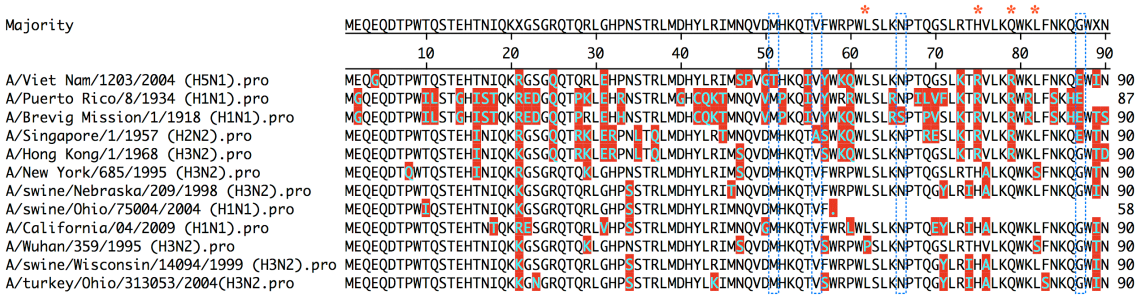



**Figure 5.1. Alignment triple reassortant H3N2 PB1-F2 used in this study.** Sw/99 and Ty/04 PB1-F2 ORF were aligned to those of the 20th century pandemic strains (1918 H1N1, 1957 H2N2, and 1968 H3N2), other H3N2 swine IAV strains, and the previously characterized PB1-F2 proteins. Boxed residue highlights amino acid residues that have been associated with increased virulence of 1918 and highly pathogenic H5N1 strains: 51(T), 56(V), 66(S), and 87(E). Asterisks indicated the positions in which pro-inflammatory mutations (P62L, H75R, Q79R, and S82L) have been found in the human H3N2 PB1-F2 lineage (Alymova, Green et al. 2011).

In order to study the PB1-F2 proteins expressed by either Sw/99 or Ty/04, we first established a reverse-genetics (RG) system for these viruses using a previously published approach (Hoffmann, Neumann et al. 2000). PB1-F2 translation from the segment 2 RNA was completely abolished by mutating the ATG start codon to ACG and introducing 2 stop codons downstream of the gene. Since Sw/99 virus is of low virulence to pigs, we introduce the N66S mutation in its PB1-F2, an important mutation for the virulence H5N1 and 1918 influenza viruses in the mouse model of infection (Conenello, Zamarin et al. 2007). The viruses used herein are summarized in Table 1. Each viral gene was cloned into the pDP-2002 vector and the WT RG viruses were rescued by transfection of 293-T and MDCk co-cultures. Because the presence of PB1-F2 in certain virus strains has been shown increase polymerase activity, we next assessed whether this phenotype could be recapitulated in viruses containing the TRIG cassette. We found no difference in polymerase activity between the WT and PB1-F2 mutants from the two different strains chosen for this study (Fig. 5.2), suggesting that PB1-F2 does not affect polymerase activity of the TR H3N2 influenza viruses studied herein.

**Table 5.1. Influenza viruses used in this study.**

| Viruses | Acronym in main text |
|---|---|
| A/turkey/Ohio/313053/04 (H3N2) | Ty/04 WT |
| A/turkey/Ohio/313053/04 (H3N2) PB1-F2 knockout | Ty/04 KO |
| A/swine/Wisconsin/14094/99 (H3N2) | Sw/99 WT |
| A/swine/Wisconsin/14094/99 (H3N2) PB1-F2 knockout | Sw/99 KO |
| A/swine/Wisconsin/14094/99 (H3N2) PB1-F2 N66S | Sw/99 N66S |

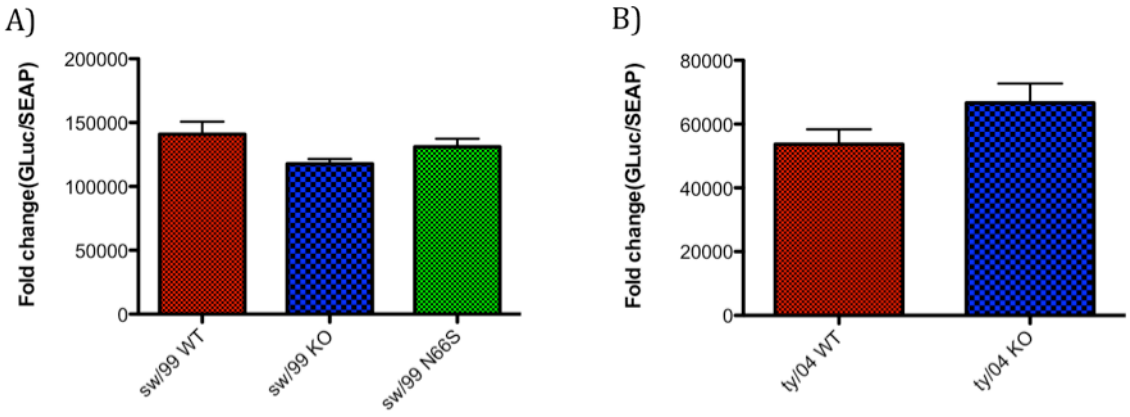



**Figure 5.2. PB1-F2 derived from TR H3N2 does not influence polymerase activity.** 293-T cells were transfected with plasmids encoding the minimal components required for viral transcription and replication (PB2, PB1, and PA polymerase subunits, NP and a vRNA influenza driven-luciferase reporter replicon expressing GLuc) derived from either Sw/99 (A), Ty/04(B). The pCMV/SEAP, plasmid was used to normalize transfection efficiency as previously described (Pena, Vincent et al.). At 24 hours post transfection, the supernatant was harvested and assayed for both luciferase and phosphatase activities. Data are expressed as polymerase activities (mean ± SEM) determined from four independent experiments.

### 5.4.2. PB1-F2 affects both the infectivity and viability of porcine alveolar macrophages in a strain-dependent fashion

Since PB1-F2 has been shown to induce cell death in immune cells (Chen, Calvo et al. 2001; McAuley, Chipuk et al. 2010), we investigated whether the presence of PB1-F2 affects the infectivity and the viability of freshly isolated porcine alveolar macrophages (PAMs). The purity of PAMs preparations was over 98%, as determined by flow cytometry using anti-porcine CD163 antibody (data not shown). PAMs derived from 5 healthy pigs were infected at MOI of 2 with each of the PB1-F2 recombinant viruses and viral yields in PAM supernatant were determined by standard $TCID_{50}$ in MDCK cells. Disruption of PB1-F2 expression significantly reduced viral yields in PAMs from 6-48 hpi, although the presence of N66S mutation had no effect in viral replication in these cells (Fig.5.3A). The impact of Sw/99 PB1-F2 in PAMs viability as well as in apoptotic cell death correlated with the replication ability of these viruses. The Sw/99 WT caused more cytotoxicity to PAMs compared to Sw/99 KO, whereas the N66S mutation had an intermediate effect (Fig. 5.3B and 5.3C). Ty/04 WT replicated to lower levels in PAMs compared to Sw/99 WT and knocking out PB1-F2 had no effect in virus growth. Overall PAMs viability was reduced upon infection with the Ty/04 isogenic viruses. Although Ty/04 KO had lower levels of apoptotic cell death as determined by TUNEL assay, overall PAM viability was not affected as assessed by the XTT method (Fig. 5.3B and 5.3C). It should be noted that cytotoxicity measured by the XTT assay was only detected when PAMs were infected at high MOI (10). Taken together, these data suggest that PB1-F2 affects the replication and viability of PAMs in a strain-dependent manner.

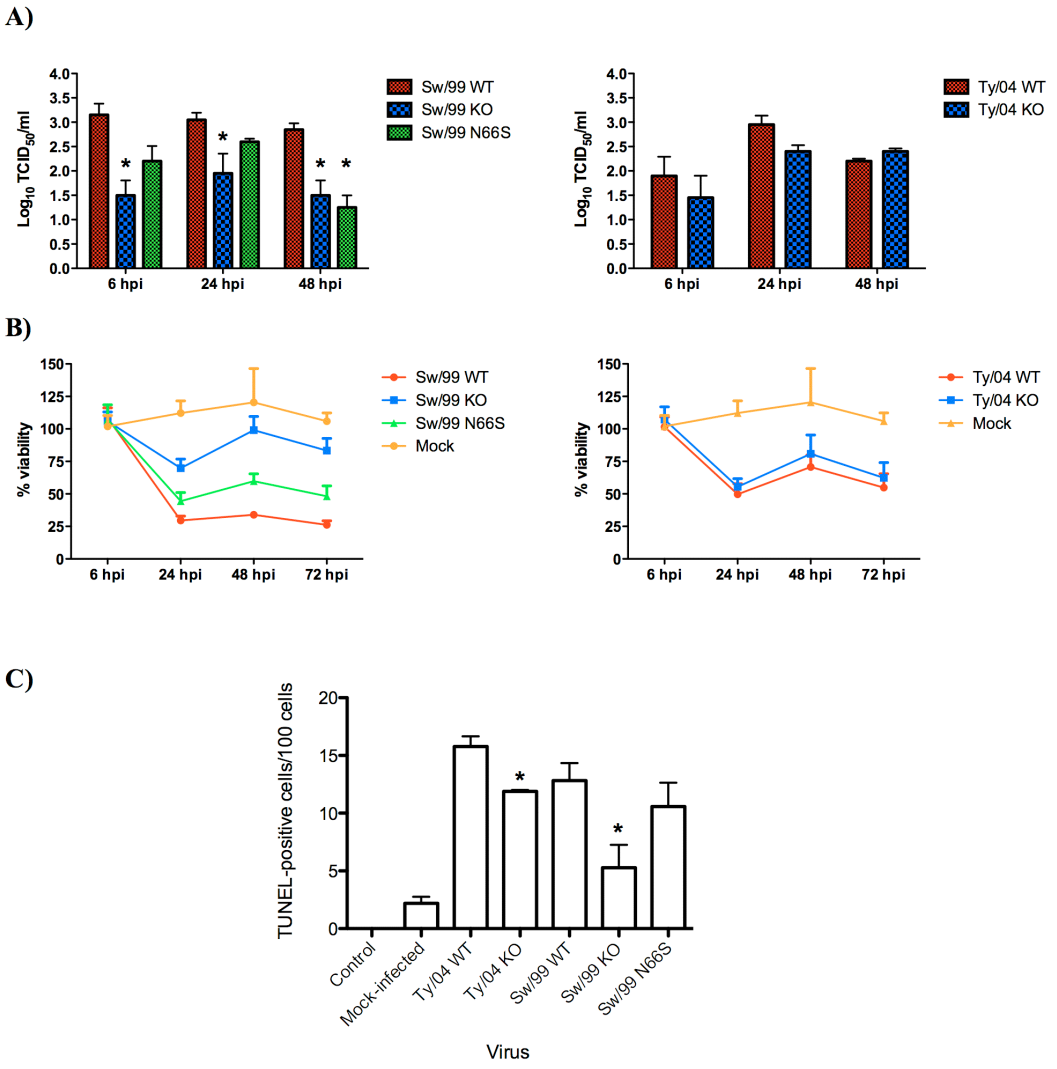



**Figure 5.3. Infectivity and viability of porcine alveolar macrophages upon infection with PB1-F2 recombinant viruses.** (A) Porcine alveolar macrophages (PAMs) derived from five healthy pigs were infected at MOI of 2 with each of the recombinant viruses and viral yields were determined by standard $TCID_{50}$ in MDCK cells. (B) Toxic effects induced by PB1-F2. PAMs were mock-infected or infected with PB1-F2 recombinant viruses at an MOI of 10 and cell viability was measured by the XTT assay at 24, 48 and 72 hpi. (C) PAMs were mock-infected or infected at MOI of 2 with the PB1-F2 recombinant viruses and assayed for apoptotic-cell death using the in situ TUNEL assay at 72 hpi. Each data point represents the mean ± SEM. Asterisks indicate that the differences are statistically significant ($p< 0.05$) between the WT and the PB1-F2 recombinant viruses.

### 5.4.3. PB1-F2 enhances replication of TR H3N2 IAV in porcine respiratory explants

It has been demonstrated that PB1-F2 increase replication of certain IAV isolates in tissue culture (McAuley, Zhang et al. 2010; Smith, Adler et al. 2011). However, cell lines do not exhibit the natural physiological conditions and cellular complexity present in the respiratory tract. In order to study the replication of PB1-F2 recombinant viruses in a relevant biological system, we developed an *ex vivo* organ culture model of the pig respiratory tract maintained an air–liquid interface. Tissue explants were prepared from nasal turbinates, trachea, proximal (close to the bronchi), and distal lung (close to the alveoli) from healthy swine donor. Knocking out PB1-F2 from Ty/04 caused mild but significant decrease in virus replication in respiratory explants. The presence of PB1-F2 (or the N66S mutation) had no impact on the replication of Sw/99 in this system (Fig. 5.4A-D). Collectively, these data indicate that PB1-F2 can modulate viral production in swine respiratory tissues but its function may be unnecessary for some SIV strains, which is consistent with the observation that approximately only 50% of SIV isolates encode a potentially functional PB1-F2 gene.

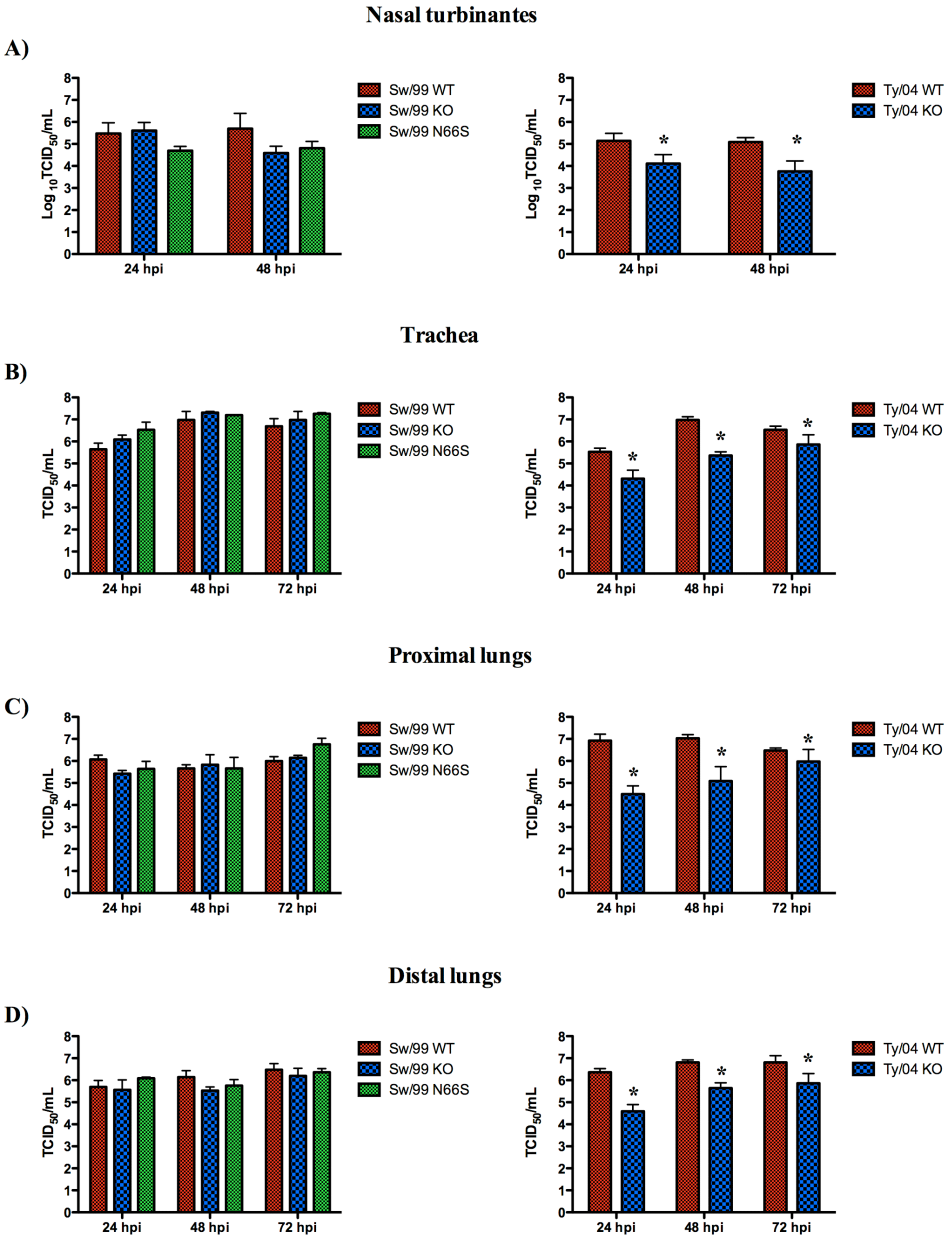



**Figure 5.4. Replication of PB1-F2 recombinant viruses in porcine respiratory explants.** Nasal turbinates (A), tracheal (B), proximal (C), and distal lung (E) explants were infected with $10^6$ $TCID_{50}$ of each viruses, The bathing media was collected at the indicated time points and titrated by the $TCID_{50}$ method in MDCK cells. Values shown are the mean ± SEM of virus titer ($log_{10}$ $TCID_{50}$/mL) from four independent experiments. * $p < 0.05$.

### 5.4.4. Deletion of PB1-F2 increases the virulence of Ty/04 to swine without affecting viral load

We next evaluated the effects of PB1-F2 in the virulence of TR H3N2 IAVs *in vivo*. Three-week old cross bred pigs were obtained from a high-health herd and separately in five groups of 10 pigs each and a control group containing 5 pigs. Animals were housed in separated isolation units and inoculated intratracheally with $10^5$ $TCID_{50}$/animal. The control group was inoculated with sterile tissue culture media. Five pigs from each of the infected group were euthanized at 24 hpi and the remaining five animals were euthanized at 3 dpi along with the control pigs. The expression PB1-F2 (or the N66S modification in sw/99) did not affect viral shedding in nasal secretions from either of the strains tested (Fig. 5.5A). Similarly, viral lung load was not affected by PB1-F2 in neither of the strains (Fig. 5.5B). At necropsy, PB1-F2 or N66S substitution had no effect in either the macroscopic (Fig.5C) or microscopic lung pathology (Fig. 5.5D) caused by sw/99. Surprisingly, knocking out PB1-F2 from the Ty/04 strain significantly increased the macroscopic and microscopic pneumonia of this virus at 3dpi (Fig. 5.5C-D), indicating that PB1-F2 is a modulator of virulence of certain IAVs in pigs.

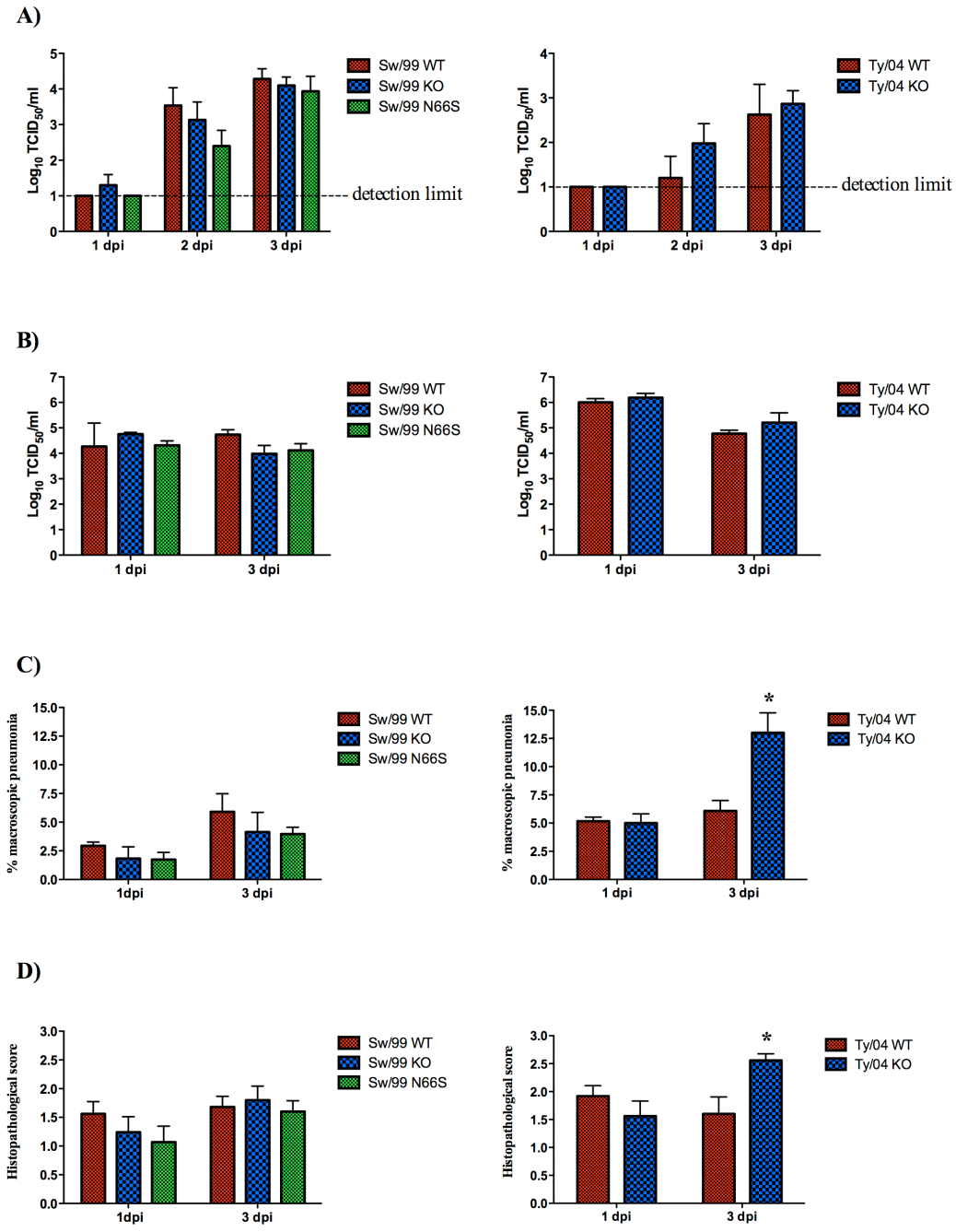



**Figure 5.5. Replication and pathology induced by PB1-F2 recombinant viruses in swine**. Groups of pigs (n=10) were infected with 105 TCID50 of PB1-F2 recombinant viruses and nasal swabs were collected from 1-3 dpi for measuring virus shedding. Five animals from each group were euthanized either at 1 or 3 dpi. The lungs were collected and processed for virus titration and pathological analysis. (A) Viral shedding in nasal secretions of pigs. (B) Pulmonary replication of PB1-F2 isogenic viruses in pigs. Values are mean ± SEM of virus titer ($log_{10}$ $TCID_{50}$/mL) in bronchoalveolar lavage fluid (BALF) collected at the indicated time points. (C) Percentage of macroscopic lung lesions (D) Histopathologic scores in the lungs. Values shown are mean ± SEM.

### 5.4.5. PB1-F2 modulates the innate immune response in swine lungs

It has been shown that PB1-F2 strongly influences the early host response during IAV infection (McAuley, Hornung et al. 2007; Le Goffic, Leymarie et al. 2011). To further characterize the effect of PB1-F2 in the pathological manifestations during infection with TR H3N2 IAV in swine, we measured the protein levels of ten porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-α, and IFN-γ) in BALF collected at 1 and 3 dpi necropsies. The amounts of the cytokines/chemokines IL-1β, IL-2, IL-4, IL-10, IL-12p70, TNF-α, and IFN-α were not affected by the PB1-F2 in either of the strains (Fig. 5.6, and data not shown). However, increased pulmonary levels of IL-6 were seen early (1 dpi) during the infection with Sw/99 WT as compared to Sw/99 KO, suggesting that PB1-F2 differentially regulates these cytokines in pigs infected with Sw/99 virus. The presence of N66S change increased the levels of IL-8 at 1 dpi compared to WT virus. Interestingly, knocking out PB1-F2 from Ty/04 augmented the pulmonary levels of IL-8 and IFN-γ at 3 dpi, which was concomitant with the development of the enhanced pneumonia in this group. Collectively, these results indicate that PB1-F2 differentially modulates the local levels cytokines and chemokines during infection with TR H3N2 in pigs and this heightened production of IL-8 and IFN-γ pro-inflammatory mediators correlated with the lung pathology developed in Ty/04 KO-infected animals.

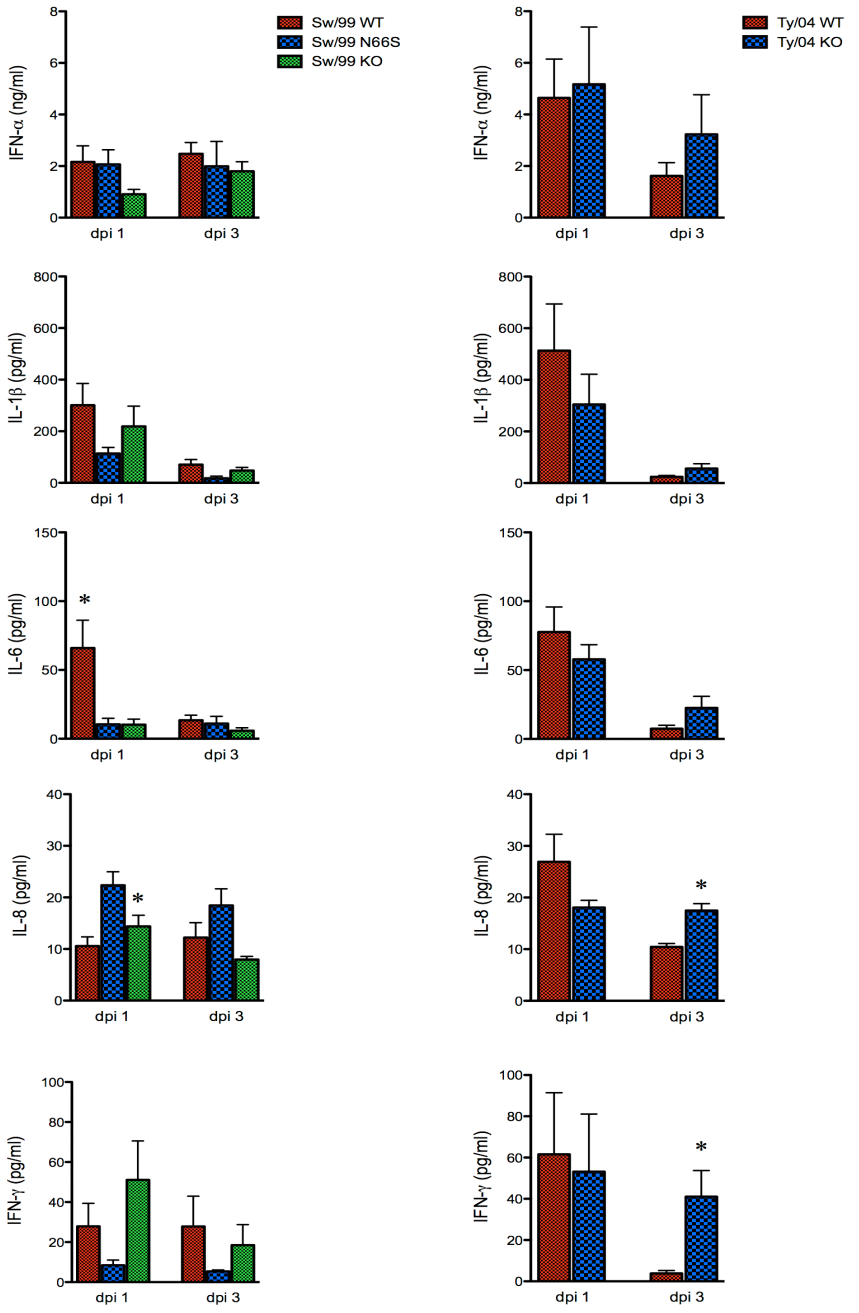



**Figure 5.6. PB1-F2 modulates the innate immune response in swine lungs.** Groups of pigs (n=10) were infected intratracheally with $10^5$ $TCID_{50}$ of PB1-F2 recombinant viruses. On days 1 and 3 post-infection, animals from each group were euthanized and the cytokines/chemokines levels in BALF were determined by ELISA. Data are shown as mean ± SEM of five animals in each challenge group. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. * $p < 0.05$ as compared to WT virus.

## *5.5. Discussion*

Triple reassortant (TR) IAVs have become endemic in swine populations in North American since their first introduction in 1998 (Zhou, Senne et al. 1999). TR swine IAV have demonstrated remarkable ability to reassort with either classical swine H1N1 or human H1N1 and H3N2 viruses, generating several different virus reassortants in the last decade. Remarkably, the 2009 pandemic H1N1 (pH1N1) resulted from genetic reassortment between TR swine IAV circulating in North America and Eurasia swine H1N1 IAV (Garten, Davis et al. 2009). In the US, pH1N1 has been reassorting with endemic TR strains at an alarming rate, emphasizing the epidemiological importance of viruses harboring the TRIG cassette (Ducatez, Hause et al. 2011).

The phenotypes associated with the presence of PB1-F2 proteins derived from TR H3N2 IAVs have not yet been elucidated. Here, we created PB1-F2 null viruses in the background of two representative lineages of swine-origin North American TR H3N2 viruses: sw/99, which belongs to the phylogenetic cluster III; and Ty/04, a representative swine-origin TR H3N2 IAV belonging to cluster IV. These viruses were chosen because they are genetically related and the Ty/04 virus is thought to have originated from the Sw/99 strain following interspecies transmission event(s). Since Sw/99 is low virulent for pigs (Pena *et al*, manuscript in preparation), we also studied the effects of the virulence marker N66S in the PB1-F2 of this strain. We focused in swine systems to evaluate the role of PB1-F2 in the context of isogenic viruses whose differences were limited to the PB1-F2 gene product. In addition to the impact of influenza infection on the swine industry and the epidemiology of IAV, conducting pathogenesis studies in pigs also offers the possibility of extrapolating the findings to humans, since there are important similarities between the two species regarding the distribution of sialic acid receptors, cytokine production, and clinical-pathological outcomes after influenza infection (Van Reeth, Van Gucht et al. 2002; Barnard 2009; Barbe, Atanasova et al. 2010; Barbe, Saelens et al. 2010; Nelli, Kuchipudi et al. 2010).

Alveolar macrophages are located at the air-tissue interface in the lungs and are the initial cells that combat inhaled microorganisms and particulate matter (Coleman 2007). IAV have been shown to infect alveolar macrophages in a variety of species, including mice, swine, and humans (Rodgers and Mims 1982; Reading, Miller et al. 2000; Seo, Webby et al. 2004; Upham, Pickett et al. 2010; van Riel, Leijten et al. 2011). In pigs, *in vivo* depletion of

alveolar macrophages by chemical treatment unveiled that these cells are indispensable for controlling IAV infection (Kim, Lee et al. 2008). Since its original discovery, PB1-F2 has been suggested to play a role in influenza virulence by destroying host immune cells such as macrophages (Chen, Calvo et al. 2001; Lamb and Takeda 2001; Zamarin, Garcia-Sastre et al. 2005). We addressed this question by infecting freshly isolated porcine alveolar macrophages (PAMs) *ex vivo* with PB1-F2 recombinant viruses. We found that the presence of PB1-F2 was required from optimal replication of the Sw/99 strain, whereas its presence did not affect Ty/04 replication in these cells (Fig. 5.3). Consistent with its enhanced replication, the viability of PAMs infected *ex vivo* was affected by the presence of PB1-F2 in the Sw/99 strain. Differences in apoptotic cell death were detected by TUNEL assay when PAMs were infected at MOI 2, but this effect was only quantifiable by XTT when PAMs were infected at MOI 10. There was no correlation between the ability of PB1-F2 recombinant viruses to replicate in PAMs and the virulence of these viruses to pigs. Disruption of PB1-F2 ORF had no effect in Sw/99 virulence as measured by viral shedding, viral lung load, and lung pathology. These results are in accordance with previous reports that showed a lack of correlation between macrophage infectivity and/or cell death *in vitro* and the course of IAV disease in mouse models (McAuley, Chipuk et al. 2010; Tate, Pickett et al. 2010). In the background of Ty/04, PB1-F2 had no effect on viral replication, apoptosis or viability of PAMs.

Expression of full-length PB1-F2 as well as the N66S substitution has been shown to increase replication of certain IAV isolates in tissue culture (McAuley, Zhang et al. 2010; Schmolke, Manicassamy et al. 2011; Smith, Adler et al. 2011). Because cell lines lack the cellular complexity and natural physiological conditions of the respiratory tract, we studied the replication of PB1-F2 recombinant viruses in porcine respiratory explants. Different groups have shown that respiratory explants are a valuable system to study influenza-host interaction in a scalable, well-defined experimental setting (Chan, Chan et al. 2010; Nunes, Murcia et al. 2010; Van Poucke, Nicholls et al. 2010), although no study has compared side-by-side viral replication *ex vivo* versus *in vivo*. Our results supported the hypothesis that PB1-F2 modulates viral replication in a strain-dependent manner. Although Ty/04 PB1-F2 did not affect polymerase activity, higher viral yields were observed in respiratory explants infected with Ty/04 WT relative to Ty/04 KO. In the backbone of Sw/99 viruses, neither PB1-F2 deletion nor N66S mutation affected viral growth kinetics in this system (Fig.6.4). Interestingly, the *ex vivo* replication of Sw/99 PB1-F2 recombinants viruses paralleled with

the levels of viral shedding in nasal secretions and lung viral load *in vivo*. On the other hand, we found no differences between Ty/04 WT and Ty/04 KO regarding viral shedding in the upper respiratory tract and BALF titers, suggesting that results obtained from *ex vivo* and *in vivo* results not always correlate with each other. Even though tissue culture explants preserve the normal tissue cytoarchitecture, it must be noted that these cells are removed from their normal in vivo environment and may thus behave different and may not reflect the effects of *in vivo* systemic factors present during infection (Grivel and Margolis 2009). These limitations should be considered when using *ex vivo* models to extrapolate conclusions obtained in these systems to *in vivo* settings.

McAuley e*t al.* demonstrated that PB1-F2 proteins from human H3N2 IAV differ profoundly in their ability to induce inflammatory responses and pathology in lungs. They found that PB1-F2 derived from the 1968 pandemic strain A/Hong Kong/1/68 (H3N2) exerts robust pro-inflammatory effects *in vivo*, whereas a later seasonal H3N2 isolate was not capable of triggering this response (McAuley, Chipuk et al. 2010). Recently, the same group demonstrated that PB1-F2 from recent H3N2 isolates lost the immunostimulatory activity through truncation or accumulations of point mutations, presumably during viral adaptation in humans. The authors mapped the pro-inflammatory mutations in PB1-F2 to P62L, H75R, Q79R, and S82L (Alymova, Green et al. 2011). Interestingly, the PB1-F2 sequences of both Sw/99 and Ty/04 presents conserved residues in these positions, which include a mix of the pro-inflammatory motifs 62(L) and 82(L) as well as the non-inflammatory mutations 75(H), 79(Q). In our swine pathogenicity studies, PB1-F2 deletion or N66S substitution in the background of Sw/99 did not alter viral-induced pneumonia in these animals. Unexpectedly, disruption of PB1-F2 ORF from the Ty/04 strain significantly aggravated both the microscopic and macroscopic pneumonia caused by this virus at 3 dpi. The degree of pneumonia in Ty/04 KO cannot be explained by improved replication efficiency of this virus since no replication variation was observed between the two Ty/04 isogenic viruses

The pro-inflammatory cytokine response is critical for recruiting effector cells to the site of an infection. However, elevated or prolonged cytokine production can also contribute to the pathological changes observed during infection. Thus, a fine balance between inflammation and immunity must be achieved in order to successfully counteract IAV infection. To better understand the mechanism of enhanced pneumonia in Ty/04 KO-infected pigs, we measured the pulmonary concentration of ten cytokines/chemokines early during the course of disease. Concomitantly with the development of severe pneumonia in this group,

there was a significant increase in the pulmonary levels of IL-8 and IFN-γ at 3 dpi as compared to the group infected with Ty/04 WT virus.

IL-8 is a major chemokine involved in neutrophil recruitment to infection sites, a vital mechanism for elimination of the assaulting microorganism. Nevertheless, this process must be tightly regulated to ensure a beneficial outcome to the host since uncontrolled neutrophil recruitment can lead to harmful release of enzymes, reactive oxygen species, and increase vascular permeability in the lungs (Dallegri and Ottonello 1997). Importantly, elevated level of IL-8 has been implicated in the pathogenesis of viral-induced bronchiolitis in humans (Koh, Jung da et al. 2007).

IFN-γ is an important immunoregulatory cytokine, which is produced by T cells, NK cells, and macrophages. IFN-γ has pleiotropic effects on various cells of the immune systems and plays an important role in T-cell–mediated lung injury (Bruder, Srikiatkhachorn et al. 2006). Influenza virus infection induces local production of IFN-γ in the respiratory tract. Experiments in gene-targeted mice showed that IFN-γ plays no significant role in protection from lethal infection (Graham, Dalton et al. 1993) and IAV has developed mechanisms to evade its antiviral activity by disrupting intracellular signaling pathways (Uetani, Hiroi et al. 2008). *In vivo* neutralization of IFN-γ with monoclonal antibodies demonstrated that this cytokine plays crucial role in the recruitment and/or retention of leucocytes in the inflammed lung parenchyma during IAV infection (Baumgarth and Kelso 1996). Recently, Le Goffic *et al.* reported that infection with the laboratory strain A/WSN/1933 (H1N1) in mice resulted in higher levels of IFN-γ relative to infection with an isogenic virus lacking PB1-F2 (Le Goffic, Leymarie et al. 2011). These results are in disagreement with our findings in pigs using a TR H3N2 AIV, in which deletion of PB1-F2 led to enhanced IFN-γ concentrations in BALF. The reason for this discrepancy is unclear, but it might be due to differences in viral strains and host system used in each study. In fact, there have been conflicting reports of the role of PB1-F2 in modulating type I interferons, suggesting that the exact role of PB1-F2 in influencing the host innate immune response remains unresolved (Le Goffic, Bouguyon et al. 2010; Dudek, Wixler et al. 2011; Varga, Ramos et al. 2011). Thus, it is possible that deregulation of the innate host defense through IL-8 and IFN-γ pathways might have played a role in the exacerbated immunopathology induced by PB1-F2 null Ty/04 virus. Should Ty/04 PB1-F2 have a direct effect in blocking or downregulating IL-8 and IFN-γ expression, this effect would explain the lessened pulmonary lesions developed in the Ty/04 WT group. These studies are beyond the scope of this work and warrant further investigation. Furthermore,

research efforts focusing on the modulation of the porcine immune system by PB1-F2 and its interaction with host factors should shed light into the molecular mechanisms of PB1-F2 in strain and host-dependent manner.

In conclusion, we have shown that PB1-F2 has pleiotropic effects in the swine host, which are expressed in a strain-dependent manner. We demonstrated that PB1-F2 is an important viral component implicated in orchestrating innate immune responses in the infected lungs. For the first time, PB1-F2 has been shown to play a detrimental role in AIV pathogenesis in mammals. This study fills a critical gap regarding the virulence determinants of influenza virus for swine. Our studies provide important insights into the impact of PB1-F2 on the virulence of TR H3N2 IAV during infection of a natural host.

# Chapter 6: Modifications in the Polymerase Genes of a Swine-Like Triple-Reassortant Influenza Virus To Generate Live Attenuated Vaccines Against 2009 Pandemic H1N1 Viruses

## *6.1. Abstract*

On June 11, 2009 the World Health Organization (WHO) declared that the outbreaks caused by novel swine-origin influenza A (H1N1) virus had reached pandemic proportions. The pandemic H1N1 (H1N1pdm) is the predominant influenza strain in the human population. It has also crossed the species barriers and infected turkeys and swine in several countries. Thus, the development of a vaccine that is effective in multiple animal species is urgently needed. We have previously demonstrated that introduction of temperature-sensitive mutations in the PB2 and PB1 genes of an avian H9N2 combined with the insertion of an HA tag in PB1 resulted in an attenuated (*att*) vaccine backbone for both chickens and mice. Because the new pandemic strain is a triple reassortant (TR) virus, we chose a swine-like TR virus isolate, A/turkey/OH/313053/04 (H3N2) (ty/04), to introduce the double attenuating modifications with the goal of producing live attenuated influenza vaccines (LAIV). This genetically modified backbone had impaired polymerase activity and restricted virus growth at elevated temperatures. *In vivo* characterization of two H1N1 vaccine candidates generated using the ty/04 *att* backbone demonstrated that this vaccine is highly attenuated in mice as indicated by the absence of signs of disease, limited replication and minimum histopathological alterations in the respiratory tract. A single immunization with the ty/04 *att*-based vaccines conferred complete protection against a lethal H1N1pdm infection in mice. More importantly, vaccination of pigs with a ty/04 *att*-H1N1 vaccine candidate resulted in sterilizing immunity upon an aggressive intratracheal challenge with the 2009 H1N1 pandemic virus. Our studies highlight the safety of the ty/04 *att* vaccine platform and its potential as a master donor strain for the generation of live attenuated vaccines for humans and livestock.

## *6.2. Introduction*

In the spring of 2009, the Centers for Disease Control and Prevention (CDC) of the United States of America announced the identification of a novel H1N1 strain (H1N1pdm) of influenza A virus causing an acute respiratory disease in humans (Dawood, Jain et al. 2009). The virus spread easily and sustainably among humans throughout the world, prompting the World Health Organization (WHO) to declare on June11$^{th}$, 2009 the first influenza pandemic of the 21$^{st}$ century (Enserink and Cohen 2009; Al Hajjar and McIntosh 2010). This new isolate was identified as a swine-origin influenza virus (S-OIV) because its RNA segments were most closely related to influenza viruses isolated from pigs in North America and Eurasia (Garten, Davis et al. 2009; Gibbs, Armstrong et al. 2009). Specifically, six of its genomic segments (PB2, PB1, PA, HA, NP, and NS) are most similar to those of triple-reassortant (TR) influenza viruses currently circulating in North American pigs, whereas the NA and M gene segments are related to prevalent Eurasian H1N1 swine influenza strains. This particular gene constellation has never been described among swine or human influenza isolates from anywhere in the world and the precise evolutionary pathway in the genesis of the pandemic H1N1 is currently unknown (Dawood, Jain et al. 2009; Garten, Davis et al. 2009; Gibbs, Armstrong et al. 2009; Smith, Vijaykrishna et al. 2009). Since 1998, the emergence of TR influenza viruses, which have their genes derived from human, swine and avian strains, have caused a dramatic change in the epidemiology of influenza in pigs in North America (Zhou, Senne et al. 1999; Karasin, Olsen et al. 2000; Karasin, Schutten et al. 2000; Olsen 2002; Karasin, Carman et al. 2006; Olsen, Karasin et al. 2006). Prior to 1997-98, swine influenza in North America was caused almost exclusively by infection with classical H1N1 viruses, a derivate of the 1918 Spanish flu that was initially isolated from pigs in 1930 (Shope 1931). Since their introduction in the late 90's, TR swine influenza viruses have become endemic in North American swine. TR strains of the H3N2, H1N2, and H1N1 subtypes predominate in the US swine population (Vincent, Ma et al. 2008). TR swine viruses have demonstrated remarkable reassortment ability with either classical swine H1N1 and human H1N1 and H3N2 viruses, generating at least seven different reassortant lineages in the last decade (Karasin, Olsen et al. 2000; Karasin, Schutten et al. 2000; Richt, Lager et al. 2003; Webby, Rossow et al. 2004; Karasin, Carman et al. 2006; Lekcharoensuk, Lager et al. 2006; Ma, Gramer et al. 2006; Olsen, Karasin et al. 2006; Ma, Vincent et al. 2007). A unique feature shared by all of these novel reassortants is the maintenance of the so-called triple reassortant internal gene (TRIG) cassette, which consists of the avian-like PB2 and PA

genes, the human-like PB1 gene and the classical swine NP, M and NS genes (Vincent, Ma et al. 2008; Ma, Lager et al. 2009). The TRIG cassette appears to accept multiple HA and NA types, which could provide a selective advantage to swine viruses possessing this internal gene constellation (Vincent, Ma et al. 2008; Ma, Lager et al. 2009). Although there have been sporadic human infections in the United States with the H1 TR swine influenza viruses, none of these events led to sustained human-to-human transmission, until the emergence of the H1N1pdm virus (Neumann, Noda et al. 2009; Shinde, Bridges et al. 2009; Vincent, Lager et al. 2010). Outbreaks of H1N1pdm in pigs in commercial swine operations have been reported in several countries, such as Canada, Argentina, Australia, Singapore, UK, Ireland, Norway, USA, Japan, and Iceland. In all incidents, epidemiological investigations have linked humans as the possible source of the infection to the pigs (Hofshagen, Gjerset et al. 2009; Howden, Brockhoff et al. 2009). Experimentally, it was established that the virus is pathogenic and transmits readily in pigs (Brookes, Irvine et al. 2009; Itoh, Shinya et al. 2009; Lange, Kalthoff et al. 2009; Weingartl, Albrecht et al. 2009; Vincent, Lager et al. 2010). It also induces clinical signs of disease and respiratory tract pathology similar to other influenza A viruses of swine (Brookes, Irvine et al. 2009; Itoh, Shinya et al. 2009; Lange, Kalthoff et al. 2009; Weingartl, Albrecht et al. 2009; Vincent, Lager et al. 2010). The natural outbreaks of H1N1pdm and laboratory studies underscore the threat that the virus poses to the swine industry.

The rapid spread of the H1N1pdm virus around the globe and its ability to cross the species barrier highlight the need for developing effective control strategies. In this regard, the development of safe and potent vaccines that are effective in more than one animal species would be highly desirable. LAIVs closely mimic natural infection and have several advantages over inactivated vaccines. LAIVs trigger cell-mediated immunity and mucosal immune responses, thereby providing longer-lasting immunity and broader antigenic coverage than conventional inactivated vaccines (Cox, Brokstad et al. 2004; He, Holmes et al. 2006; Horimoto and Kawaoka 2009). In the United States, a trivalent live attenuated influenza vaccine (FluMist®) containing two type A viruses (H1N1 and H3N2) and a type B virus has been licensed for use in humans since 2003 (Ambrose, Luke et al. 2008; Ambrose, Yi et al. 2008). Moreover, LAIV human influenza vaccines have been used for many years in Russia without the emergence of new influenza virus reassortants, thus demonstrating the stability of these attenuating phenotypes (Klimov, Egorov et al. 1995; Marsh, Watson et al. 2003; Ambrose, Luke et al. 2008; Ambrose, Yi et al. 2008; Chen and Subbarao 2009). In veterinary medicine, a LAIV equine influenza virus vaccine (Flu Avert® I.N. Vaccine;

Intervet) has been developed for horses and is available commercially in North America (Paillot, Hannant et al. 2006). Field experience with both the human and the equine influenza live vaccines have demonstrated the safety, effectiveness, and the genetic and phenotypic stability of these vaccine platforms (Chambers, Holland et al. 2001; Lunn, Hussey et al. 2001; Townsend, Penner et al. 2001; Paillot, Hannant et al. 2006; Belshe, Ambrose et al. 2008; Block, Yogev et al. 2008; Chen and Subbarao 2009; Rhorer, Ambrose et al. 2009; Talaat, Karron et al. 2009).

In swine medicine, however, temperature-sensitive LAIVs have not yet been developed, despite their demonstrated safety and efficacy for other animal vaccines. More recently, live attenuated vaccines based on deletions in the NS1 viral protein have been shown to provide protection in swine (Richt, Lekcharoensuk et al. 2006; Vincent, Ma et al. 2007). Currently, commercially available swine influenza vaccines are based on inactivated whole virus preparations of the H1N1 and H3N2 subtypes. The use of vaccination has become a common practice to control swine influenza in the US. Nonetheless, these commercially available vaccines provide limited protection against the antigenically diverse influenza viruses that circulate in North American pigs, and consequently, forced swine producers to use autogenous inactivated vaccines with the hope of achieving better antigenic matching (Vincent, Lager et al. 2007). However, it is important to note that the use of inactivated vaccines has been associated with enhanced pneumonia when immunized pigs were challenged with divergent viruses (Vincent, Lager et al. 2007). Thus, the development of LAIV swine influenza vaccines has the potential to circumvent the drawbacks associated with commercial vaccines and improve both the homotypic and heterosubtypic protection in pigs against this important swine pathogen. Furthermore, the testing of newly developed LAIV influenza vaccines in swine offers the possibility of translating the findings to human medicine, given the similarity of influenza clinical signs, pathology and distribution of sialic acid receptors between the two species (Barnard 2009; Nelli, Kuchipudi et al. 2010).

With the aim of developing LAIV temperature-sensitive influenza vaccines against the H1N1pdm virus, we have used reverse genetics to introduce attenuation markers in the polymerase genes of a swine-like TR H3N2 influenza virus, A/turkey/Ohio/313053/04 (H3N2) (ty/04) (Tang, Lee et al. 2005). We chose this isolate because it grows well in both eggs and cell culture-based substrates, displays a broad host range and has internal genes similar to the H1N1pdm virus. These features highlight the potential of this backbone to induce strong immune responses in multiple animal species and possible cross-protection to circulating TR strains in swine. We found that the genetically modified ty/04 backbone (ty/04

*att*) had impaired polymerase activity and viral growth at elevated temperatures. *In vivo* characterization in mice of two H1N1 vaccine candidates generated using this backbone demonstrated that this vaccine is attenuated as indicated by the absence of any signs of disease upon vaccination, absent or limited replication in the respiratory tract, and minimum histopathological alterations in the lungs. More importantly, a single low dose of the H1N1 ty/04 *att*-based vaccines provided complete protection against a lethal H1N1pdm challenge in mice. Subsequently, we chose one of the ty/04 *att*-H1N1 vaccine candidates for further testing in swine and found that the vaccine was safe and conferred sterilizing immunity upon an aggressive intratracheal challenge of pigs with the 2009 H1N1 pandemic virus. Thus, introduction of genetic signatures for *att* in the backbone of a swine-like TR influenza virus resulted in highly attenuated and efficacious live influenza vaccines with promising applications in both human and veterinary medicine.

## *6.3. Material and Methods*

### 6.3.1. Ethics Statement

Animal studies were conducted under ABSL-3 conditions approved by USDA and performed according to protocols R-09-93 "Transmissibility of Influenza A Viruses", R-08-16 Transmissibility of Influenza A Viruses in Swine", R-09-55 "Evaluation of attenuation and protection efficiency of live attenuated influenza vaccines", and R-09-68 "Evaluation of the pathogenesis of swine-like human H1N1 and live attenuated vaccines in a mouse model" approved by the Institutional Animal Care and Use Committee of the University of Maryland. In addition, swine studies were performed under Animal Care and Use Protocol 3950 "Influenza A virus pathogenesis and host response in swine" approved by the USDA-ARS Animal Care and Use Committee, Ames, Iowa. Animal studies adhere strictly to the US Animal Welfare Act (AWA) laws and regulations.

### 6.3.2. Cell lines and virus strains

Human embryonic kidney cells (293-T) were cultured in OptiMEM I (GIBCO, Grand Island, NY) containing 5% FBS and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and antibiotics. A/turkey/Ohio/313053/04 (H3N2) (ty/04) has been previously described and it was kindly provided by Yehia Saif, Ohio State University, Wooster, OH) (Tang, Lee et al. 2005). A/California/04/09 (H1N1) (Ca/04) was kindly provided by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. The lethal mouse-adapted Ca/04 (ma-Ca/04) was generated in our laboratory by adapting the wild type Ca/04 in both DBA/2 and BALB/c mice through two serial lung passages (Ye, Sorrell et al. 2010). Recombinant viruses used in this paper were generated from cloned cDNAs and are described below and in Table 6.1. All experiments using the pandemic H1N1 or its wild type reassortants were performed under Animal Biosafety Level 3 (ABSL-3) conditions approved by the US Department of Agriculture (USDA).

**Table 6.1. Influenza viruses used in this study.**

| Viruses | Acronym in main text |
|---|---|
| A/California/04/09 (H1N1) [a] | Ca/04 |
| mouse-adapted A/California/04/09 (H1N1) | ma-Ca/04 |
| A/turkey/Ohio/313053/04 (H3N2) [c] | ty/04 WT |
| Reverse genetics A/turkey/Ohio/313053/04 (H3N2) [d] | ty/04 RG |
| A/turkey/Ohio/313053/04 (H3N2) *att* [e] | OH 2:6 ty/04 *att* |
| Surface genes from A/New York/18/09 (H1N1) in ty/04 *att* backbone [f] | NY 2:6 ty/04 *att* |
| Surface genes from A/Netherlands/602/09 (H1N1) in ty/04 *att* backbone [g] | NL 2:6 ty/04 *att* |
| Surface genes from A/Netherlands/602/09 (H1N1) in ty/04 WT backbone [h] | NL 2:6 ty/04 WT |
| A/Netherlands/602/09 (H1N1) | NL WT |

[a] Genbank taxonomy ID*2:6*41501
[b] Mouse-adapted A/California/04/09 (H1N1) that is lethal for BALB/c mice.
[c] Genbank taxonomy ID*:* 533026
[d] A/turkey/Ohio/313053/04 (H3N2) entirely generated from cloned cDNA.
[e] A/turkey/Ohio/313053/04 (H3N2) carrying temperature sensitive mutations in both PB2 and PB1 and an HA tag in the carboxyl-terminus of PB1
[f] 2:6 reassortant containing the surface genes of A/New York/18/09 (H1N1) (genbank taxonomy ID2:643545) and the internal genes from the attenuated ty/04 *att.*
[g] 2:6 reassortant containing the surface genes of A/Netherlands/602/09 (H1N1) (genbank taxonomy ID2:643212) and the internal genes from the attenuated ty/04 *att.*
[h] 2:6 reassortant containing the surface genes of A/Netherlands/602/09 (H1N1) (genbank taxonomy ID2:643212) and the internal genes from unmodified ty/04 WT.
[i] A/Netherlands/602/09 (H1N1) (genbank taxonomy ID2:643212) entirely generated from cloned cDNA.

6.3.3. Generation of recombinant viruses

The A/turkey/Ohio/313053/04 (H3N2) (ty/04) strain was generated by reverse genetics (RG) using a previously described method (Hoffmann, Neumann et al. 2000). Briefly, viral RNA was extracted from stock virus using the RNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Reverse transcription was carried out with the uni-12 primer (5′-AGCAAAAGCAAAGG-3′) and AMV reverse transcriptase (Promega, Madison, WI, USA) and the viral genes were PCR amplified using a set of universal primers (Hoffmann, Stech et al. 2001). Purified PCR products were digested with either BsaI or BsmBI and cloned into the pDP2002 vector, which is derived from the pHW-2000 vector (26) in which a spacer sequence of 444 base pairs was cloned between the two BsmBI sites to allow visual monitoring of vector digestion efficiency through agarose gel electrophoresis.The resulting clones were fully sequenced and compared to the WT viral consensus sequence. Primer sequences are available upon request. The entire plasmid set for the A/Netherlands/602/09 (H1N1) (NL WT) was kindly provided by Ron A. Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands, whereas the HA and the NA RG plasmids for the A/New York/18/09 (H1N1) (NY) were kindly provided by Ruben Donis, Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA.

The genetic signatures for attenuation of an avian influenza virus were previously described (Song, Nieto et al. 2007). Here, the same modifications were introduced into the PB2 and PB1 genes of ty/04. Briefly, the *ts* mutations in PB1 and PB2 were introduced by site-directed mutagenesis using a commercially available kit (Stratagene, La Jolla, CA), whereas the HA tag epitope was introduced by subcloning this sequence from the WF10 *att* (Song, Nieto et al. 2007) into the PB1 gene of ty/04 to generate ty/04 *att*. The recombinant WT viruses as well as the 2:6 surface gene reassortants were generated by transfecting the RG plasmids into co-cultured 293T and MDCK cells as previously reported (Hoffmann, Neumann et al. 2000). NL 2:6 ty/04 WT is a 2:6 reassortant with the surface genes from the NL virus and the internal genes from ty/04 wild type virus. NY 2:6 ty/04 *att* and NL 2:6 ty/04 *att* are surface gene reassortants between the NY and NL viruses, respectively and the ty/04 *att* internal genes. All viruses were amplified in MDCK cells to produce viral stocks.

### 6.3.4. Viral ribonucleoprotein (vRNP) reconstitution assay to study polymerase activity

A model viral RNA (vRNA), consisting of the *Gaussia* Luciferase (GLuc) open reading frame flanked by the non-coding regions of the influenza NS segment (pGLuc-NS) was used to assess polymerase activity in a minigenome reconstitution assay (Fig. 1A). Briefly, 293T cells were seeded in 6-well plates and transfected with 1 μg of the reporter plasmid along with 1 μg of each of the pDP2002 plasmids encoding PB2, PB1, PA and NP using the TransIT-LT1 (Mirus, Madison, WI) reagent following the recommendations of the manufacturer. In addition, the pCMV/SEAP plasmid, which encodes a secreted alkaline phosphatase gene, was cotransfected into the cells to normalize the transfection efficiency. At the indicated time points, supernatant from transfected cells were harvested and assayed for both luciferase and secreted alkaline phosphatase activities using the BioLux™ *Gaussia* Luciferase Assay Kit (NEB, Ipswich, MA) and the Phospha-Light™ Secreted Alkaline Phosphatase Reporter Gene Assay System (A&D, Foster City, CA) according to the manufacturers' protocol. Relative polymerase activity was calculated as the ratio of luciferase and SEAP luminescence for three independent experiments.

### 6.3.5. Replication of recombinant viruses at different temperatures

The temperature sensitive (*ts*) phenotypes of wt and *att* viruses were evaluated by titrating the stock viruses at 33, 35, 37, 39, and 41°C by the $TCID_{50}$ method in MDCK cells as previously described (Jin, Lu et al. 2003; Joseph, McAuliffe et al. 2008; Chen, Santos et al. 2009). Replicates of 10 wells were used for each dilution. Cells were incubated at the respective temperatures for 3 days and the presence of virus in each well was determined by HA assay. The *ts* phenotype of a virus was defined by a 2 $\log_{10}$ reduction in titer at 39°C compared to its titer at 33°C (Song, Nieto et al. 2007).

6.3.6. Immunization and challenge studies in mice

Seven-week-old female BALB/c mice (Charles River Laboratories, Frederick, MD) were anaesthetized with isofluorane prior to intranasal inoculation. Mice were inoculated with 50 µl $10^5$ tissue culture infectious dose 50 ($TCID_{50}$) of the recombinant viruses in PBS/mouse. Each experimental group contained 10 animals. Group 1 received PBS alone as a vaccine negative control. Groups 2, 3, 4 and 5 were inoculated with NY 2:6 ty/04 *att* ; NL 2:6 ty/04 *att*; NL 2:6 ty/04 WT; and NL WT, respectively. Groups 4 and 5 served as positive controls for the vaccine and as a reference to evaluate the attenuation of the ty/04 *att*-based vaccines. Mice were bled using the submandibular bleeding method (Golde, Gollobin et al. 2005) prior to inoculation and at days 7, 21, 28 and 42 after immunization. At 3 days post-inoculation (dpi), 3 animals from each group were humanely euthanized and tissues were harvested for virus titration and histopathologic evaluation.

Mice (n=7) were challenged at 21 dpi with 20 mouse lethal dose 50 ($MLD_{50}$) of the lethal ma-Ca/04 by the intranasal route. At 5 days post challenge (5 dpc), 3 mice from each group were euthanized and their lungs collected for histopathologic analysis and to measure levels of challenge virus. Tissue homogenates were prepared in PBS, clarified by centrifugation, and stored at -70ºC until use. Clinical signs of disease, body weight, and mortality were monitored daily in the remaining mice.

6.3.7. Attenuation phenotype and efficacy of ty/04 att-based vaccines in swine

Swine studies were conducted in the high-containment facilities of the National Animal Disease Center (NADC), Ames, IA following protocols approved by the NADC and University of Maryland Animal Care and Use Committees. In both studies, three-week old cross bred pigs were obtained from a high-health herd free of SIV and porcine reproductive and respiratory syndrome virus. All pigs were treated with ceftiofur crystalline free acid (Pfizer Animal Health, New York, NY) to reduce bacterial contaminants prior to the start of the experiment. Animals that received the inactivated Ca/04 vaccine were housed under biosafety level 2 (ABSL2) conditions and those that received the NY 2:6 ty/04 *att* vaccine or challenge Ca/04 virus were maintained under ABSL3 conditions.

6.3.8. Safety of ty/04 att-based vaccines in pigs

25 pigs were randomly divided into five treatment groups (n=5) and housed in separate isolation rooms. To further evaluate the safety of the ty/04 *att* backbone in swine, groups of pigs were intranasally inoculated with $10^5$ $TCID_{50}$/animal of either OH 2:6ty/04 *att* (a virus that carries the surface genes of ty/04 WT and ty/04 *att* internal genes) or with NY 2:6ty/04 *att* vaccines diluted in 2ml of MEM. Two other groups were similarly inoculated with ty/04 WT and ty/04 RG and served as controls for comparing the attenuation phenotype of the modified ty/04 *att* backbone, whereas a fifth group was mock-vaccinated with PBS alone. Clinical observations and rectal temperatures were recorded individually on day −2 through day 0 in order to establish normal baseline values for each animal. Nasal swabs were collected before vaccination and daily during three days as previously described (Vincent, Ciacci-Zanella et al. 2010; Vincent, Lager et al. 2010). Following inoculation, rectal temperature and clinical signs of disease were monitored daily until the terminus of the experiment (3 dpi) when the animals were humanely euthanized and necropsy was performed.

6.3.9. Efficacy of H1N1 ty/04 att vaccine in pigs

40 pigs were randomly divided in four groups of ten pigs each (Table 4). Group 1 was vaccinated with $10^5$ $TCID_{50}$/animal of NY 2:6 ty/04 *att* through intranasal route whereas group 2 was vaccinated intramuscularly with 2 ml of an adjuvanted UV-inactivated Ca/04 vaccine (UVadj-Ca/04) as previously described (Vincent, Ciacci-Zanella et al. 2010). Briefly, the inactivated experimental vaccine was prepared from the WT Ca/04 at 8 HA units per 50 µL (or $10^5$ $TCID_{50}$/ml) with inactivation by ultraviolet irradiation and addition of a commercial adjuvant (Emulsigen D, MVP Labs) at a v:v ratio of 4:1 virus to adjuvant. Group 3, non-vaccinated and challenged (NV+Ca/04), and group 4, non-vaccinated, mock-challenged (NV+Mock), were also included in the vaccine trial. At two-weeks after primary immunization, pigs were boosted using the same vaccines, dose and route as described for primary vaccination. Fourteen days post boost (dpb), pigs from groups 1-3 were challenged intratracheally with 2 ml of $1\times10^5$ $TCID_{50}$ of Ca/04 as previously described (Vincent, Ciacci-Zanella et al. 2010). Following challenge, pigs were monitored daily for hyperthermia and clinical signs of disease for five days, at which time the animals were euthanized and

necropsied. Virus shedding in nasal secretions was measured at 3 and 5 dpc. Blood samples were collected at day -3 (pre-bleed), at 14 dpv, at 0 dpc and 5 dpc for analysis of the humoral antibody response against Ca/04.

6.3.9. Pathologic examination of swine lungs after vaccination and challenge

At necropsy, lungs were removed in toto and evaluated to determine the percentage of the lung affected with purple–red, consolidated lesions that are typical of influenza virus infection in pigs. The percentage of the lung affected with pneumonia was visually estimated for each lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume as previously described (Halbur, Paul et al. 1995). Each lung was then lavaged with 50 ml MEM to obtain bronchoalveolar lavage fluid (BALF). Tissue samples from the trachea and right cardiac lung lobe and other affected lobes were taken for histopathologic examination. Lung sections were given a score from 0 to 3 to reflect the severity of bronchial epithelial injury using previously described methods (Richt, Lager et al. 2003).

6.3.10. Hemaglutination inhibiton (HI) assay

Serum samples were treated with receptor-destroying enzyme (Accurate Chemical and Scientific Corp., Westbury, NY) to remove nonspecific inhibitors and the anti-viral antibody titers were evaluated using the HI assay method outlined by the WHO Animal Influenza Training Manual (WHO/CDS/CSR/NCS/2002.5). HI assays were performed using turkey red blood cells (RBC) and NY 2:6 ty/04 att and the wild type Ca/04 as the HI antigens. $Log_2$ transformations were analyzed and reported as geometric mean of titers (GMT).

6.3.11. Quantification of antibody isotypes in porcine BALF samples

The presence of Ca/04-specific IgG and IgA antibodies in porcine BALF was detected by ELISA assays as previously described (Vincent, Ciacci-Zanella et al. 2010). Briefly, concentrated Ca/04 virus was resuspended in Tris–EDTA basic buffer, pH 7.8, and diluted to an HA concentration of 100 HA units/50 µl. Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with 100 µl of antigen solution and incubated at room temperature overnight. Plates were blocked for 1 h with 100 µl of 10% BSA in PBS and washed three times with 0.05% Tween 20 in PBS prior to addition of test BALF. The assays were performed on each BALF sample in triplicate. Antibody levels are reported as mean ODs, and the mean ODs of the different treatment groups are compared after averaging the

triplicate sample of each animal. Peroxidase-labeled goat anti-swine IgA (Bethyl, Montgomery, TX) or IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were used as secondary antibodies. Secondary antibodies were peroxidase-labeled goat anti-swine IgA (Bethyl, Montgomery, TX) or IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) Secondary antibodies were peroxidase-labeled goat anti-swine IgA (Bethyl, Montgomery, TX) or IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

6.3.12. Titration of virus stocks and virus present in biological samples

Viral stocks and virus present in animal samples (clarified tissue homogenates, nasal swabs or BALF) were titrated on MDCK cells and the TCID50/ml was determined by the method of Reed and Muench (Reed and Muench 1938). Briefly, samples were serially diluted 10-fold in serum-free media containing antibiotics and 1 µg/ml TPCK-trypsin (SIGMA, Saint Louis, MO). Next, 100 or 200 µl of the inoculum was overlaid onto confluent monolayers of MDCK cells seeded in 96-well plates. The cells with sample were incubated for 3 days and the endpoint viral titer was determined by an HA assay or immunostaining with an anti-influenza A nucleoprotein monoclonal antibody as previously described (Vincent, Ciacci-Zanella et al. 2010).

6.3.13. Histopathology

Lung and trachea samples were fixed in 10% buffered formalin and embedded in paraffin following standard histopathologic procedures. Sections of 5-µm thickness were cut from the lung and stained with hematoxylin and eosin (H&E). The degree of microscopic lesions was determined and representative pictures were taken from each slide.

6.3.14. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student t-test, whereas multiple comparisons were carried out by analysis of variance (ANOVA) using Tukey's post hoc test, unless otherwise specified. Correlations were determined with bivariate correlations procedures (Pearson's correlation procedure). The differences were considered statistically significant at $p < 0.05$.

## *6.4. Results*

### 6.4.1. Swine-like TR influenza att backbone has impaired polymerase activity at elevated temperatures

The molecular features that control the ts phenotype of the master donor virus (MDV) A/Ann Arbor/6/60 (H2N2) have been mapped to five mutations distributed in the PB2 (N265S), PB1 (K391E, E581G and A661T), and NP (D34G) segments (Jin, Lu et al. 2003). In previous studies, we showed that these genetic signatures can be transferred to an H9N2 avian influenza virus and impart the same ts phenotype. However, the ts avian influenza virus was not sufficiently attenuated in chickens, which raised potential safety concerns (Song, Nieto et al. 2007). Thus, an additional genetic modification, an HA tag epitope in the C-terminus of the PB1 gene was introduced to enhance the ts phenotype in vitro and the att phenotype in birds and mammals (Song, Nieto et al. 2007; Hickman, Hossain et al. 2008). Moreover, viruses carrying the ts loci along with the HA tag, herein named as att, were both genetically and phenotypically stable, a critical feature in the development of live att influenza vaccines.

In this study, we wanted to ascertain whether our dual attenuation approach could be introduced into the swine-like TR genetic backbone of A/turkey/Ohio/313053/04 (H3N2) (ty/04) strain. The ty/04 strain is an ideal candidate for an att vaccine backbone because the virus has been shown to replicate in several animal species such as chickens, turkeys and swine (Yassine, Al-Natour et al. 2007). We altered the PB2 and PB1 genes of ty/04 by incorporating ts mutations in both genes and also incorporated the HA tag into PB1 (Fig.6.1A and B). Additionally, the WT ty/04 PB1 contains aspartic acid at position 581, in contrast to the WT PB1 of A/Ann Arbor/6/60, which encodes glutamic acid.

The ts effect of mutations in the polymerase complex of ty/04 was analyzed at different temperatures, using a viral ribonucleoprotein (vRNP) reconstitution assay as previously described (Fig.6.1A). 293-T cells were co-transfected with plasmids encoding the ty/04 PB2, PB1, PA, and NP genes and the influenza replicon carrying a secreted luciferase (GLuc). Transfection efficiency was normalized using a plasmid encoding the secreted alkaline phosphatase (SEAP) gene. The extent of influenza polymerase activity is expressed as the relative fold change of GLuc vs. SEAP. Transfections were performed at different

temperatures (33, 35, 37, 39, and 41°C) and the supernatants were harvested 24 hours post-transfection for quantification of reporter gene expression. Introduction of ts and att mutations in the PB2 and PB1 genes of ty/04 led to significantly lower polymerase activities than that of WT genes at non-permissive temperatures (Fig.6.1C). In addition, the temperature in which the different backbones reached maximal polymerase activity, as indicated by luciferase activity also differed among the different constructs. The highest polymerase activity of the ty/04 att was detected at 35°C, whereas the peak for the ty/04 WT was at 39°C and the ts was between 35-37°C (Fig.6.1C). More importantly, introduction of the HA epitope in PB1 had a combined deleterious effect in combination with the four ts mutations in PB1 and PB2 since the att backbone had the most marked reduction in polymerase activity at temperatures above 37°C. Taken together, these results indicate that the ts phenotype displayed by the modified human and avian influenza strains can be transferred to a swine-like TR backbone and that inclusion of an HA epitope in PB1 enhances this phenomenon in human cells.

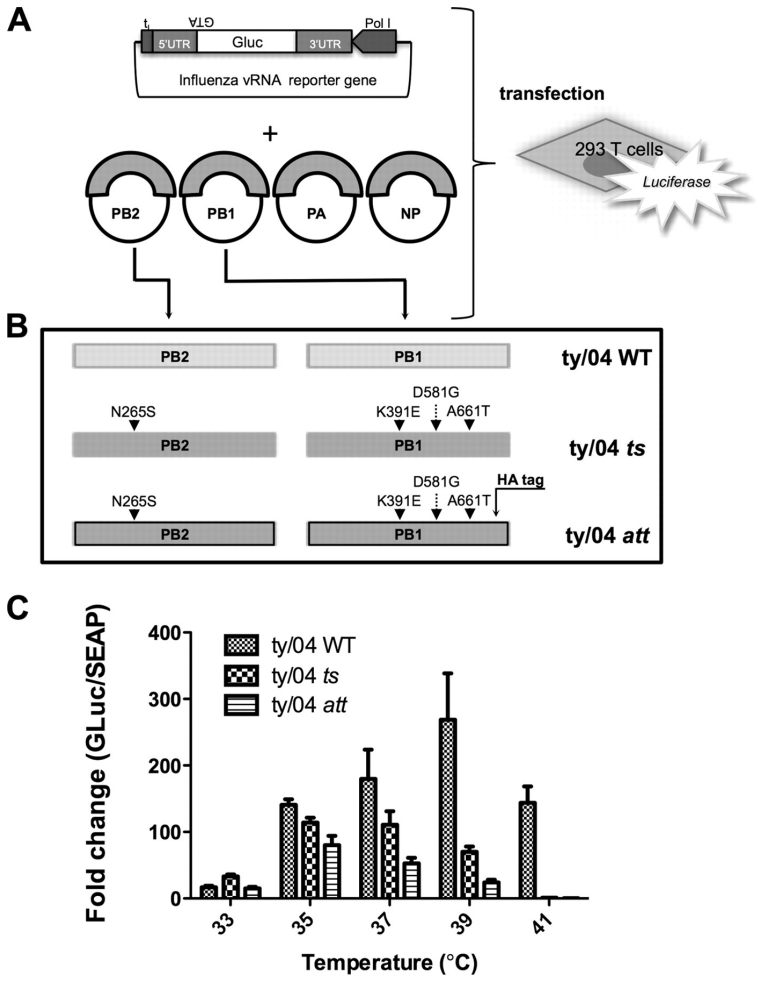



**Figure 6.1.Viral ribonucleoprotein reconstitution assay to study the polymerase activities of ty/04 WT and its mutants.** (A) 293-T cells were transfected with plasmids encoding the minimal components required for viral transcription and replication (the PB1, PB2, and PA polymerase subunits, NP, and a vRNA influenza virus-driven luciferase reporter replicon). The influenza virus reporter plasmid contains the open reading frame of Gaussia luciferase (GLuc) flanked by the 5' and 3' untranslated regions (UTR) of the influenza virus NS segment. This cassette was inserted between the human polymerase I promoter (Pol I) and terminator (tI) sequences. The presence of luciferase protein in the supernatant is measured by a luciferase assay. (B) Schematic representation of the ty/04 PB2 and PB1 constructs for the generation of ts and att mutant viruses. Site-directed mutagenesis was used to introduce 1 temperature-sensitive (ts) mutation into the PB2 gene (N265S) and 3 ts mutations into the PB1 gene (K391E, D581G, and A661T) of A/turkey/Ohio/313053/04 (H3N2) to generate ty/04 ts. The PB1 gene was further modified by incorporating an HA tag into the carboxy terminus of PB1 to generate ty/04 att. (C) Polymerase activities of WT ty/04 and its mutants at different temperatures. 293-T cells were transfected with 1 μg each of the influenza virus-driven luciferase reporter plasmid and the PB2, PB1, PA, and NP plasmids and were incubated at different temperatures as shown. pCMV/SEAP, which encodes secreted alkaline phosphatase, was cotransfected into the cells to normalize the transfection efficiency. At 24 h

posttransfection, the supernatant was harvested and assayed for both luciferase and phosphatase activities. Normalized polymerase activities (means ± standard errors) were determined from three independent experiments.

### 6.4.2. Genetically modified ty/04 att vaccines display restricted growth at non-permissive temperatures

In order to study the effects of the att mutations in the context of the viral life cycle, we compared the replication of WT and att H1N1pdm viruses in MDCK cells at different temperatures. Four different 2:6 reassortant viruses were tested: NY 2:6 ty/04 att, NL 2:6 ty/04 att, NL 2:6 ty/04 WT, and NL WT (Table 2). The NL 2:6 ty/04 WT and NL WT viruses replicated equally well at temperatures ranging from 33° to 39°C (Table 6.2). In agreement with the minigenome assay, the two H1N1 ty/04 att viruses had at least a 100-fold reduction in virus titers at 39°C compared to 33°C, confirming the ts phenotype of this genetically modified vaccine backbone. In addition, the ty/04 att viruses were unable to replicate at 41°C in contrast to the NL 2:6 ty/04 WT, and NL WT which showed significant growth at 41ºC (about 4-5 log10 lower than at 33ºC). Thus, the two H1N1 ty/04 att-vaccines displayed the expected ts phenotypes in a canine cell line.

**Table 6.2. Replication of recombinant viruses at different temperatures.**

| Viruses | 33°C | 35°C | 37°C | 39°C | 41°C |
|---|---|---|---|---|---|
| NY 2:6 ty/04 *att* | 6.0 | 6.0 | 5.0 | **4.0** | BLD |
| NL 2:6 ty/04 *att* | 6.0 | 6.0 | 6.0 | **4.0** | BLD |
| NL 2:6 ty/04 WT | 7.8 | 8.3 | 8.0 | 7.8 | 4.2 |
| NL WT | 7.1 | 6.7 | 6.2 | 6.5 | 2.1 |

MDCK cells were inoculated with WT or att viruses and incubated for 3 days at increasing temperatures (33, 35, 37, 39, and 41°C). The presence of the virus in each well was detected by an HA assay and the log10 titer was determined by TCID50. Shown are representative data of two independent experiments. Values in bold indicate that the virus is temperature sensitive (100-fold

reduction in virus titer at 39°C as compared to 33°C). BLD: below the detection limit (0.6 log10 TCID50/ml).

### 6.4.3. Genetically modified ty/04 att vaccines are attenuated in mice

To evaluate whether the reduction in polymerase activity and virus replication at non-permissive temperatures by the ty/04 att-based viruses would translate into an attenuated phenotype in vivo, we assessed weight loss, the levels of viral replication in the respiratory tract and the extent of pulmonary pathology in BALB/c mice inoculated with these viruses. Groups of ten mice were inoculated with 105 TCID50 of the recombinant viruses (Fig 6.2) through the intranasal route. At 3 dpi, 3 animals from each group were humanely euthanized and organs were collected for virus titration and histopathology. Body weight changes during 12 dpi were monitored daily as a parameter of influenza morbidity. The 2 ty/04 att vaccines were highly attenuated in mice as demonstrated by the absence of clinical disease signs and no changes in body weight. Conversely, mice inoculated with the WT ty/04 backbone (NL 2:6 ty/04 WT) showed significant body weight loss (≤20%) by 7 dpi, although they eventually recovered. As expected, noticeable reduction in body weight was also observed when mice received the NL WT, and recovery was slightly delayed in comparison to the NL 2:6 ty/04 WT group (Fig.2 A). In examining tissue tropism and viral replication, the NY 2:6 ty/04 att virus was not detected in any of the organs sampled (nasal cavity, trachea, and lungs). Lung sections from the NY 2:6 ty/04 att group were indistinguishable from the mock-inoculated controls at the microscopic level, corroborating that the ty/04 att LAIV is highly attenuated (Fig. 6.2C, D). Although mice inoculated with NL 2:6 ty/04 att showed no signs of disease upon vaccination, there was limited viral replication in the lower respiratory tract of these animals. However, replication in the trachea was detected only in a single individual (17 $TCID_{50}$/g of tissue) and the titers in the lungs were significantly lower than from those obtained in the NL 2:6 ty/04 WT group (Fig. 6.2B). Only mild histopathological alterations were detected in the lungs of a single mouse inoculated with the NL 2:6 ty/04 att virus, suggesting that pulmonary replication of this att vaccine virus does not lead to significant pathological damage in the lungs (Fig 6.2B, E). In contrast, mice inoculated with NL 2:6 ty/04 WT had high viral replication ($10^4$ to $10^6$ $TCID_{50}$) in the entire respiratory tract and moderate histopathological alterations in the lungs characterized by necrotizing bronchitis, bronchiolitis and varying degrees of hemorrhage (Fig. 6.2B, F). Infection with NL WT resulted in 10 to 100-fold higher viral titers in the respiratory tract ($10^5$ to $10^7$ $TCID_{50}$) compared to the NL 2:6 ty/04 WT group (Fig 6.2B). Consistently, the NL WT-inoculated

group showed more severe pathology in the lungs as compared to NL 2:6 ty/04 WT (Fig. 6.2G), suggesting that the internal genes from the H1N1pdm are more virulent in mice than the internal genes from the North American TR viruses. Together, the double attenuation strategy resulted in a swine-like TR backbone that displayed desirable safety properties in the mouse influenza model.

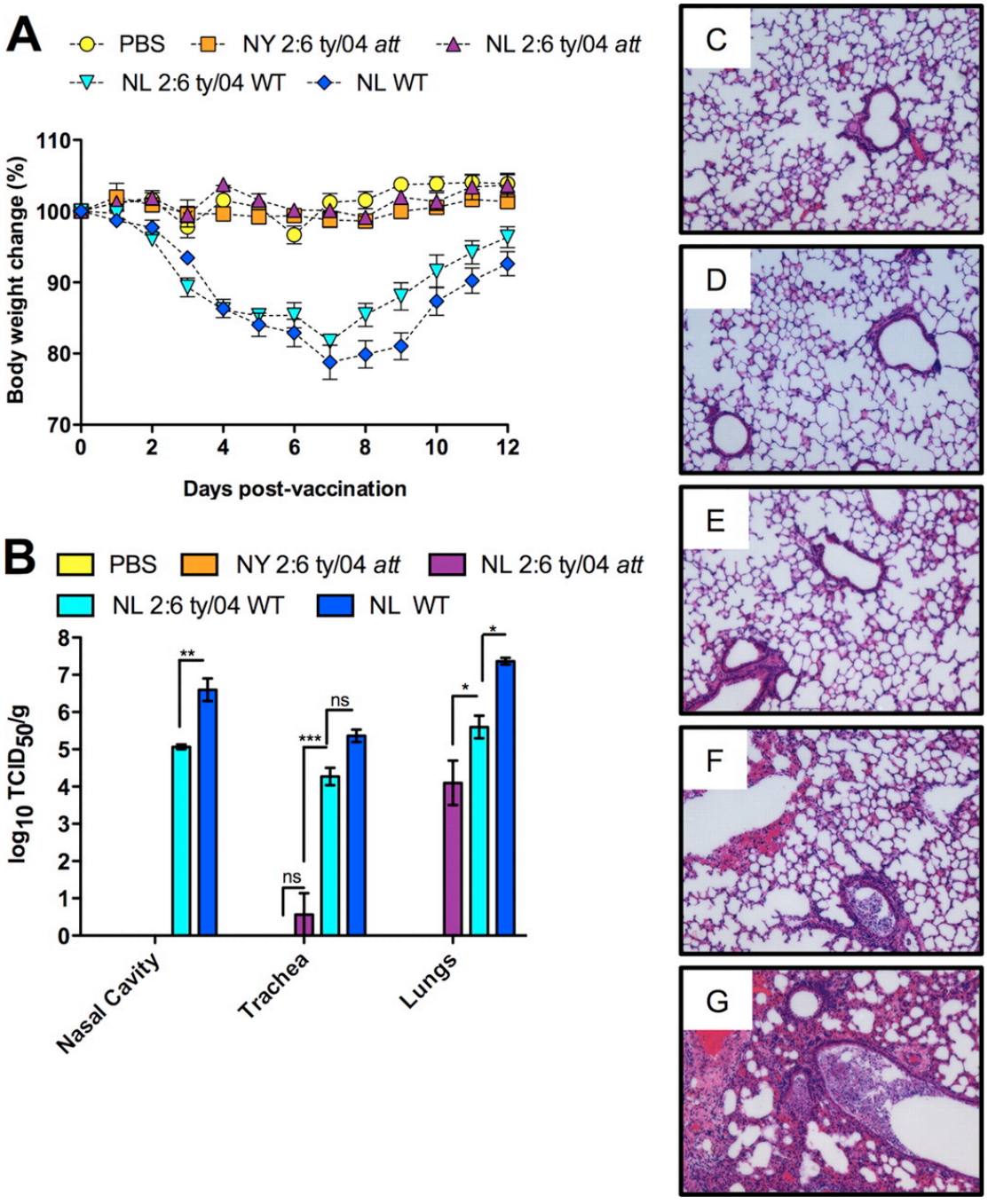


**Figure 6.2.Safety of ty/04 *att* H1N1 vaccines in BALB/c mice.** Groups of 10 mice each were inoculated intranasally with $10^5$ $TCID_{50}$s of the recombinant virus. One group received PBS and served as a vaccine negative control. (A) Percentage of change in body weight as a measure of morbidity following intranasal inoculation of mice with either a ty/04 WT or a ty/04 *att* virus. Mice were weighed daily for 12 days. (B) Replication and tissue tropism of WT and *att* H1N1 viruses in the respiratory tracts of mice. At 3 dpi, three animals from each group were euthanized, and virus titers in the respiratory tract were determined by standard $TCID_{50}$ in MDCK cells. $Log_{10}$ titers for each group are plotted as means ± standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. (C, D, E, F, and G) Lung histopathology of mice inoculated with ty/04 WT or ty/04 *att* viruses at 3 dpi. A representative image from each treatment group is shown. (C) Lung of a control mouse showing normal bronchiole and alveoli. (D and E) Lungs from animals inoculated with NY 2:6 ty/04 *att* or NL 2:6 ty/04 *att*, respectively, are indistinguishable from those of mock-infected animals. (F) Lung of a mouse infected with the NL 2:6 ty/04 WT virus, showing moderate histopathological lesions, including necrotizing bronchiolitis, the presence of cell debris, and various degrees of hemorrhage. (G) Lung of a mouse infected with NL WT, displaying more-severe histopathology characterized by necrotizing bronchiolitis, the presence of cell debris and inflammatory infiltrates, hyperemia, and hemorrhage.

### 6.4.4. The ty/04 att-based vaccines are attenuated in swine

In order to further evaluate the safety of the ty/04 att vaccine backbone, groups of pigs (n=5) were inoculated intranasally with ty/04 att vaccines containing either H1N1 or H3N2 surfaces. The H1N1 surface genes were derived from the A/New York/18/09 (H1N1), whereas the H3N2 genes were from the A/turkey/Ohio/313053/04 (H3N2) isolate (Table 6.1). Ty/04 WT and ty/04 RG viruses were included as non-attenuated virus controls. Clinical signs of disease and rectal temperatures were monitored and nasal swabs were collected daily to quantify virus shedding in nasal secretions. At 3 dpi, pigs were euthanized, the degree of lung pathology was determined and the presence of virus in BALF samples titrated. Pigs inoculated with non-attenuated ty/04 viruses developed a febrile response (> 40° C) that peaked 24 hpi (Fig. 6.3A) and shed large amounts of virus (3-5 $log_{10}$ $TCID_{50}$/ml) in nasal secretions (Fig. 6.3B). Similarly, viral titers in BALF collected at 3 dpi ranged from $10^5$ to $10^6$ $TCID_{50}$/ml (Fig. 6.3C). At necropsy, the lungs from animals inoculated with these viruses had up to 15% of the total lung area (mean % pneumonia of 7.5 and 7.4 for ty/04 WT and ty/04 RG, respectively) displaying pneumonic lesions typical of influenza infection in pigs (Fig. 6.3D). In general, the gross lesions were marked, plum-colored consolidated areas located mainly in the cranioventral lobes of the lung. Microscopically, lesions in lungs were typical of influenza virus infection in pigs and were characterized by multifocal to widespread necrotizing bronchitis and bronchiolitis, light peribronchiolar lymphocytic

cuffing, and mild multifocal interstitial pneumonia and varying degrees of alveolar involvement (Fig. 6.3E). Clinical signs of disease, lung pathology and viral replication in biological samples were indistinguishable between the Ty/04 WT and RG viruses, confirming that the ty/04 generated through RG displays a phenotype identical to WT virus. In contrast, none of the animals inoculated with H3N2 or H1N1 ty/04 att viruses developed fever, cough or other clinical signs following vaccination, indicating that the ty/04 att viruses were safe for administration to pigs (Fig. 6.3A). Correspondingly, there was 100-1,000 fold less virus shedding from the nose of pigs vaccinated with ty/04 att viruses as compared to unmodified ty/04 viruses. In general, NY 2:6 ty/04 att–vaccinated pigs shed less virus than H3N2:6 ty/04 att inoculated pigs (Fig. 6.3B). In addition, viral titers in BALF were significantly reduced ($p < 0.01$) in ty/04 att-vaccinated pigs as compared to ty/04 WT-infected pigs (Fig. 6.3C). Although both vaccines caused mild gross and microscopic lesions in the lungs, the percentage of lung involvement was not significantly different from mock-vaccinated pigs ($p > 0.05$, Dunnett's test), corroborating the clinical findings that these vaccines are sufficiently attenuated in pigs (Fig. 6.3D, E). Histopathologically, nasal turbinates and trachea obtained from pigs immunized with either vaccine were similar to control animals, as opposed to the WT-inoculated pigs (Fig. 6.3E). Thus, modified ty/04 viruses displayed att phenotype in swine, in agreement with the mice studies.

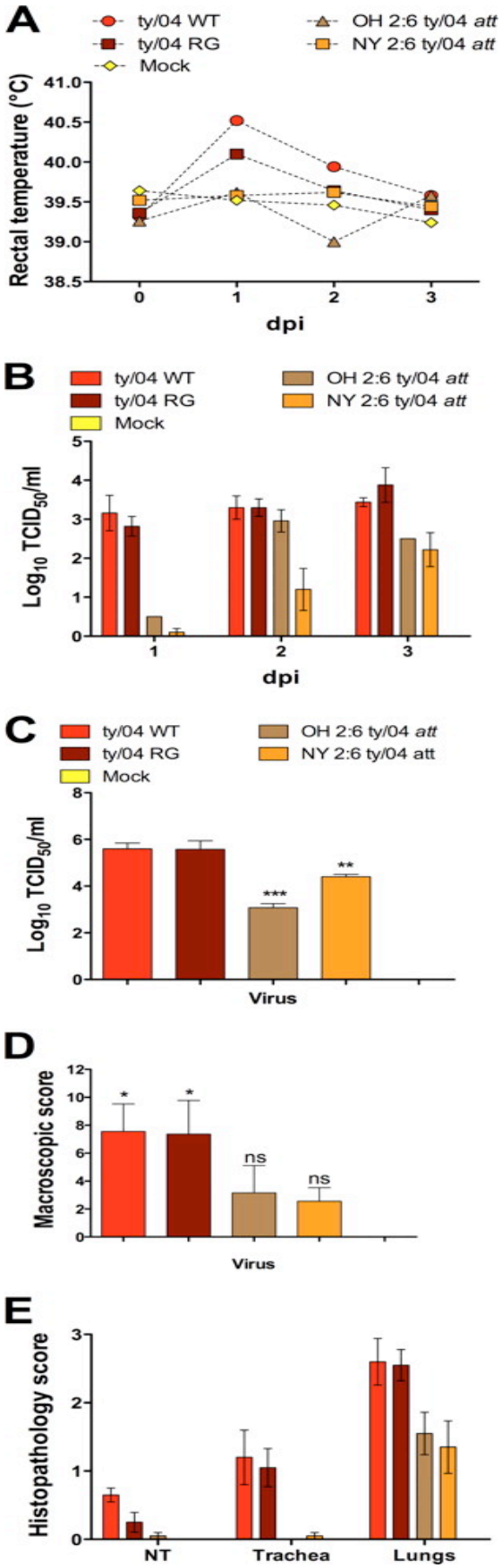

A
ty/04 WT
ty/04 RG
Mock
OH 2:6 ty/04 att
NY 2:6 ty/04 att
Rectal temperature (°C)
41.0
40.5
40.0
39.5
39.0
38.5
0
1
2
3
dpi
B
ty/04 WT
ty/04 RG
Mock
OH 2:6 ty/04 att
NY 2:6 ty/04 att
Log10 TCID50/ml
5
4
3
2
1
0
1
2
3
dpi
C
ty/04 WT
ty/04 RG
Mock
OH 2:6 ty/04 att
NY 2:6 ty/04 att
Log10 TCID50/ml
8
6
4
2
0
***
**
Virus
D
Macroscopic score
12
10
8
6
4
2
0
*
*
ns
ns
Virus
E
Histopathology score
3
2
1
0
NT
Trachea
Lungs

**Figure 6.3. Attenuation phenotype of H1N1 and H3N2 ty/04 *att* viruses in swine.** Groups of pigs (n=5) were randomly divided into five treatment groups and inoculated intranasally with $10^5$ $TCID_{50}$/animal of either H3N2:6ty/04 *att* or with NY 2:6 ty/04 *att* vaccines diluted in 2 ml of MEM. Two other groups were similarly inoculated with either ty/04 WT or with a WT virus generated from plasmid DNA (ty/04 RG) to compare the attenuation phenotype of the modified ty/04 *att* backbone. A fifth group was mock vaccinated with PBS alone (Mock). Nasal swabs and rectal temperature readings were taken daily. At 3 dpi, all animals were euthanized to measure viral replication and lung pathology. (A) Daily mean rectal temperatures of pigs following inoculation with WT or ty/04 *att* viruses. (B) Viral shedding in nasal secretions of pigs. (C) Viral titers in bronchoalveolar lavage fluid (BALF) collected at necropsy. Viral titers in nasal swabs and BALF were determined by $TCID_{50}$ on MDCK cells. Values are shown as the mean ± SEM. * $p< 0.05$ ; ** $p< 0.01$; *** $p< 0.001$ as compared to ty/04 WT control by Dunnett's test. (D) Percentage of macroscopic lung lesions and (E) Histopathologic scores of nasal turbinates, trachea and lungs of swine inoculated with WT or ty/04 *att* viruses at 3 dpi. Lesions were compared to sham inoculated group by Dunnett's test. * $p< 0.05$; n.s. not significant**.**

6.4.5. Vaccination with H1N1 ty/04 att-based vaccines confers complete protection in mice against a lethal H1N1pdm challenge

Recent pathology studies have shown that H1N1pdm infections can lead to fatal viral pneumonia in a small percentage of cases (Perez-Padilla, de la Rosa-Zamboni et al. 2009; Soto-Abraham, Soriano-Rosas et al. 2009; Gill, Sheng et al. 2010). Thus, we have developed a lethal mouse model of H1N1pdm (Ye, Sorrell et al. 2010) that recapitulates the severe viral pneumonia induced by the novel H1N1 in human patients. Hence, we used this lethal model of H1N1 severe pneumonia to test whether the ty/04 att vaccines can induce protective immunity against lethal H1N1pdm infection. To determine the robustness of protection induced by the two H1N1 ty/04 att vaccines, mice were intranasally challenged with 20 $MLD_{50}$ of the lethal mouse-adapted Ca/04 at 21 dpv. Groups of mice immunized with WT H1N1 viruses (NL WT and NL 2:6 ty/04 WT) were also included as a positive control for the vaccine. Again, body weights were measured prior to challenge infection and daily after challenge. At 5 dpc, the lungs from three animals of each group were collected for histopathology and virus titration. All the H1N1 ty/04 att-vaccinated animals were completely protected against the lethal ma-Ca/04 infection as demonstrated by the absence of clinical signs of disease and no changes in body weight after challenge (Fig. 4A, B). More importantly, mice vaccinated with NL 2:6 ty/04 att had neither lung pathology nor detectable replication of the challenge virus (Fig. 6.4A, B, C, D). The group that received the NY 2:6 ty/04 att vaccine showed a significant reduction in the level of challenge virus isolated from lungs by 5 dpc in contrast to the mock-vaccinated mice (Fig. 6.4C). Additionally, pulmonary histopathology was detected only in one individual in this group, which agreed with the clinical findings (Fig 4E). As expected, the groups that were immunized with the WT H1N1 viruses were completely protected from challenge (Fig 6.4A, B, C) and had neither viral replication nor pathological alterations in the lungs (Fig. 6.4F, G). In contrast, all unvaccinated control mice developed severe signs of disease such as lethargy, rough coat, dehydration, and up to 25 % of body weight loss. The control mice died within 5-6 dpc (Fig. 6.4A, B). Examination of the lungs of the mock-immunized group revealed severe pneumonia characterized by extensive hemorrhagic lesions, necrotizing bronchitis and bronchiolitis, interstitial edema, and the presence of cell debris and inflammatory infiltrates in the alveoli (Fig. 6.4H). Infection by the ma-Ca/04 caused much more severe bronchopneumonia as compared with the lesions induced by the NL WT or the NL 2:6 ty/04 WT upon immunization (compare Figs. 6.4H with 6.2G).

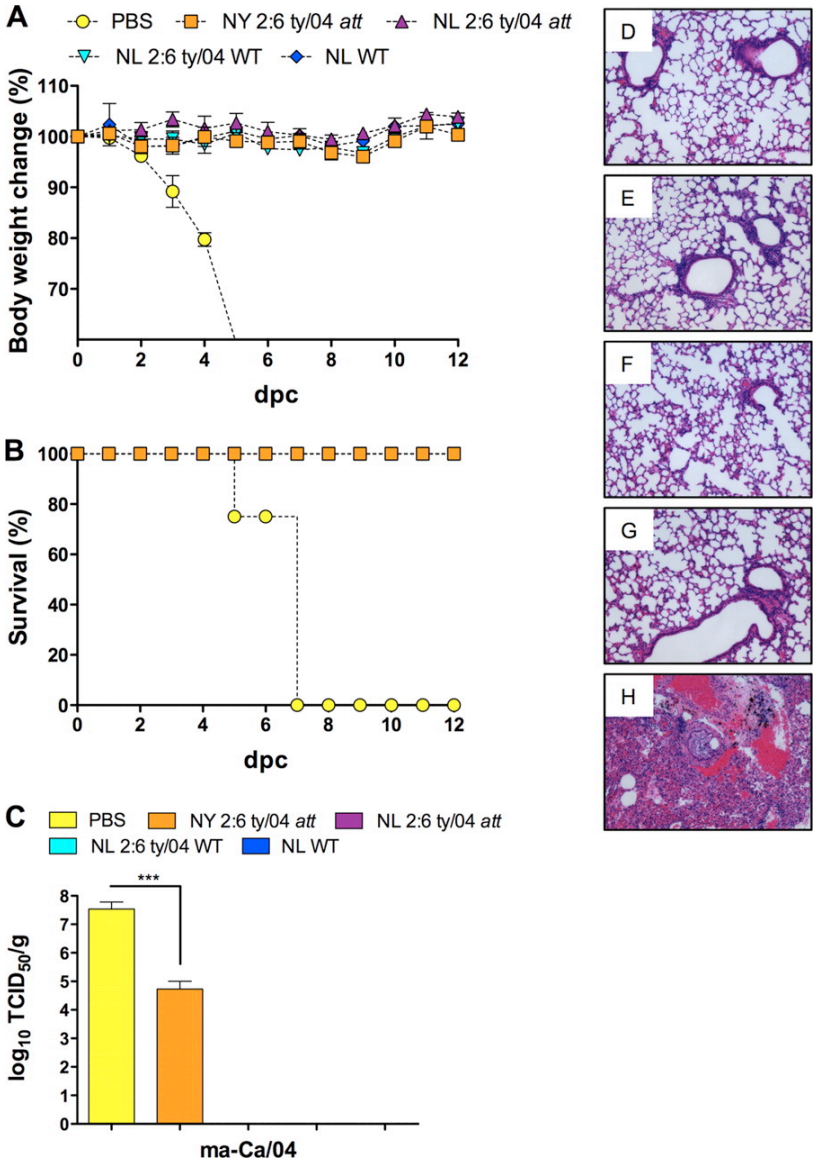



**Figure 6.4. Protective efficacy of ty/04 *att* H1N1 vaccines against lethal H1N1pdm challenge in mice.** Groups of mice (n=7) were vaccinated with H1N1 recombinant viruses containing either *att* or WT backbones. A control group was mock vaccinated with PBS. All animals were challenge 21 days later with 20 $MLD_{50}$ of the lethal mouse-adapted Ca/04 by the intranasal route. At 5 dpc, the lungs from three animals were collected and processed for virus titration and histopathology. (A) Percent body weight change of naïve and vaccinated mice after lethal challenge. (B) Survival rates of naïve and vaccinated mice after lethal challenge. (C) Pulmonary replication of the challenge virus in the lungs of mice. Values are mean ± SEM. *** p< 0.001 between PBS and NY 2:6 ty/04 *att.* (D, E, F, G, and H) Lung pathology following a lethal ma-Ca/04 challenge. Immunized animals showed no significant histopathological changes. D) Mice immunized with NL 2:6 ty/04 *att*. E) Mice immunized with NY 2:6 ty/04 *att*. F) Mice immunized with NL 2:6 ty/04WT. G) Mice immunized with NL WT. H) Mock vaccinated mice had severe bronchopneumonia after lethal challenge that was characterized by extensive hemorrhagic lesions, necrotizing bronchitis and bronchiolitis,

interstitial edema, and the presence of cell debris and inflammatory infiltrates in the alveoli and airways.

In order to evaluate the immune responses induced by the ty/04 att vaccines that protected mice against lethal H1N1pdm challenge, we measured the levels of anti-Ca/04 antibodies in the sera of immunized mice using a HI assay (Table 6.3). In general, the protection rate correlated with the level of the HI antibody titer measured on the day of challenge (21 dpv). At 0 dpc, the geometric mean titer (GMT) against Ca/04 was slightly lower in the NY 2:6 ty/04 att vaccine as compared to the NL 2:6 ty/04 att group, though the difference was not statistically significant. As expected, the highest GMT was induced by the non-attenuated viruses NL 2:6 ty/04 WT and NL WT. Mock-vaccinated animals had undetectable HI antibodies. We also found a positive correlation (Pearson's correlation, $r2=0.82$, $p<0.05$) between the invasiveness of the vaccines/viruses, i.e. the ability of the H1N1 ty/04 att or WT viruses to replicate in the respiratory tract, and the levels of circulating HI antibodies. The least invasive vaccine, NY 2:6 ty/04 att, induced lower HI antibodies as compared with the more invasive NL 2:6 ty/04 att vaccine. The highest HI titer was elicited by the NL WT. Based on this correlation analysis, 82% of the variance in HI titers at 21 dpv could be explained by variation in the ability of the viruses to replicate in the lungs. Collectively, these results suggested that a single low dose inoculation of the ty/04 att LAIV could prevent histopathological alterations in the lungs and provide complete protection against lethal pandemic H1N1-induced pneumonia.

**Table 6.3. Immunogenicity of the ty/04 *att* H1N1 vaccines in mice.**

| Groups | 0 dpv | 21 dpv | 21 dpc |
|---|---|---|---|
| PBS | <10 | <10 | N/A |
| NY 2:6 ty/04 *att* | <10 | 25 | 1076 |
| NL 2:6 ty/04 *att* | <10 | 89 | 1810 |
| NL 2:6 ty/04 WT | <10 | 718 | 2560 |
| NL WT | <10 | 1016 | 2560 |

Hemagglutination inhibiton (HI) titers are given as the reciprocal of the highest dilution of serum that showed activity against the A/CA/04/09(H1N1). Values are given as the geometric mean titer (GMT. N/A: not applicable-animals were dead at this time point.

### 6.4.6. Vaccination with H1N1 ty/04 att-based vaccines provides sterilizing immunity against an aggressive H1N1pdm challenge in pigs

The NY 2:6 ty/04 att was selected for a vaccination/challenge trial in swine because it was the most attenuated vaccine in mice and had limited shedding in pigs after vaccination. Because only inactivated influenza vaccines are currently licensed for use in the U.S. swine industry, we also compared side-by-side the efficacy of the NY 2:6 ty/04 att with an experimental Ca/04 inactivated adjuvanted vaccine (UVAdj-Ca/04). Thus, at two weeks after boost immunization, pigs were challenged with $10^5$ $TCID_{50}$ of the virulent Ca/04 by intratracheal inoculation and followed for 5 days. The clinical performance in pigs of the H1N1 vaccines is summarized in Table 4. Non-vaccinated and challenged (NV+Ca/04) animals developed clinical signs of disease after challenge and typical macroscopic lung lesions of influenza infection by 5 dpc. In addition, all 10 pigs from this group shed virus in the nose at both 3 and 5 dpc and replicated virus in BALF at 5 dpc. Two doses of the UVAdj-Ca/04 vaccine provided satisfactory protection as determined by the reduced lung pathology and limited challenge virus replication in both the upper and lower respiratory tract.

However, 2 pigs in this group did shed virus in the nose at day 5 and 1 animal had detectable virus in BALF. Remarkably, none of the animals vaccinated with NY 2:6 ty/04 att had detectable infectious virus in nasal secretions or in the lungs, suggesting that replication of the challenge virus was abolished at the site of infection. Macroscopically, discrete lung lesions were detected in 50% of the animals immunized with NY 2:6 ty/04 att vaccine, however the percentage was lower than the group immunized with the inactivated vaccine in which 90% of the animals developed limited gross pulmonary pathology upon challenge. Nevertheless, in both vaccine groups there was a significant reduction ($p<0.001$) in the percentage of macroscopic lung pathology compared to the NV+Ca/04 group. Microscopic changes associated with influenza infection in the lungs were reflective of the macroscopic pneumonia. As expected, non-vaccinated, mock-challenged (NV+Mock) controls remained healthy throughout the study and had neither significant macroscopic nor microscopic lesions in the lungs.

Serum samples were also collected at the day of challenge and Ca/04-specific antibodies were examined by an HI assay. All pigs were negative for antibodies (HI titer <10) at the start of the experiment. At the day of challenge, 8 out of 10 pigs had detectable HI antibodies against Ca/04 in the inactivated vaccine group, whereas 7 out of 10 animals were HI positive in NY 2:6 ty/04 att group. The mean HI antibody titers measured at the day of challenge in both vaccine groups were approximately the same (Table 6.4).

**Table 6.4. Clinical performance of H1N1 ty/04 *att*-based vaccine in swine after challenge with pandemic H1N1**

| Group | HI titer [a] | Macroscopic pneumonia [b] | Microscopic pneumonia (0-3) [c] | Nasal swabs [d] | | BALF 5 dpc [e] |
|---|---|---|---|---|---|---|
| | | | | 3 dpc | 5 dpc | |
| NY 2:6 ty/04 *att* | 16.2 (7/10) | 0.3 ± 0.1* | 0.5 ± 0.1* | 0 ± 0 * | 0 ± 0* | 0 ± 0* |
| UVAdj-Ca/04 | 20.0 (8/10) | 1.2 ± 0.6* | 0.3 ± 0.2* | 0 ± 0 * | 0.4 ± 0* | 0.3 ± 0.3* |
| NV+Ca/04 | <10 (0/10) | 5.5 ± 1.3 | 1.5 ± 0.4 | 2.2 ± 0.4 | 2.8 ± 0.2 | 3.9 ± 0.2 |
| NV+Mock | <10 (0/10) | 0.2 ± 0.1* | 0 ± 0* | 0 ± 0* | 0 ± 0* | 0 ± 0* |

NV+Ca/04, non-vaccinated, challenged positive control group; NV+Mock, non-vaccinated, non-challenged negative control group.
[a] Geometric mean HI titer against Ca/04 at the day of challenge. Numbers within parenthesis represents the number of animals that seroconverted/total animals per group.
[b] Percentage of macroscopic lung lesions given as mean score ± SEM.
[c] Microscopic pneumonia scores given as mean score ± SEM.

[d] Average viral titer ($log_{10}$) measure as $TCID_{50}$ per ml of fluid present in nasal swabs collected at 3 and 5 days post-challenge (dpc)
[e] Average viral titer ($log_{10}$) measure as $TCID_{50}$ per ml of bronchoalveolar lavage fluid (BALF) collected at 5 days post-challenge (dpc).
* Significantly different from NV+Ca/04 control group at $P < 0.05$.

To characterize the immunoglobulins present in the lower respiratory tract of swine, total levels of Ca/04-specific IgG or IgA isotype antibodies were measured by ELISA in BALF samples collected at necropsy (5 dpi). The data presented in Fig. 6.5 shows the mean of the isotype response in each group. Levels of Ca/04-specific IgG were similar between the two vaccinated groups (Fig. 6.5A). In contrast, IgA levels in BALF from pigs in the NY 2:6 ty/04 att group were significantly higher than in the inactivated vaccine group (Fig. 6.5B). Taken together, these results suggest that NY 2:6 ty/04 att vaccine is immunogenic in pigs and provides sterilizing immunity against pandemic H1N1 challenge.

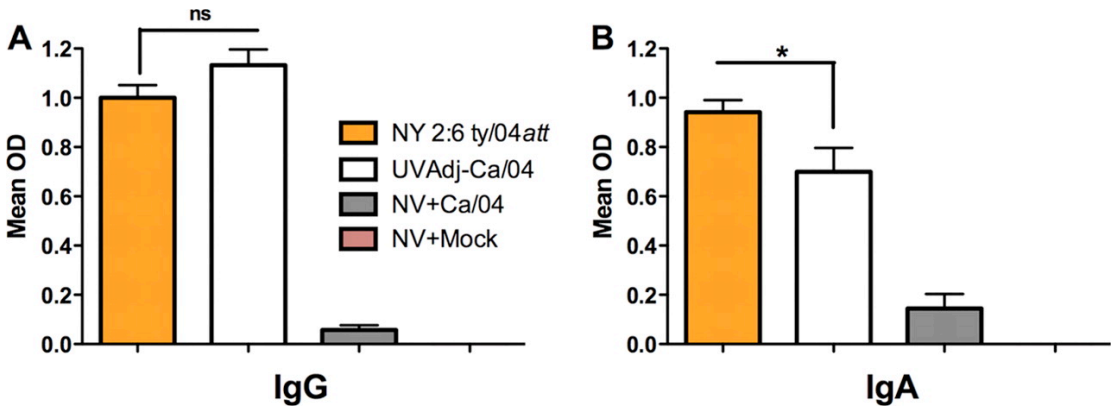



**Figure 6.5. Antibody isotype profiles in BALF samples of vaccinated pigs after challenge with H1N1pdm.** The levels of Ca/04-speficic IgG (A) and IgA (B) antibodies present in BALF samples were quantified by ELISA. Groups of pigs (n=10) were vaccinated twice with either the NY 2:6 ty/04 *att* or with an inactivated Ca/04 vaccine, challenged with H1N1pdm at 2 weeks post-boost and necropsied at 5 dpi when BALF was collected. Non-vaccinated pigs challenged with the Ca/04 virus (NV+Ca/04) and non-vaccinated mock challenge pigs (NV+Mock) were included as controls. Results are given as the mean OD ±standard error of the mean. * $p < 0.05$ between the NY 2:6 ty/04 *att* and UVAdj-Ca/04 vaccine by Student t-test.

## *6.5.Discussion*

The 2009 H1N1 pandemic caused by a triple reassortant influenza virus has spread worldwide and caused profound economic, health, and social impacts. This virus is the predominant circulating influenza virus among people in both northern and southern hemispheres (Al Hajjar and McIntosh 2010) and has the ability to infect pigs experimentally and under natural conditions (Brookes, Irvine et al. 2009; Howden, Brockhoff et al. 2009; Lange, Kalthoff et al. 2009; Weingartl, Albrecht et al. 2009; Vincent, Lager et al. 2010). Therefore, the development of alternative influenza vaccines for use in humans and animals would play an important role in mitigating the effects of the evolving H1N1 pandemic. Inactivated swine influenza vaccines are available for pigs; however, they do not provide either adequate immunity or cross-protection (Vincent, Lager et al. 2008). This poor cross-protection associated with the use of inactivated vaccines was highlighted in a recent study, which reported only limited serologic cross-reactivity between sera from pigs vaccinated with contemporary H1 swine influenza viruses and the pandemic H1N1 2009 isolates (Vincent, Ciacci-Zanella et al. 2010). These findings are of major significance for the current scenario, given the possibility that 2009 H1N1 viruses could become established in swine. In the present study, we addressed this issue and developed temperature-sensitive LAIV Influenza vaccines for use in pigs. Moreover, we used mouse and swine models to test the efficacy of our vaccine platform in protecting against severe H1N1pdm-induced pneumonia that has been reported in some human fatalities (Perez-Padilla, de la Rosa-Zamboni et al. 2009; Soto-Abraham, Soriano-Rosas et al. 2009; Gill, Sheng et al. 2010).

In this study, we have used reverse genetics and molecular biology techniques to modify the polymerase genes of a swine-like TR influenza virus with the goal of developing alternative influenza vaccines for use in more than one animal species. We have previously shown that attenuation of an avian influenza strain required both the ts mutations found on the PB2 and PB1 genes of the A/Ann Arbor/6/60 (H2N2) human strain and incorporation of an HA tag epitope in the carboxy-terminus of PB1 (Song, Nieto et al. 2007). Here, we studied the effects of the ts and att (ts+ HA tag) mutations in the polymerase activity of a triple reassortant influenza virus whose polymerase genes are from avian (PB2 and PA), human (PB1) and swine (NP) origin. We demonstrated that introduction of ts mutations into the PB2 and PB1 subunits of a TR influenza virus severely impaired polymerase activity in human cells (293T) at non-permissive temperatures. This effect on polymerase function was

enhanced by the covalent attachment of an HA epitope in PB1. These results are in agreement with our previous studies in which incorporation of an HA tag in PB1 synergistically enhances the attenuating effects of the ts mutations in the context of an avian influenza strain (Song, Nieto et al. 2007; Hickman, Hossain et al. 2008). It is unclear how the ts loci combined with the HA tag modulates polymerase activity in elevated temperatures. In addition, the mechanisms responsible for the restricted replication of the ts influenza viruses at the non-permissive temperature have not been completely elucidated. A recently proposed mechanism to explain the ts phenotype involves multiple defects in replication of the MDV A/Ann Arbor/6/60 harboring the ts loci. These defects include decreased vRNA synthesis, blockage of viral RNP export from nucleus, reduced incorporation of M1, and production of virions with irregular morphology at 39º C (Chan, Zhou et al. 2008).

To gain further insight into the phenotypic alterations caused by the att mutations in viruses containing the TRIG cassette, we investigated the replication of WT and att H1N1 viruses at different temperatures. In agreement with the RNP reconstitution assay and our previous reports (Song, Nieto et al. 2007; Hickman, Hossain et al. 2008), the growth of the ty/04 att viruses was highly restricted at the non-permissive temperatures. Thus, introducing the double mutations from H9N2 avian virus into a divergent TR influenza strain was sufficient to transfer the ts phenotype.

The next step for evaluating live att influenza vaccines would be to test whether the vaccines were safe for use in mice and other animal models. Consistent with our in vitro data, all the H1N1 ty/04 att-based vaccines were sufficiently attenuated in mice, unlike their WT H1N1 counterparts that caused clinical disease and pulmonary pathology. However, there were differences in the extent of attenuation depending on the origin of the surface protein genes. Although none of the ty/04 att vaccines caused clinical disease or major histopathological changes in mice, animals inoculated with NL 2:6 ty/04 att had higher levels of replication of vaccine virus in the lungs as compared with the group immunized with NY 2:6 ty/04 att. These findings were not unexpected since our group and others have reported limited replication of LAIV (att or ts) upon intranasal inoculation of mice and ferrets (Hickman, Hossain et al. 2008; Joseph, McAuliffe et al. 2008; Chen and Subbarao 2009; Chen, Santos et al. 2009). The difference in attenuation between the two vaccines suggests that the surface genes from A/Netherlands/602/09 carry virulent markers that might be absent in A/New York/18/09. In fact, a recent study reported a difference in virulence between Ca/04, which is more closely related to A/New York/18/09, and A/Netherlands/602/09

(Manicassamy, Medina et al. 2010). These authors reported that A/Netherlands/602/09 was lethal to C57B/6 mice as opposed to Ca/04, even though their genomes differ only in eight amino positions. Future studies investigating the contributions of individual amino acids for the delayed clearance of the NL 2:6 ty/04 att as compared to NY 2:6 ty/04 att will help to pinpoint virulence determinants in surface glycoproteins of the H1N1pdm viruses.

We next evaluated the safety and the efficacy of the double-mutant live att influenza vaccines in pigs. Although other strategies such as truncations of NS1 (Solorzano, Webby et al. 2005; Richt, Lekcharoensuk et al. 2006; Vincent, Ma et al. 2007) and elastase growth-restriction (Masic, Babiuk et al. 2009; Masic, Booth et al. 2009) have been developed for attenuating live influenza viruses for use in swine, these technologies are relatively new and are not as well-characterized as the ts vaccines. In addition to the impact of influenza infection on the swine industry, testing influenza vaccines in pigs also offers the possibility of extrapolating the findings to humans, since there are important similarities between the two species regarding the distribution of sialic acid receptors, cytokine production, and clinical-pathological outcomes after influenza infection (Van Reeth, Van Gucht et al. 2002; Barnard 2009; Barbe, Atanasova et al. 2010; Barbe, Saelens et al. 2010; Nelli, Kuchipudi et al. 2010). Data from our safety studies showed that both the H3N2 and H1N1 ty/04 att vaccines were attenuated in pigs as evidenced by the absence of clinical signs of disease upon intranasal vaccination, reduced viral replication in the respiratory tract and minimal pathological changes in the lungs. The decreased replication of the ty/04 att vaccines in the upper respiratory tract of pigs is consistent with previous reports showing that the cold-adapted, temperature sensitive A/Ann Arbor/6/60 influenza vaccines replicate to moderate levels in the nasal cavities of ferrets (Joseph, McAuliffe et al. 2008; Chen and Subbarao 2009; Chen, Santos et al. 2009; Chen, Lamirande et al. 2010) and with NS1-truncated H3N2 influenza vaccines in pigs that also had limited replication in the nasal cavity (Solorzano, Webby et al. 2005). Although the ty/04 att vaccines were detected in BALF samples, the level of viral replication was significantly reduced in comparison to unmodified virus and, more importantly, caused no overt clinical signs and only minimal pathology in the lungs. A minimal amount of replication is likely beneficial for eliciting T-cell responses to internal genes that may provide heterologous cross-protection. These findings are also consistent with safety studies of truncated NS1-based vaccines in swine that showed limited replication and pathology in the lower respiratory tract (Solorzano, Webby et al. 2005). Moreover, a detailed toxicological evaluation of the LAIV trivalent seasonal human influenza or H5N1 LAIV in

ferrets showed that the virus replicated in the lungs and caused discrete to moderate bronchointerstitial inflammation at 3 dpv after intranasal administration and these adverse effects correlated with the origin of the surface genes, the dose and the volume of the vaccine (Jin, Manetz et al. 2007). Since we used a large volume (2 ml) in our vaccination experiments, the present study confirms and extends the safety studies of Jin and colleagues in ferrets (Jin, Manetz et al. 2007). It has been shown that intranasal administration of volumes larger than 0.2 ml results in replication of the LAIV A/Ann Arbor/6/60 vaccine in the lower respiratory tract of ferrets (Jin, Manetz et al. 2007), but the authors also highlighted that the ferrets studies did not accurately reflect the administration of this vaccine in humans, in which intranasal delivery of 0.5 ml inoculum containing radiolabeled albumin failed to reach the lower respiratory tract (Bryant, Brown et al. 1999). Thus, our safety studies in swine could be more relevant than the ferret model to evaluate adverse effects of live att influenza vaccines for use in humans.

One of the most challenging tasks in producing effective live attenuated vaccines is to achieve an adequate balance between safety and efficacy. By introducing the att modifications into the polymerase genes of a swine-like TR strain, this desirable balance was achieved. The vaccines were attenuated in both mice and pigs and, more importantly, elicited protective immunity in both species. In mice, the ty/04 att-based H1N1 proved to be efficacious against a lethal infection with H1N1pdm. The mice challenge model used in our vaccine trials attempted to recapitulate the severe pneumonia induced by the pandemic H1N1 in certain human patients. All vaccinated animals survived the lethal challenge and had no signs of morbidity following challenge; even though one vaccinated group had limited viral replication in the lungs. The incomplete clearance of challenge virus in the lungs of the immunized mice was not unexpected. Previously, we reported that mice vaccinated with the avian att backbone and challenged with a lethal dose of H5N1 had viral replication in the lungs, although the animals were completely protected from disease (Hickman, Hossain et al. 2008). Our results are in agreement with this report, and of note, the limited pulmonary replication of the challenge virus did not cause significant pathologic changes, correlating with the clinical findings. The ty/04 att-based H1N1 vaccine was also immunogenic in swine and provided sterilizing immunity upon an aggressive challenge with pandemic H1N1 as opposed to an experimental Ca/04 inactivated vaccine, which elicited protective but not sterilizing immunity in all animals. Although serum HI antibody titers were modest in the att H1N1 vaccinated group at the day of challenge, high levels of Ca/04-specific IgA and IgG

were detected in the lungs of all pigs in the group. The local antibody response is likely correlated with the protection from challenge with Ca/04, although we cannot exclude the contribution of T-cell mediated responses to the sterilizing immunity provided by vaccination with the live att H1N1 virus.

In the face of influenza pandemics that have the ability to overcome the species barriers such as the 2009 H1N1, the supply of vaccines for use in agriculture could be jeopardized. Our cell culture-based live att H1N1 vaccines could be an attractive alternative for this possible pandemic vaccine shortage. Because the ty/04 att live vaccines developed here are efficacious in multiple species, are easier to manufacture than inactivated vaccines and do not require adjuvants, our study represents a major advance in vaccine development for the 2009 H1N1 pandemic.

In conclusion, we demonstrated that the double attenuating mutations first implemented for an H9N2 avian influenza strain could be successfully transferred to a heterologous nonattenuated swine-like triple reassortant influenza virus. Thus our second generation of live att influenza vaccines based on modifications of the PB2 and PB1 genes of ty/04 expresses desirable biological traits in vitro, retains its safety properties in vivo, and can induce excellent protection against aggressive H1N1 challenges in more than one animal species. Our studies highlight the attenuation of the ty/04 att vaccine platform and its potential as a master donor strain for the generation of live attenuated vaccines with applications in both human and veterinary medicine.

# Chapter 7: Shuffling of the Influenza Genome: A Novel Approach to Influenza-Based Vectors and Live-Attenuated Vaccines

## *7.1. Abstract*

H5N1 and H9N2 avian influenza subtypes top the World Health Organization's (WHO) list with the greatest pandemic potential. Inactivated H5N1 vaccines have induced limited immune responses and, in the case of live attenuated vaccines, there are safety concerns regarding the possibility of reassortment between the H5 gene with other circulating influenza viruses. In this study, we shuffled the genome of an avian H9N2 influenza virus, A/Guinea Fowl/Hong Kong/WF10/99 (H9N2), to allow insertion and expression of foreign genes. Subsequently, this strategy was used to generate an influenza-vectored H5 vaccine to address the limitations of H5N1 vaccines. Shuffling of the influenza genome was achieved by expressing the NEP/NS2 gene from a single open reading frame (ORF) downstream of the PB1 gene, and the transgene was cloned downstream of a truncated form of the NS1 protein. Avian influenza viruses (H9N2) expressing reporter genes and the entire ORF of the H5 hemagglutinin were generated by reverse genetics. *In vitro* studies demonstrated that these vectors had impaired polymerase activity and viral replication. Further *in vivo* characterization of the shuffled influenza virus vector showed that this strategy completely attenuated a ressortant virus containing 6 genes from a highly pathogenic H5N1 and the 2 modified segments from the shuffled vector (PB1-NEP/NS2 and NS1-GFP) in chickens. More importantly, immunization with the H9N2-H5 virus protected against lethal H5N1 challenges in both mice and ferrets, whereas the H9N2-GFP control vector did not. Taken together, our results demonstrate that the shuffled influenza virus vector is highly attenuated *in vivo* and provides efficient protection against lethal H5N1 virus challenges. These vectors have great potential for the development of improved vaccines against influenza as well as other pathogenic agents.

## *7.2. Introduction*

The use of viral vectors for the delivery of traceable reporter genes and bioactive molecules has broad applications in the fields of gene therapy and infectious disease. A number of live recombinant viral vectored vaccines have been licensed for veterinary use and many are in clinical development for humans (Draper and Heeney 2010). These vaccines combine some of the positive features of DNA vaccines with those of live attenuated vaccines. Similar to DNA vaccines, viral vectors deliver the nucleic acid encoding protein immunogens into target host cells, but with the added advantage of inducing more robust immune responses, which are elicited by replication of the viral entity (Draper and Heeney 2010). Since the development of reverse genetic methods for segmented negative sense RNA viruses, influenza A viruses (IAV) have also been considered as potential vaccine vectors (Hoffmann, Neumann et al. 2000).

IAV are single-stranded RNA viruses with negative polarity belonging to the family O*rthomyxoviridae*. Its genome contains eight RNA segments which encode 10-12 viral proteins. During viral assembly, the vRNAs are selectively packaged into virions in the form of viral ribonucleoprotein complexes. The packaging signals in each of the vRNAs consist of the untranslated regions (UTR) at both the 3' and 5' terminal sequences, and regions of the adjacent coding sequence. Two major viral glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA), are present in the viral envelope (Hutchinson, von Kirchbach et al. 2010).

The engineering of IAV as vaccine vectors offers several advantages: (i) IAV induce strong cellular and humoral immune responses both systemically and at the mucosal surfaces(Garulli, Kawaoka et al. 2004), (ii) influenza does not have a DNA phase during replication, eliminating safety concerns regarding integration of viral DNA into the host genome, (iii) IAV have 16 different HA and 9 different NA surface protein genes, which readily permits reconstitution of the vector surface proteins, making boost vaccinations feasible(Fouchier, Munster et al. 2005), and (iv) IAV are well characterized with attenuated strains already used as vaccines for humans and livestock (Belshe, Edwards et al. 2007). However, most of the IAV vectors developed to date have either been unstable or tolerated only short gene inserts(Manicassamy, Manicassamy et al. 2010). Thus, both the instability of the transgene and the insert size limitation has prevented IAV vectors from being used as vaccines(Martínez-Sobrido and García-Sastre 2007).

H9N2 avian influenza virus and highly pathogenic avian influenza virus (HPAIV) H5N1 subtypes top the World Health Organization's (WHO) list with the greatest pandemic potential. The development of effective vaccines against these subtypes is an essential component of the WHO's global strategy for pandemic preparedness (WHO 2010). Furthermore, with the emergence of the swine-origin pandemic H1N1 in the spring of 2009, there is heightened concern that these viruses may reassort with the pandemic virus to produce a highly pathogenic virus or a novel strain that readily infects and transmits in humans. Inactivated vaccines, particularly in the context of HPAIV H5N1 are poorly immunogenic and often require the addition of an adjuvant and/or boosting to induce an immune response. LAIV vaccines provide broad cross-protective responses and do not require the use of adjuvants as they mimic a natural infection (Johnson, Feldman et al. 1985; Belshe, Mendelman et al. 1998). However, with respect to the development of vaccine candidates against H5N1 influenza strains, live-attenuated vaccines (LAIV) have the potential to reassort with seasonal influenza viruses (Suguitan, McAuliffe et al. 2006; Treanor, Campbell et al. 2006). This creates a significant safety concern as vaccination may inadvertently result in the generation of novel pandemic viruses of the H5 subtype on a seasonal virus background.

In this study, we used reverse genetics to rearrange the IAV genome and generate influenza vectors that stably express foreign genes. Specifically, the NEP/NS2 protein was expressed as a discrete single open reading frame (ORF) downstream of the PB1 gene, and the foreign gene of interest was cloned downstream of the NS1 gene under the transcriptional control of the NS promoters. We have successfully rescued avian IAV (H9N2) expressing enhanced green-fluorescent protein (GFP), secreted *Gaussia* Luciferase (GLuc), and the entire ORF of the HA from a prototypic highly pathogenic H5N1 virus A/Viet Nam/1203/04 (H5N1) strain. Expression of the transgenes was maintained during serial passages in mammalian cells, confirming the stability of the engineered vectors. Importantly, high levels of expression of both HA proteins (H9 and H5) were detected in cells infected with the H9N2 virus expressing the H5 ORF (H9N2-H5). *In vitro* studies demonstrated that these vectors had impaired polymerase activity and viral replication. *In vivo* characterization of the shuffled influenza virus vector demonstrated that this strategy was able to attenuate a ressortant virus containing six genes from a highly pathogenic H5N1 and the two modified segments from the shuffled vector in chickens. More importantly, immunization of mice and ferrets with the H9N2-H5 virus protected against lethal H5N1 challenges, whereas the H9N2-GFP control

vector did not. Our results demonstrate that the shuffled influenza virus vector is highly attenuated in vivo and provides efficient protection against lethal H5N1 virus challenges. Furthermore, because the H5 ORF expressed in this construct does not code for a functional genomic RNA, reassortment of the influenza H5 HA is very unlikely to occur. Therefore, these vectors have great potential for the development of improved vaccines against influenza and other pathogenic agents as well as facilitate studies of influenza pathogenesis in several biological systems.

## *7.3. Material and Methods*

### 7.3.1. Ethics Statement

Vaccination studies were conducted under BSL-2 condition, whereas virus challenge with HPAIV H5N1 was performed under Animal Biosafety Level 3 (ABSL-3) conditions approved by USDA. Animal studies were performed according to protocols R-09-93 "Transmissibility of Influenza A Viruses", and R-09-55 "Evaluation of attenuation and protection efficiency of live attenuated influenza vaccines", approved by the Institutional Animal Care and Use Committee of the University of Maryland. Animal studies adhere strictly to the US Animal Welfare Act (AWA) laws and regulations.

### 7.3.2. Viruses and cell lines

Human embryonic kidney cells (293-T) were cultured in OptiMEM I (GIBCO, Grand Island, NY) containing 10% FBS and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich) and antibiotics.

The avian influenza virus, A/Guinea fowl/Hong Kong/WF10/99 (H9N2) [hereafter H9N2 WT], has been previously described (Song, Nieto et al. 2007). The HPAIV A/Viet Nam/1203/04 (H5N1) [hereafter H5N1 WT] was a kind gift from the Centers for Disease and Control (CDC).

Recombinant viruses used in this paper were generated from cloned cDNAs and are described below and in Table 1.All the viruses were propagated in 7-10 day old embryonated hens eggs and titrated by at least one of the following methods: egg infectious dose 50% ($EID_{50}$), tissue culture infectious dose 50% ($TCID_{50}$), or mouse lethal dose 50% ($MLD_{50}$).

### 7.3.3. Generation of recombinant viruses by reverse genetics

The eight plasmid reverse genetic system for H9N2 WT has been previously described and it is based in the bidirectional plasmid vector pDP2002 (Song, Nieto et al.

2007). The HA and NA genes from the H5N1 WT strain were cloned into pDP2002 vector. The ΔH5 HA plasmid encodes the HA segment from H5N1 WT, which has been further modified by the removal of the encoded polybasic cleavage site. To generate H9N2 WT with ΔNS1, the NS segment was modified so it encodes a carboxy-terminal-truncated NS1 protein product comprising amino acids 1 to 99 and an unmodified NEP/NS2 protein.

Shuffling of the influenza genome was accomplished by expressing the NEP/NS2 protein from a single polypetide downstream of the PB1 gene. Foreign genes of interest are cloned downstream of a full-length or truncated NS1 gene between two AarI cloning sites (or other compatible type III restriction endonucleases) so that there is no introduction of exogenous sequences. Processing of the PB1-NEP/NS2 and NS1-foreign gene proteins is achieved by the in frame incorporation of the foot-and-mouth disease virus (FMDV) 2A cis-acting hydrolase elements (CHYSEL) (de Felipe 2004) downstream of PB1 and NS1, respectively. The corresponding packaging signals previously determined for RNA segments 2 and 8 were maintained to achieve efficient vRNA incorporation into virions (Fujii, Fujii et al. 2005; Liang, Hong et al. 2005). All the plasmid constructs and recovered recombinant viruses were fully sequenced to confirm their identity.

### 7.3.4. Minigenome assay

The minigenome assay was performed as described previously (Pena, Vincent et al.). The PB1 attenuated plasmid (PB1 *att*) used here as a control, has been previously published and contains the K391E, E581G, and A661T mutations and an HA tag sequence fused in frame with the C-terminus of the PB1 protein (Song, Nieto et al. 2007).

### 7.3.5. Growth kinetics

MDCK cells were seeded in 6-well plates and infected in triplicate with each of the viruses described in Table 1 at a multiplicity of infection (MOI) of 0.01. Following 1 hour adsorption, the monolayers were washed 3 times with PBS and 2 mL of OptiMEM media containing 1 μg/ml TPCK-trypsin was added. Plates were incubated at 37°C and cell culture supernatant was harvested at 0, 12, 24, 48, 72, and 96 hours post-infection (hpi). The titer of virus released into the cell culture supernatant at each time point was determined by $TCID_{50}$ in MDCK cells.

### 7.3.6. Immunoelectron microscopy

Recombinant viruses were purified by sucrose density gradient centrifucation. Purified viruses were adsorbed to formvar/ silicon monoxide-coated nickel grids (Electron Microscopy Sciences, Hatfield, PA). The grids were blocked in PBS containing 0.2% BSA and incubated with in-house produced mouse monoclonal antibodies specific for the H5 or H9 HA. Grids were washed in blocking solution and incubated in goat anti-mouse IgG antiserum conjugated to 6 nm gold beads (Aurion, Costerweg 5, The Netherlands). The grids were then negatively stained with 2 % phosphotungstic acid (PTA) for 3 min, dried, and examined under a transmission electron microscope.

### 7.3.7. Mouse studies

Five-week-old female BALB/c mice (Charles River Laboratories, Frederick, MD) were anaesthetized with isofluorane prior to intranasal inoculation. Mice were inoculated with 50 µl $10^5$ egg infectious dose 50 ($EID_{50}$) of the recombinant viruses diluted in PBS. A boost immunization was given in half of the animals two weeks after the first inoculation. Each experimental group contained 30 animals. Mice were divided into 4 groups as follows: a) PBS (negative control) ; b) H9N2-GFP(vector control) ; c) H9N2-H5 (test vaccine); and d) ΔH5N1(positive control for the H5N1 vaccine). At 2 weeks post-vaccination (or 2 weeks post-boost for animals immunized twice), each group was divided into 3 subgroups (n=10) and intranasally challenged with either 20, 200, or 2000 mouse lethal dose 50% ($MLD_{50}$) of the highy pathogenic A/Viet Nam/1203/04 (H5N1) strain. Mice were bled using the submandibular bleeding method (Golde, Gollobin et al. 2005) prior to inoculation and at several time points after immunization to evaluate the immunogenicity of the vaccines. At 5 days post challenge (5 dpc), 3 mice from each subgroup were euthanized and their lungs were collected for histopathologic analysis and to measure levels of challenge virus. Tissue homogenates were prepared in PBS, clarified by centrifugation, and stored at -70ºC until use. Clinical signs of disease, body weight, and mortality were monitored daily throughout the experiment to evaluate vaccine safety and efficacy. Mice presenting ≥25% body weight loss were humanely euthanized and counted as have succumbed from the infection.

To assess the ability of the viruses to replicate in the respiratory tract of mice, 16 additional BALB/c mice (n=4 per group) were inoculated with the same viruses above. Three

days post-inoculation (dpi), all animals were euthanized and their tissues were harvested for virus titration and histopathologic evaluation.

### 7.3.8. Ferret studies

Twelve female Fitch ferrets, 3 to 6 months-old, were purchased from Triple F Farms (Sayre, PA) and randomly divided in four groups of three animals each. Animals were seronegative for IAV, as determined by blocking NP ELISA. Prior to vaccination, ferrets were housed in a BSL2 facility and monitored for 5 to 7 days to measure body weight and establish baseline body temperatures. A subcutaneous implantable temperature transponder (Bio Medic Data Systems,Seaford, DE) was placed in each ferret for identification and temperature readings. Ferrets were intranasally immunized twice two weeks apart with 500 µl containing $10^5$ $EID_{50}$ of the recombinant viruses diluted in PBS, using the same viruses as for murine. At 2 weeks after boost, ferrets were lethally challenged with $10^6$ $EID_{50}$ of the HPAIV H5N1 A/Viet Nam/1203/2004 strain. Body weight changes, clinical signs of disease including fever and mortality were monitored daily throughout the experiment as parameters for vaccine evaluation. Nasal washes were collected for seven days after each vaccination and for nine days post-challenge to quantify virus shedding. Blood samples were collected at 0,14, and at 28 dpv and a final bleed was performed at 15 dpc.

### 7.3.9. Chicken studies

To determine the pathogenicity of a shuffled HPAIV H5N1 (Table 7.1) was evaluated in chickens. Two-week-old specific-pathogen-free leghorn chickens were inoculated intravenously (n=2) or through a combination of natural routes (intranasal, intraocular, oral, and intratracheal) [n=5] with a total of $10^7$ $EID_{50}$ of the shuffled HPAIV H5N1 and morbidity and mortality was followed for 10 days after inoculation.

### 7.3.10. Serological analysis

Serum samples were treated with receptor-destroying enzyme (Accurate Chemical and Scientific Corp., Westbury, NY) to remove nonspecific receptors and the anti-viral antibody titers were evaluated using an HI assay outlined by the WHO Manual for the

laboratory diagnosis and virological surveillance of influenza (WHO 2011). H9N2 WT and ΔH5N1 were used in these assays.

### 7.3.11. Stability of shuffled vectors

The stability of the shuffled H9N-GFP virus was assessed by serially passaging the virus in MDCK cells at a low MOI. Viruses obtained from MDCK passages 1, 5, and 10 were further amplified in embryonated eggs, and used infect fresh MDCK cells with each passage at 1:100 dilution and measuring the levels of GFP transgene expression by fluorescence microscopy.

### 7.3.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student t-test, whereas multiple comparisons were carried out by analysis of variance (ANOVA) using Tukey's post hoc test, unless otherwise specified. The differences were considered statistically significant at $p < 0.05$.

## *7.4. Results*

### 7.4.1. Generation of shuffled influenza virus vectors expressing reporter genes

The IAV RNA segment 8 codes for two proteins: NS1, a nonstructural protein that inhibits the host's antiviral response, and NEP/NS2, an structural component of the viral particle. NEP/NS2 interacts with the cellular export machinery, participates in the assembly of virus particles, and is involved in the regulation of IAV transcription and replication. While NS1 is produced from unspliced RNA, NEP/NS2 is expressed from spliced mRNA (Robb, Smith et al. 2009). In this study, we used reverse genetics to rearrange the genome of an avian IAV, A/Guinea Fowl/Hong Kong/WF10/99 (H9N2) (Song, Nieto et al. 2007). We chose this strain because it grows well in eggs and tissue culture and has been shown to replicate in several animal species such as mice, chicken and ferrets without previous adaptation (Song, Nieto et al. 2007; Hickman, Hossain et al. 2008).

Shuffling of the influenza genome was accomplished by expressing the NEP/NS2, an essential viral protein, from a single polypetide downstream of the PB1 gene and introducing the foot and mouth disease virus (FMDV) 2A autoproteolytic cleavage site between them to allow cotranslational release of a processed NEP/NS2 from the upstream PB1-2A polyprotein. Removing NEP/NS2 from RNA segment 8 resulted in additional cloning space in this segment. The expression of the transgenes of interest was achieved by cloning their gene downstream of NS1 protein. The FMDV 2A was cloned between NS1 and the transgene to enable discrete expression of the foreign protein. The corresponding packaging signals previously determined for RNA segments 2 and 8 were maintained to optimize incorporation of the modified vRNA into virions (Fujii, Fujii et al. 2005; Liang, Hong et al. 2005). As proof of principle for this technology, the secreted Gaussia luciferase (GLuc), the enhanced green fluorescent protein (GFP) (Fig. 7.1A, and Table 7.1) was cloned into this vector and successful virus rescue was achieved for all these transgenes. Recombinant IAV virus vectors reached titers in the order of 6-7 $Log_{10}$ $EID_{50}$/mL after amplification in embryonated hens eggs. Expression of both GFP and GLuc reporter genes was readily detected either in the cytoplasm or in the supernant, respectively, of infected MDCK cells (Fig. 7.1B and 7.1C).

**Table 7.1. Influenza viruses used in this study.**

| Viruses | Genome modification | Transgene | Virus subtype | Acronym |
|---|---|---|---|---|
| A/Guinea fowl/Hong Kong/WF10/99 (H9N2) [a] | none | none | H9N2 | H9N2 WT |
| A/Viet Nam/1203/04 (H5N1) [b] | none | none | H5N1 | H5N1 WT |
| Unshuffled H9N2-ΔNS1 [c] | NS1 truncation | none | H9N2 | ΔH9N2 |
| Shuffled H9N2-ΔNS1-GFP [d] | NS1 truncation and shufflng | GFP | H9N2 | H9N2-GFP |
| Shuffled H9N2-ΔNS1-GLuc [e] | NS1 truncation and shufflng | GLuc | H9N2 | H9N2-GLuc |
| Shuffled H9N2-ΔNS1-ΔH5 Orf [f] | NS1 truncation, shufflng, and modified cleavage site in HA | Modified H5 HA | H9N2-H5 | H9N2-H5 |
| Surface genes from ΔH5N1 in unshuffled H9N2-ΔNS1 backbone [g] | NS1 truncation and modified cleavage site in HA | none | H5N1 | ΔH5N1 |
| Shuffled H5N1 WT [h] | shufflng | GFP | H5N1 | none |

[a] Genbank taxonomy ID: 221116
[b] Genbank taxonomy ID: 284218
[c] A/Guinea fowl/Hong Kong/WF10/99 (H9N2) with NS1 truncation at position 99.
[d] A/Guinea fowl/Hong Kong/WF10/99 (H9N2) with NS1 truncation at position 99 with genome shuffling and expressing GFP.
[e] A/Guinea fowl/Hong Kong/WF10/99 (H9N2) with NS1 truncation at position 99 with genome shuffling and expressing GLuc.
[f] A/Guinea fowl/Hong Kong/WF10/99 (H9N2) with NS1 truncation at position 99 with genome shuffling and expressing H5 HA ORF derived from A/Viet Nam/1203/04 (H5N1) . The polybasic cleavage site present in the H5 HA was modified.
[g] 2:6 reassortant containing the surface genes of A/Viet Nam/1203/04 (H5N1) and the internal genes A/Guinea fowl/Hong Kong/WF10/99 (H9N2) with NS1 truncation at position 99. The polybasic cleavage site present in the H5 HA was modified.
[h] 2:6 reassortant containing 6 genes of A/Viet Nam/1203/04 (H5N1) and the segment 2 and 8 derived from the shuffled A/Guinea fowl/Hong Kong/WF10/99 (H9N2). Shuffled segment 2 corresponds to PB1-2A-NEP/NS2 and modified segment 8 refers to a full-length NS1-2A-GFP.

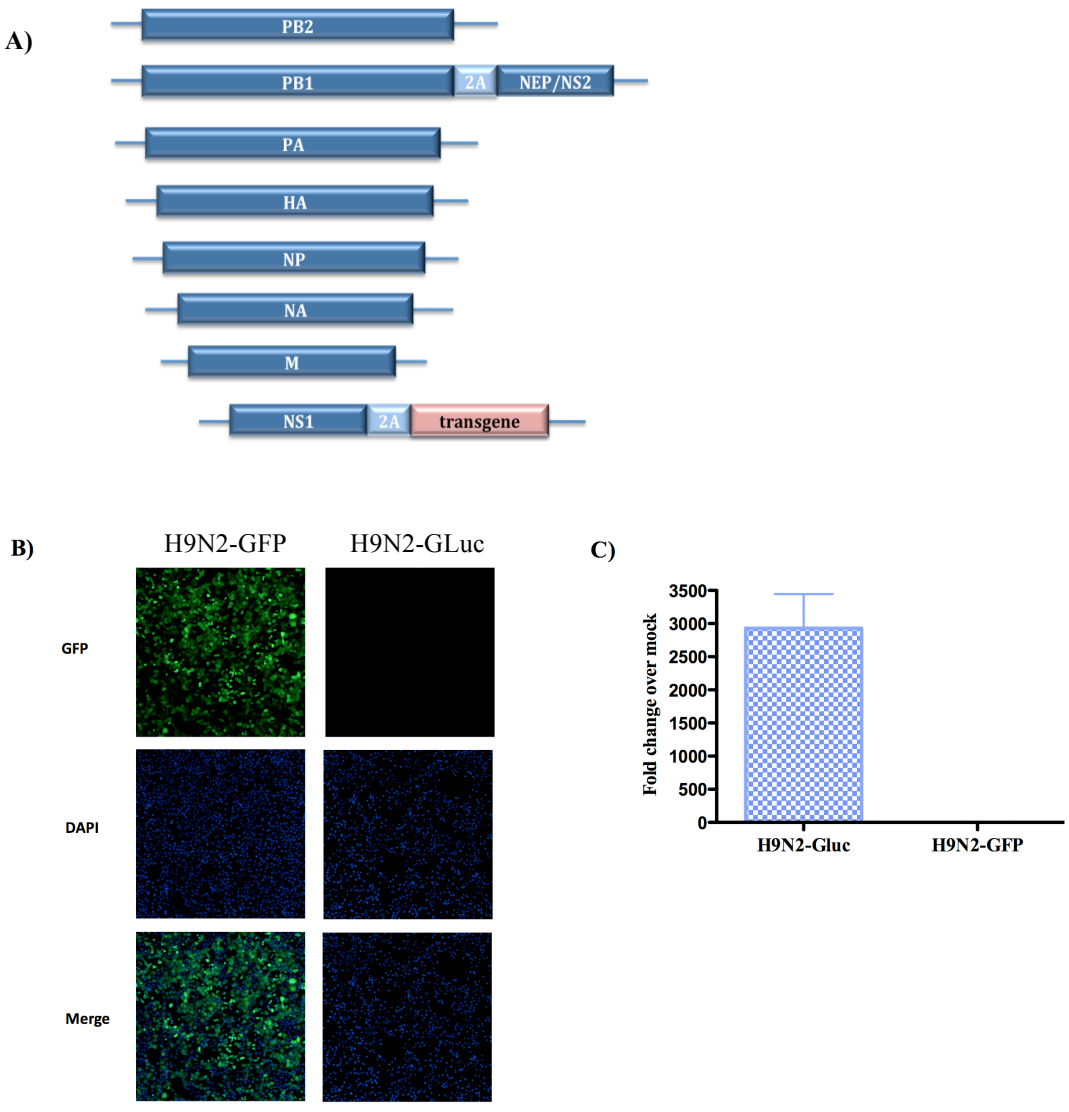



**Figure 7.1. Shuffled H9N2 influenza A viruses expressing reporter genes. (**A) Schematic representation of shuffled influenza viruses. NEP/NS2 protein is expressed from a single ORF (solid box) downstream of the PB1 gene, whereas the foreign antigen of interest is expressed downstream of the NS1 gene. The packaging signals span both the untranslated region (solid line) and a part of the ORF at both ends each RNA segment. Two recombinant viruses were made expressing either eGFP (A), GLuc (B) downstream of a truncated NS1 gene. The viruses were rescued by reverse genetics and used to infect MDCK cells. At 24 hpi, GFP expression was detected by confocal microscopy (A) and GLuc was detected in tissue culture supernatant by a luficerase assay (B).

### 7.4.2. The H5 hemagglutinin expressed from segment 8 is incorporated into the envelope of a shuffled H9N2 virus

With the goal of developing shuffled influenza virus as a vaccine vector, the HA ORF, a type I membrane protein, from the A/Viet Nam/1203/04 (H5N1) isolate was cloned downstream of a truncated NS1 gene in the background of an H9N2 virus. The H5 HA was genetically modified to remove its multi-basic cleavage site, a domain associated with high pathogenicity phenotype of the HPAIV (Basler and Aguilar 2008). Similarly to the viruses expressing reporter genes, the recombinant H9N2-H5 virus was successfully rescued and propagated in embryonated eggs, a substrate approved for human vaccines. To our knowledge, this is the first report that successfully recovered an eight-segmented IAV expressing two different HA proteins. Cells infected with the H9N2-H5 virus expressed high levels of both HA subtypes as determined by IFA using monoclonal antibodies (mAb) against the H5 and H9 HA, respectively (Fig. 7.2).

Previous studies have demonstrated the surface incorporation of two different HAs (H1 and H3) by a recombinant influenza virus containing nine RNA segments (Gao, Lowen et al. 2010). We next sought to determine whether the H5 HA could be incorporated into our eight –segmented H9N2 shuffled influenza vector. The H9N2-H5 virus showed typical influenza virus morphology, consistent of pleomorphic particles and roughly spheroidal virions with approximately 100 nm in diameter (Fig. 7.2 and data not shown). Immunogold electron microscopy using an anti-H5 and anti-H9 specific mAb revealed that the H5 and the H9 HAs were incorporated into virus (Fig 7.2, small insets). As expected, H5 and H9 control virus only reacted with the respective mAb. These results indicate that shuffled IAV vectors retained the typical virion morphology and that the H5 HA expressed from segment 8 was incorporated into the surface of the shuffled H9N2-H5 virus.

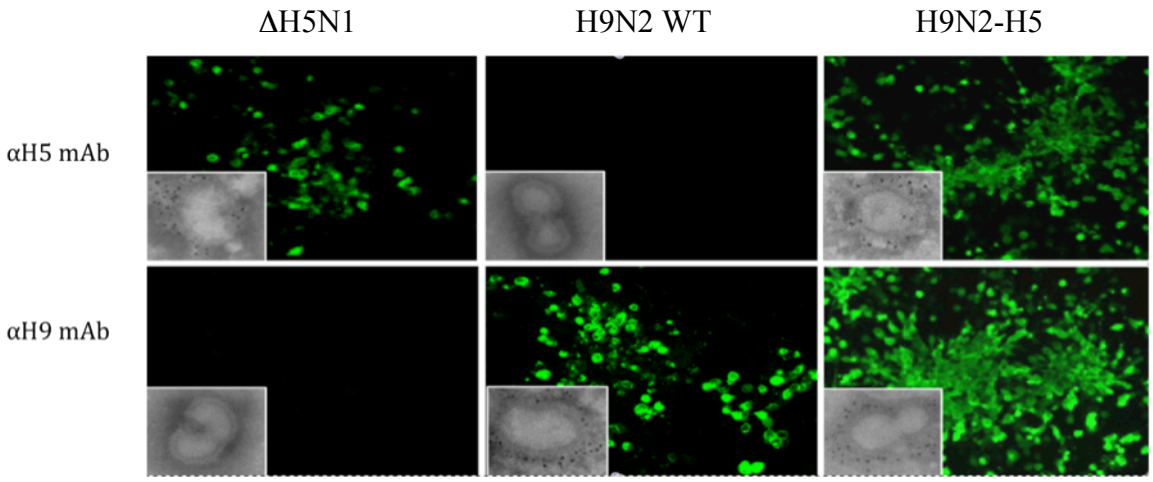



**Figure 7.2. Novel influenza vector expressing H9 and H5 HA proteins on viral surface.** A recombinant H9N2 virus was made expressing the H5 HA ORF and rescued by reverse genetics and used to infect MDCK cells. Expression of the H9 and the H5 HA was confirmed by IFA and TEM (small insets) using monoclonal antibodies specific for these antigens and compared to either a H5N1 or H9N2 wild type virus. TEM, black dots correspond to immunogold reactions for either H5 or H9 HA proteins, please note incorporation of H5 HA into virus particles.

### 7.4.3. Genome shuffling leads to impaired polymerase activity and reduced viral growth *in vitro*

In order to study the effects of the incorporation of FMDV 2A and NEP/NS2 downstream of the PB1 gene in viral polymerase activity, we performed viral ribonucleoprotein (vRNP) reconstitution assay to measure polymerase activity at different temperatures (33, 35, 37, 39, and 41°C) as previously described (Pena, Vincent et al.). 293-T cells were co-transfected with plasmids encoding the wild type PB2, PB1 (or PB1-2A-NEP/NS2), PA, and NP genes and the influenza vRNA reporter construct carrying a secreted luciferase (GLuc). Transfection efficiency was normalized using a plasmid encoding the secreted alkaline phosphatase (SEAP) gene. Polymerase activity was monitored by the ratio of Gluc/SEAP. These experiments revealed that the presence of PB1-2A-NEP/NS2 led to significantly lower polymerase activities than that of WT genes at the different temperatures studied (Fig. 7.3A). These results suggested that the strategy used to rearrange the IAV genome decreases viral polymerase activity.

To evaluate whether the reduction in polymerase activity viruses would translate into attenuated viral replication, the growth properties of the shuffled vectors H9N2-GFP and H9N2-H5 were evaluated in MDCK cells infected at MOI 0.01. In agreement with the minigenome assay, shuffled viruses harboring a truncated NS1 had 10-100 fold reductions in virus titer as compared to unshuffled viruses containing the same NS1 modification. Both WT and shuffled viruses reached maximum viral titers at 24 hours postinfection (Fig. 7.3B). Together, these results indicate that shuffled viruses can undergo multiple cycles of replication in MDCK cells, although the maximum virus titer is reduced by 10 fold as compared to the WT isogenic virus.

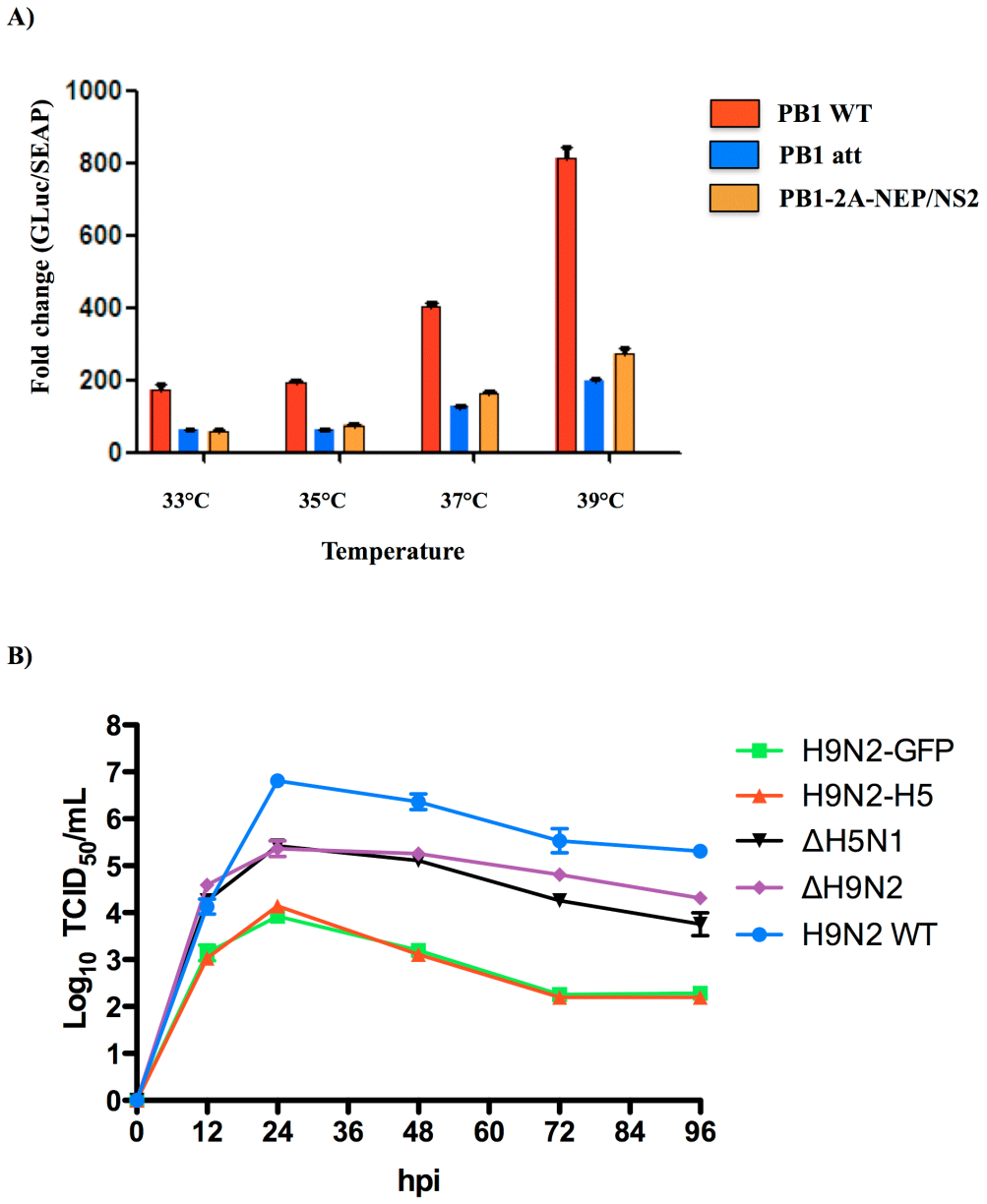



**Figure 7.3.** ***In vitro*** **characterization of shuffled influenza vectors**. (A) Minigenome assay. 293-T cells were transfected with 1 µg each of the influenza driven-luciferase reporter plasmid and PB2, PB1, PA and NP plasmids and incubated at different temperatures as shown. pCMV/SEAP, which encodes secreted alkaline phosphatase was cotransfected into the cells to normalize transfection efficiency. The WT PB1 and an attenuated PB1 gene (PB1 att) were used here as a controls. At 24 hours post-transfection, the supernatant was harvested and assayed for both luciferase and phosphatase activities. Normalized polymerase activities (mean ± SE) were determined from three independent experiments. (B) Multicycle step growth curve. MDCK cells were seeded in 6-well plates and infected in triplicates with each of the viruses described in table 1 at a multiplicity of infection (MOI) of 0.01. Supernatants were harvested at the indicated time points and titrated in MDCK cells by $TCID_{50}$.

### 7.4.4. Stability of shuffled influenza vectors

We next examined the stability of the H9N2-GFP during serial passage of this vector in MDCK cells at a low MOI. Because of the low titers obtained from MDCK cells, viruses obtained at passages 1, 5, and 10 were further amplified in embryonated eggs, and evaluated for stability. The phenotypic stability was assessed by infecting MDCK cells with 1:100 dilution of each viral passage and measuring the levels of GFP transgene expression. High expression levels of GFP were detected up to 10 passages, suggesting that shuffled IAV maintains foreign gene transcripts after several passages in tissue culture.

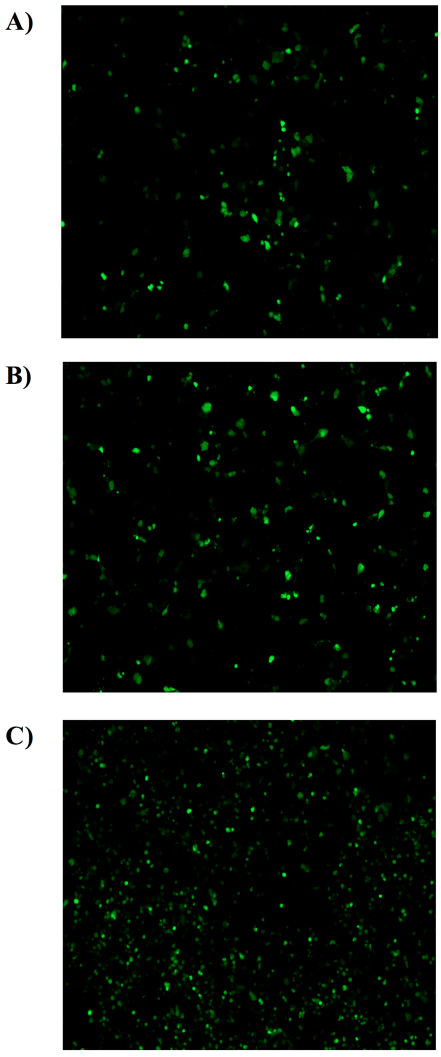



**Figure 7.4. Stability of shuffled influenza vectors.** Stability of shuffled H9N2-GFP was assessed by serially passaged in MDCK cells and using the viruses from passages 1 (A), 5 (B) and 10 (C) to infect MDCK cells at 1:100 dilution and measuring the levels of GFP expression at 24 hpi.

### 7.4.5. Genome shuffling strongly attenuates influenza vectors *in vivo*

To evaluate the safety profile of shuffled IAV vectors, we tested these vectors in mice, ferrets, and chickens. BALB/c mice (n=14) were intranasally inoculated with $10^5$ $EID_{50}$ /mouse of either H9N2-GFP or H9N2-H5 shuffled vectors. Body weight loss and the levels of viral replication in the respiratory tract were used as parameters of morbidity. An additional group was mock vaccinated with PBS alone, and a fourth group was inoculated with virus containing the surface genes derived from a low pathogenic A/Viet Nam/1203/04 (which had the polybasic cleavage site in HA removed) in the backbone of the H9N2 virus containing a truncated NS1 (hereafter referred to as ΔH5N1) as non-shuffled virus control.

The ability of the viruses to replicate in the respiratory tract of mice was assessed by measuring viral load from the lungs of mice (4 from each group) at 3 dpi. The shuffled H9N2 vectors were highly attenuated in mice as demonstrated by the absence of clinical disease signs and no change in body weight after vaccination. Conversely, mice inoculated with the ΔH5N1 unshuffled virus showed significant body weight loss (≤ 20%) by 8 dpi, although they eventually recovered. (Fig.7.5A). In examining tissue tropism and viral replication, the shuffled H9N2 vectors viruses were not detected in any of the organs sampled (nasal cavity, trachea, and lungs). Consistent with the clinical data, mice inoculated with the ΔH5N1 had high virus titers in the respiratory tract (Fig.7.5B).

To obtain more detailed information about the safety of the shuffled IAV vectors, an attenuation study was conducted in ferrets (n=3) using the same treatment groups and viral doses used for the mice studies. Clinical evaluations performed after vaccination indicated no clinical signs of disease, no body weight changes, or no fever in the vaccinated groups relative to mock animals (Fig.7.5C-D), confirming the safety features of shuffled viruses in the ferret model. Virus shedding was not detected in any of the ferrets groups after vaccination or boost (data not shown).

As the H5 HA is expressed from the shuffled H9N2-H5 virus as a chimeric HA segment with the packaging signals of the NS gene, the possibility of reassortment of the H5 HA is remote (Gao and Palese 2009). Nevertheless, genetic ressortment could take place if a circulating IAV exchanges both RNA segments 2 and 8 with the shuffled IAV. Therefore, we addressed this question by rescuing an H5N1 virus containing 6 WT RNA segments (1,3,4,5,6, and 7) from the HPAIV A/Viet Nam/1203/04 (H5N1) strain and 2 segments (PB1-2A-NEP/NS2 and NS1-2A-GFP, which harbors a full-length NS1) derived from the shuffled

H9N2 virus. The A/Viet Nam/1203/04 (H5N1) strain was isolated from a fatally infected human patient and is one of the most virulent HPAIV isolated to date (Govorkova, Rehg et al. 2005). The pathogenicity of shuffled HPAIV H5N1 was evaluated in chickens, a highly susceptible host for the HPAIV. Two-week-old specific-pathogen-free leghorn chickens were inoculated intravenously or through all natural routes at once (intranasal, intraocular, oral, and intratracheal) with a total of $10^7$ $EID_{50}$ of the shuffled HPAIV H5N1 and morbidity and mortality was followed for 10 days after inoculation. Remarkably, none of the chickens died or developed clinical signs of disease upon inoculation (Fig.7.5E and data not shown),. This suggests that even if the shuffled virus reassorted with a HPAIV H5N1, the resulting virus would be completely attenuated. Taken together, these results indicate that shuffled-based IAV vectors exhibits desirable safety features in three animal species.

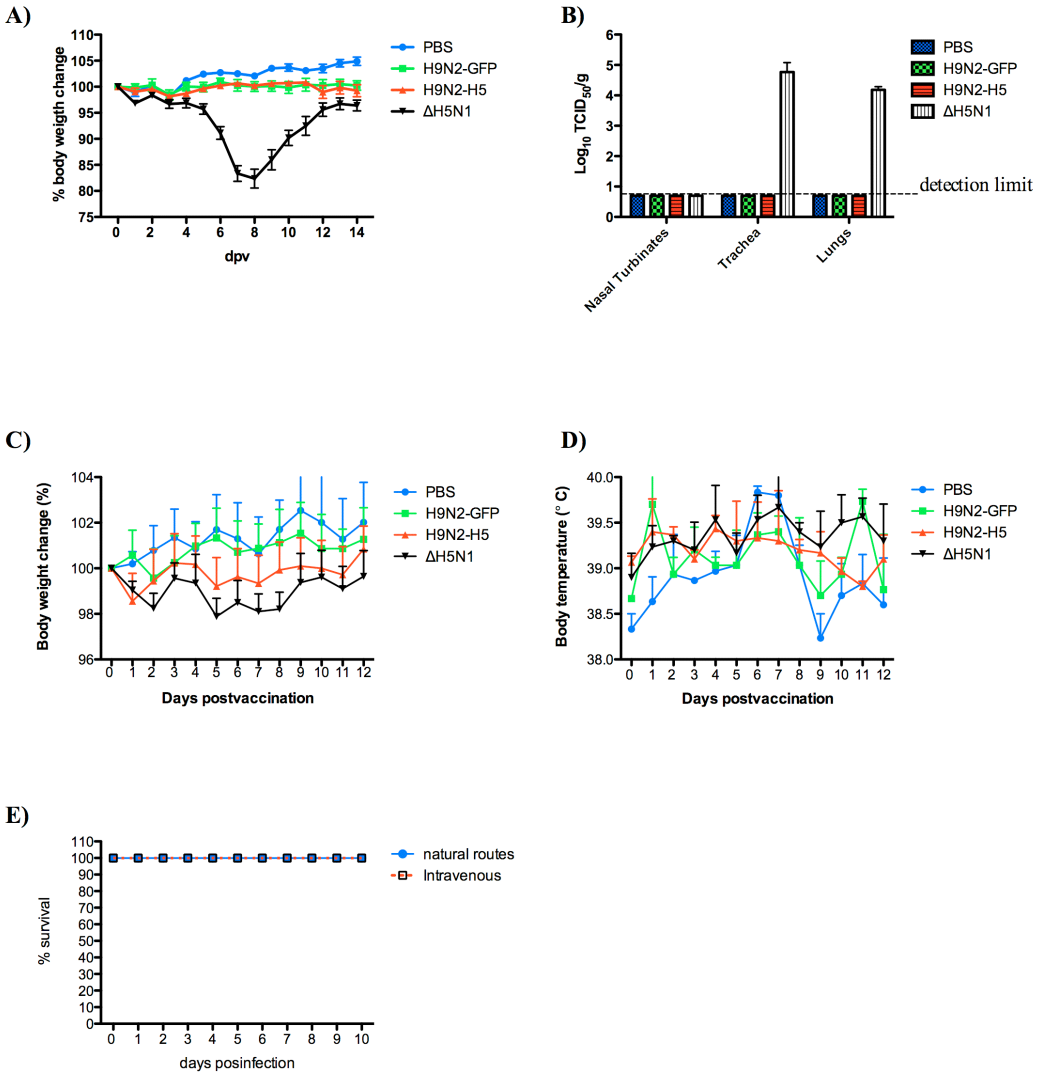



**Figure 7.5.** ***In vivo*** **attenuation of shuffled influenza viruses**. (A) Safety studies in mice. Body weight changes following intranasal inoculation of BALB/c mice (n=10) with $10^5$ $EID_{50}$ /mice of the recombinant viruses. (B) Viral replication in the respiratory tract of mice. BALB/c mice (n=4) were inoculated with $10^5$ $EID_{50}$ and viral titers in the indicated organs at 3 dpi were determined by $TCID_{50}$ . The lower limit of detection (0.6 $TCID_{50}$ /mL) of the assay is indicated by the dashed horizontal line. (C and D) Attenuation in ferrets. Ferrets (n=3) were intranasally inoculated with $10^5$ $EID_{50}$ /animal with the recombinant viruses and body temperature(C) and body weight (D) were recorded daily. (E) Safety studies in chickens. SPF leghorn chickens were inoculated intravenously (n=2) or through natural routes (intranasal, intraocular, oral, and intratracheal) [n=5] with $10^7$ $EID_{50}$ of the shuffled HPAIV H5N1 and survival was followed for 10 days after inoculation.

### 7.4.6. Shuffled H9N2-H5 virus vector provides protection against highly pathogenic H5N1 challenges in mammals

Given the epidemiological relevance of HPAIV H5N1 virus for public health and the fact that vaccination is a mainstay of WHO's pandemic preparedness plan, we assessed the ability of the shuffled H9N2-H5 virus to induce a protective immune response against one of the world's toughest pathogen: the HPAIV H5N1.

**Protective efficacy in mice.** Groups of BALB/c mice (n=30) were intranasally vaccinated once or twice with $10^5$ $EID_{50}$/animal with the following: a) PBS (negative control); b) H9N2-GFP (vector control) ; c) H9N2-H5 (test vaccine); and d) ΔH5N1(positive control for the H5N1 vaccine). At 2 weeks post-vaccination (or 2 weeks post-boost for animals immunized twice), each group (n=30) was divided into 3 subgroups (n=10) and challenged with either 20, 200, or 2000 mouse lethal dose 50% ($MLD_{50}$) of the virulent A/Viet Nam/1203/04 (H5N1) strain. Clinical signs of disease, weight loss and survival were monitored for 15 days post-challenge (dpc). At 5 dpc, three mice from each group were euthanized and their lungs collected for histopathologic analysis and to measure levels of the H5N1 challenge virus.

A single dose of the H9N2-H5 vector provided complete protection from morbidity and mortality following lethal challenges with 20 and 200 $MLD_{50}$ of HPAIV H5N1. The results are summarized in Fig. 7.6. The subgroup challenged with 2000 $MLD_{50}$ lost an average of ≤10% of body weight by 7 dpc, but all the animals recovered with the exception of a single mouse that succumbed to infection. This was in contrast to the outcome observed in the group immunized with the H9N2-GFP vector control. In this group, a single immunization was not fully protective since all animals developed clinical disease and lost body weight with all challenge doses. The mortality rate in the singly immunized H9N2-GFP group was 14.3%, 85.7%, and 100% for the 20, 200, or 2000 $MLD_{50}$ challenge doses, respectively. Additionally, protection against pulmonary viral replication mirrored the clinical performance of the vaccines. All the H9N2-GFP inoculated mice that received a single vaccine dose had high virus titers in the lungs across all challenge doses, whereas the H9N2-H5 vaccine provided sterilizing immunity against 20 $MLD_{50}$ challenge and only 1, and 2 individuals had detectable virus replication in the lungs following 200, or 2000 $MLD_{50}$ challenges, respectively. Nevertheless, viral titers in these groups were lower than in the H9N2-GFP group. At the 2000 $MLD_{50}$ challenge, viral lung load, morbidity and mortality

rates in the H9N2-GFP was indistinguishable from the PBS inoculated group, demonstrating that the cross-protective immunity elicited by H9N2-GFP control, but not by H9N2-H5, could be overcome by an overwhelming challenge.

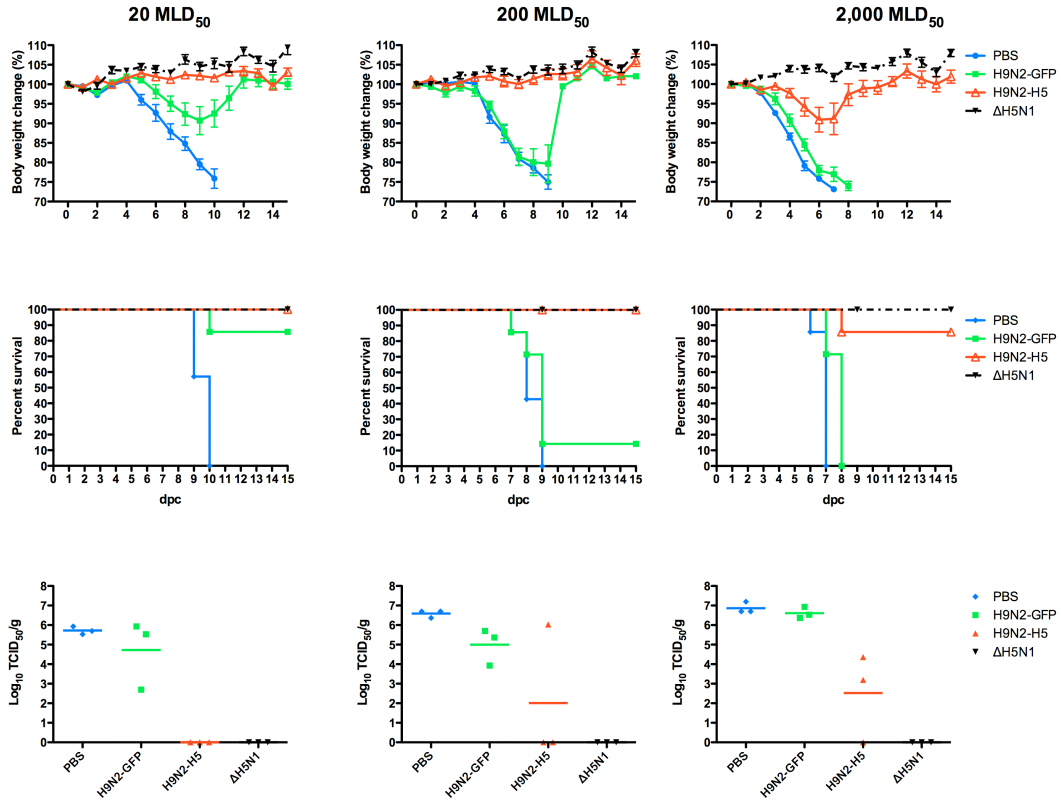



**Figure 7.6. Protective efficacy of H9N2-H5 shuffled vector in mice after a single immunization.** BALB/c mice (n=30) were inoculated with $10^5$ $EID_{50}$ /mice of the recombinant viruses. At 2 weeks post-vaccination, each treatment group was divided into 3 subgroups (n=10) and challenged with either 20, 200, or 2000 mouse lethal dose 50% ($MLD_{50}$) of the virulent A/Viet Nam/1203/04 (H5N1) strain by the intranasal route. Clinical outcome in animals receiving one dose of the H9N2-H5 vaccine. Percent body weight change (upper panels), survival (middle panels), and virus titers in lung homogenates (lower panels).

Importantly, a second dose of H9N2-H5 vaccine resulted in complete clinical protection and sterilizing immunity in all individuals regardless of the challenge dose used. The results from this trial are summarized in Fig. 7.7. In the H9N2-GFP group boost vaccination elicited protection from morbidity against 20 $MLD_{50}$ challenge. However, these animals were not protected from replication of the H5N1 virus. The mean viral titer in this group was approximately $10^3$ $TCID_{50}$/g of lung tissue. Challenge with 200 and 2000 $MLD_{50}$ overcame the cross-protective immunity induced by the H9N2-GFP vector as indicated by 28.5% and 71.5% mortality rates, respectively, and high levels of replicating challenge virus in the lungs ($10^5$ to $10^6$ $TCID_{50}$/g of lung). Collectively, these results demonstrated that the shuffled H9N2-H5 vector is suitable for prime/boost vaccination protocols as boost vaccination enhanced protection. The PBS control mice had high viral lung load ($10^{5.5}$ to $10^7$ $TCID_{50}$/g) and all animals died within 7-10 dpc, depending on the challenge dose used. As expected, immunization with the ΔH5N1 induced sterilizing immunity irrespective of the challenge dose. However, the protective immunity induced by ΔH5N1 needs to be counterbalanced by the pathogenicity of this virus to BALB/c mice, which almost reached the humane endpoint for euthanasia (25% body weight loss) upon immunization.

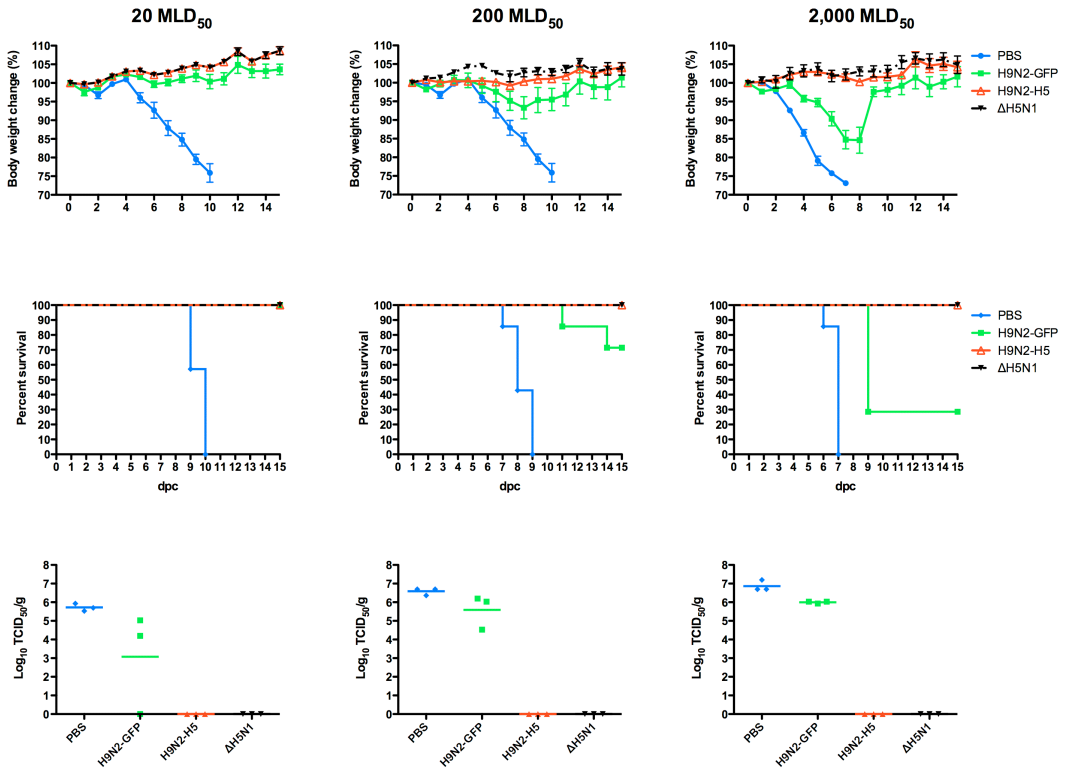



**Figure 7.7. Protective efficacy of H9N2-H5 shuffled vector in mice after a boost immunization.** BALB/c mice (n=30) were inoculated twice with $10^5$ $EID_{50}$ /mice of the recombinant viruses. At 2 weeks post-boost, each treatment group was divided into 3 subgroups (n=10) and challenged with either 20, 200, or 2000 mouse lethal dose 50% ($MLD_{50}$) of the virulent A/Viet Nam/1203/04 (H5N1) strain by the intranasal route. Clinical outcome in animals receiving two dose of the H9N2-H5 vaccine. Percent body weight change (upper panels), survival (middle panels), and virus titers in lung homogenates (lower panels).

**Protective efficacy in ferrets.** To gain more insight into the efficacy of the H9N2-H5 vector, we conducted a vaccine-challenge study in ferrets, an established outbred model of IAV (Matsuoka, Lamirande et al. 2009), using the same treatment groups as for the mouse studies. The ferrets (n=3) were vaccinated intranasally twice on days 0 and 14 with $10^5$ $EID_{50}$/animal and challenged at 2 weeks post-boost with a lethal dose ($10^6$ $EID_{50}$/ferret) of A/Viet Nam/1203/2004 (H5N1) strain. This challenge was extremely severe (equivalent to 10,000 ferret lethal dose 50% for this H5N1 strain (Mahmood, Bright et al. 2008)) and was chosen with the objective to discriminate the protective responses induced by the H9N2-H5 vector from the cross-protection induced by the H9N2-GFP vector control. Virus shedding post-challenge was measured in nasal washes collected from 1-9 dpc. Following challenge, 2 out of 3 ferrets vaccinated with the H9N2-H5 virus were completely protected against death and disease. In contrast, 3 out of 3 ferrets in the H9N2-GFP group developed severe clinical manifestations of infection and succumbed between 9-13 dpc (Fig. 7.7A). In the H9N2-H5 group, the single ferret (# 426) that succumbed to infection showed initially no clinical disease, but shed high amounts of virus, which eventually led to development of clinical signs, and required euthanasia by 13 dpc. Even though the protected H9N2-H5 ferrets were co-housed with the unprotected ferret, these animals had an average of 10-fold reduction in viral shedding compared to the ferrets from the H9N2-GFP group. PBS-inoculated animals had 2-3 log higher viral titer in nasal wash samples than H9N2-H5 (Fig. 7.7B). These animals developed severe clinical disease, which characterized by fever, lethargy, anorexia, body weight loss and diarrhea. PBS-inoculated animals had uncontrolled viral replication from 1-9 dpi and the animals either succumbed to infection or were euthanized at the humane endpoints. ΔH5N1 vaccinated ferrets did not show any clinical signs of disease after the challenge, although low levels virus shedding was detected in nasal washes collected from 2 ferrets between 1 and 3 dpc (Fig. 7.7B). Collectively, these results suggest that the shuffled H9N2-H5 vector can protect mice and ferrets against severe HPAIV H5N1challenges and that this protective immunity can be enhanced by a boost vaccination.

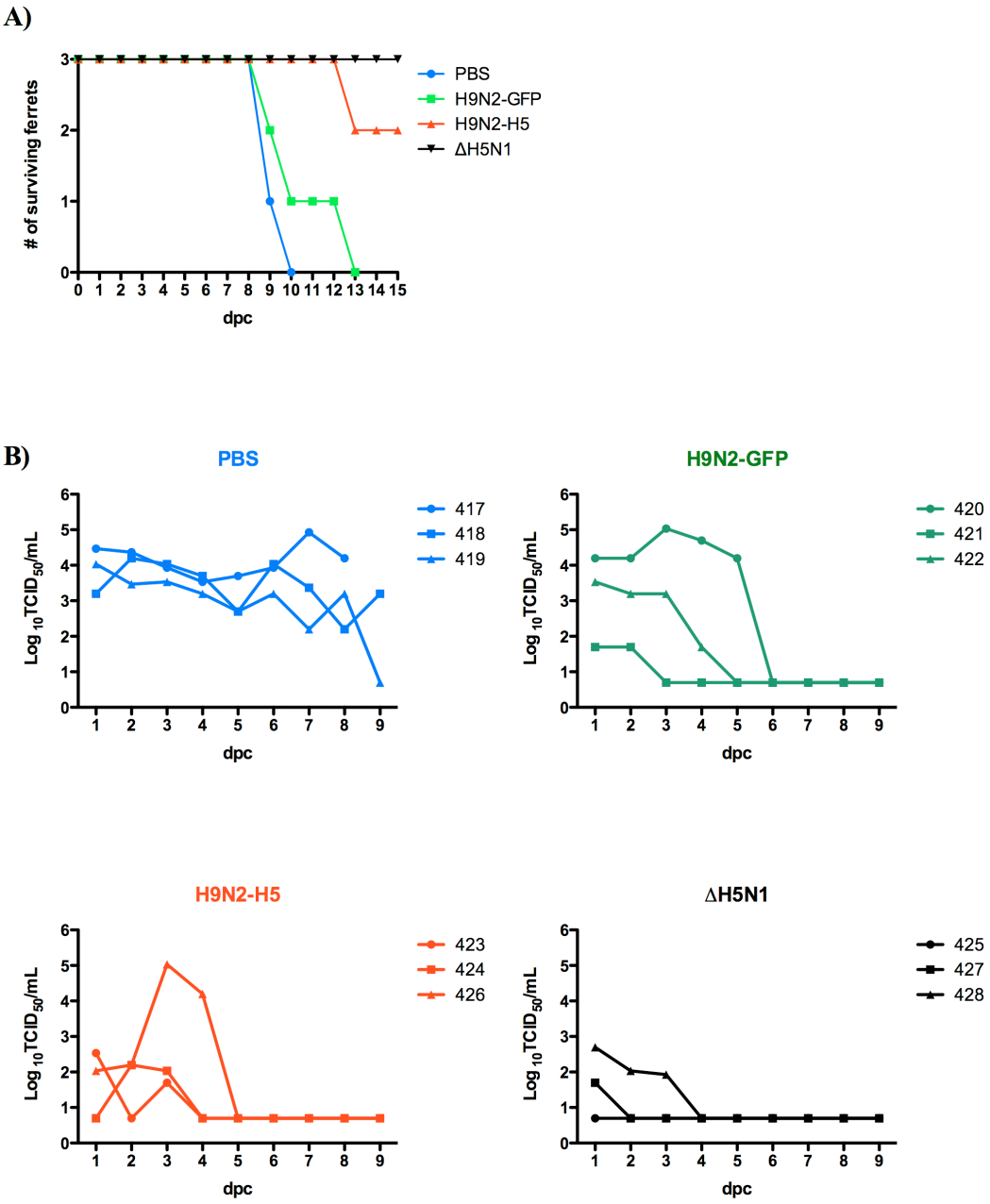



**Figure 7.8. Protective efficacy of H9N2-H5 shuffled vector in ferrets.** 6-8 months old female ferrets (n=3) were vaccinated with $10^5$ $EID_{50}$ of the recombinant viruses and boosted 2 weeks later using the same virus and dose. Two weeks after boost, ferrets were challenged with $10^6$ $EID_{50}$ /ferret (10,000 $FLD_{50}$) of A/Viet Nam/1203/2004 (H5N1) and protection was evaluated. Nasal washes were collected for nine days post-challenge to assess challenge virus shedding (A) Survival upon challenge (B) Virus shedding in nasal secretions. The lower limit of detection (0.6 $TCID_{50}$ /mL. Names on the top of each chart indicates the vaccine treatment group.

### 7.4.7. Serum antibody responses against H9N2 vector and H5 HA transgene

The ability of sera from vaccinated ferrets to neutralize H5N1 and H9N2 viruses was assessed by HI tests. No detectable HI antibody titers against the H5N1 virus were detected prior to challenge in any of the groups (Table 7.2). Interestingly, when the humoral response was evaluated against the H9N2 virus, all animals inoculated with the H9N2-GFP vector control had high HI titers, whereas only a single animal from H9N2-H5 virus seroconverted against H9N2. Neither PBS nor ΔH5N1 inoculated ferrets had detectable antibody titers against the H9N2 virus (Table 7.3).

**Table 7.2. Immunogenicity in ferrets of shuffled influenza vaccines against H5N1 virus.**

| Groups | 0 dpv | 14 dpv | 14 dpb/0 dpc | 15 dpc |
|---|---|---|---|---|
| PBS | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 | N/A, N/A, N/A |
| H9N2-GFP | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 | N/A, N/A, N/A |
| H9N2-H5 | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 | 40,20, N/A |
| ΔH5N1 | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 | 40,40,160 |

Hemagglutination inhibiton (HI) titers are given as the reciprocal of the highest dilution of serum that showed activity against the ΔH5N1 virus. HI titers for each animal are shown.. The limit of the detection of this technique is 10. N/A: not applicable-animals were dead at this time point.

**Table 7.3. Immunogenicity in ferrets of shuffled influenza vaccines against H9N2 virus.**

| Groups | 0 dpv | 14 dpv | 14 dpb/0 dpc | 15 dpc |
|---|---|---|---|---|
| PBS | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 | N/A, N/A, N/A |
| H9N2-GFP | <10,<10,<10 | 320,320,160 | 160,160,160 | N/A, N/A, N/A |
| H9N2-H5 | <10,<10,<10 | <10,<10,<10 | <10,40,<10 | <10,40, N/A |
| ΔH5N1 | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 | <10,<10,<10 |

Hemagglutination inhibiton (HI) titers are given as the reciprocal of the highest dilution of serum that showed activity against the H9N2 WT virus. HI titers for each animal are shown.. The limit of the detection of this technique is 10. N/A: not applicable-animals were dead at this time point.

## *7.5. Discussion*

With the advent of reverse genetics system for IAV, several groups have devised strategies to express foreign peptides or proteins from the IAV genome. Among all the viral proteins, the surface glycoproteins HA and NA have been successfully engineered for expression of small protein epitopes (Li, Mueller et al. 2005; Martínez-Sobrido and García-Sastre 2007; Martina, van den Doel et al. 2011). Nevertheless, neither HA nor NA glycoproteins are optimal targets for the insertion of foreign sequences because of insert size constraints and the fact that boost immunizations could be limited by pre-existing immunity to the vector or the transgene itself. Additionally, expression of foreign genes has also been achieved by fusion or co-expression of foreign proteins downstream of the NS1 protein, but expression of NEP/NS2 is still dependent of splicing of segment 8 RNA (Ferko, Stasakova et al. 2001; Kittel, Ferko et al. 2005; Sereinig, Stukova et al. 2006; Wolschek, Samm et al. 2011). Recently, Manicassamy and collaborators adapted a previously developed strategy to express NEP/NS2 as a single ORF (Basler, Reid et al. 2001) and constructed IAV expressing GFP reporter gene fused to viral NS1 protein via a linker sequence. However, the stability of the GFP-expressing virus was compromised during multicycle replication (Manicassamy, Manicassamy et al. 2010).

In this study, we rearranged or shuffled the genome of IAV with the goal of expanding its genome coding capacities and improving the stability of the IAV vector. Using this strategy, rescue of shuffled IAV carrying up to 1.7 Kb of foreign sequences (H5 ORF) downstream of a truncated NS1 protein was successfully carried out. Interestingly, viruses expressing GFP and GLuc from full-length NS1 were effectively recovered, but it was not possible to rescue a virus that stably maintained the H5 ORF cloned downstream of full-length NS1, indicating that the maximum packaging capacity of the shuffled IAV may have been reached. By cloning GFP and the secreted Gluc reporter genes it was possible to demonstrate that both the cytosolic and secretory pathways can be accessed using this technology. The high levels of GLuc expression in tissue culture supernatant suggested that shuffled IAV might have the potential to deliver soluble molecules secreted by pathogenic microorganisms, such as bacterial toxins.

As a proof of principle for the shuffled influenza-based vector platform, we expressed the entire H5 HA ORF in the eighth segment of shuffled H9N2 IAV vector and tested the safety and the efficacy of this vector against a prototypical HPAIV H5N1 viruse, the A/Viet Nam/1203/04 (H5N1) strain. The shuffled viruses were innocuous after

administration in two mammalian species. More importantly, inoculation of chickens with a shuffled virus rescued in the background of the wild type A/Viet Nam/1203/04 (H5N1) was completely attenuated in chickens, a highly susceptible host for HPAIV H5N1 viruses. The H9N2-H5 vaccine proved to be very efficacious against robust challenges with HPAIV H5N1 in both mice and ferrets. Unlike LAIV that are currently licensed in the USA, that rely on a small number of attenuating mutations (Jin, Lu et al. 2003), genome shuffling may allow a more stable attenuation phenotype since it involves more dramatic changes in the structure of IAV genome. In fact, we have previously demonstrated that transferring the genetic signatures from the LAIV A/Ann Arbor/6/60 (H2N2) to the H9N2 avian influenza virus used in the current study imparted the temperature sensitive (*ts*) phenotype but the vaccine was not sufficiently attenuated in mice or chickens, which raises potential safety concerns (Song, Nieto et al. 2007; Hickman, Hossain et al. 2008).

To gain further insight into the phenotypic alterations caused by the genome shuffling, we evaluated growth characteristics and polymerase activity derived from these viruses. Comparison of growth curves of the recombinant virus showed that shuffled virus grew to substantially lower levels than isogenic virus without NEP/NS2 shuffling. The incorporation of the H5 HA did not enhance virus replication since H9N2-GFP and H9N2-H5 had similar titers and growth kinetics. The impaired growth of shuffled viruses could be explained mechanistically by lower polymerase activity displayed by the PB1-2A-NEP/NS2 as compared to the WT PB1 gene in a side-by-side vRNP reconstitution assay. The mechanisms responsible for the decreased activity of shuffled polymerase complex as well as the impaired virus growth are not completely understood. Nevertheless, we have previously shown that incorporating an eight amino acid HA tag epitope in the C-terminus of PB1 reduced viral replication and polymerase activity (Pena, Vincent et al. ; Song, Nieto et al. 2007). Thus, it is possible that the 18 amino acid long FMDV 2A tag in PB1 contributes to the attenuated polymerase activity and viral growth. Additionally, the expression of NEP/NS2 from a single ORF in segment 2 may alter the levels of transcription and replication of the shuffled virus relative to their WT counterparts. NEP/NS2 has been shown to modulate the relative amounts of cRNA, vRNA, and mRNA in vRNP reconstitution assays and in the context of viral infection (Robb, Smith et al. 2009). Furthermore, the effect that the FMDV 2A fused to the carboxyl terminus of NS1 have in viral replication and the ability of the IAV to counteract the host innate response still need to be explored. Lastly, the exact boundaries of the packaging signals for IAV are not yet properly defined and may be subtype or even strain-specific. In this study, shuffled H9N2 IAV vectors were engineered respecting the

length of packaging signals previously reported for H1N1 strains (Liang, Hong et al. 2005). Fine mapping of the optimum cis-acting sequences required for H9N2 vRNA incorporation into influenza virus particles may allow improvements in virus yield and stability of foreign genes. Hence, the phenotypic alterations exhibited by shuffled vectors may be caused by multiple defects in several steps of the viral life cycle. Further study of the mechanisms of attenuation of shuffled IAV may shed light onto basic molecular constraints that control IAV replication.

The use of replication-competent vectors mirrors the natural infection and has the advantage of a prolonged transgene expression profile and the possibility of amplifying the infection, and consequently transgene delivery, to neighboring cells. However, a significant concern with the administration of replication-competent virus vectors derived from known human pathogens, such as the IAV, is the risk of residual virulence due to incomplete attenuation of the wild type virus. Thus, stringent safety requirements must be met for the application of replication competent vectors in human medicine. The pathogenicity of the shuffled IAV developed here was evaluated in three established animal models for IAV: mice, ferrets, and chickens. In mice, resistance to IAV is partially controlled by alleles at the *Mx* locus on chromosome 16. The Mx protein is an interferon-induced guanosine triphosphatase that confers variable levels of resistance to orthomyxoviruses infection. The *Mx* allele is absent in influenza virus-susceptible mice strains such as BALB/c because of frameshift mutation or premature truncation of the Mx protein (Staeheli, Grob et al. 1988). Because of its high susceptibility, we chose this strain to evaluate the safety profile of the shuffled IAV. Single nucleotide polymorphisms (SNPs) in the *Mx* gene have been reported in humans and were associated with increased susceptibility to viral infection (Suzuki, Arase et al. 2004; He, Feng et al. 2006; Yin, Peng et al. 2006), highlighting the relevance of our safety studies for patients harboring these SNPs. When administered to BALB/c mice, the unshuffled ΔH5N1 virus raised some safety concerns in this highly susceptible mouse strain since the animals developed significant disease and the virus replicated to high titers in the respiratory tract. In contrast, the shuffled IAV vectors, which had the truncation in the interferon antagonist NS1 protein as in the ΔH5N1, were completely attenuated in mice. This was demonstrated by the absence of clinical signs of disease upon vaccination and the undetectable virus replication and pathology in the respiratory tract. The pathogenicity of the ΔH5N1 virus in BALB/c mice is in agreement with previous studies that demonstrated attenuation but residual virulence of NS1 deletion containing viruses for this mouse strain

(Talon, Salvatore et al. 2000). Correspondingly, immunization of ferrets with shuffled vectors resulted in asymptomatic infection of the respiratory tract with no virus shedding. In order for shuffled vectors to reassort with WT IAV, both RNA segments 2 and 8 must be exchanged with the circulating virus. We foresaw a worst-case scenario in which this reassortment event would occur with a HPAIV H5N1 strain containing, among other virulent motifs, the polybasic cleavage site in the HA. Nevertheless, our data in chickens suggest that genome shuffling results in an H5N1 virus completely innocuous to this highly susceptible natural host. These preclinical studies performed in three highly susceptible animal species indicate that shuffled IAV vectors are highly attenuated *in vivo* and may be suitable for people bearing immune deficiencies as well as for the young and the elderly.

One of the challenges in developing viral-vectored vaccines is to sufficiently attenuate the virus without seriously affecting its ability to function as a useful viral vector, e.g. being immunogenic against the target pathogen. Using aggressive HPAIV H5N1 challenges in mice and ferret models, the efficacy of shuffled vectors was established. In mice, a single low dose of the H9N2-H5 vaccine provided complete clinical protection against challenges with up 200 $MLD_{50}$. When the group was challenged with 2,000 $MLD_{50}$, this vaccine decreased viral lung load by 10,000 fold as compared to the H9N2-GFP vector control and just one individual out of seven died. Subjects immunized with the vector control and challenged with the same dose experienced 100% mortality. A second administration of the H9N2-H5 vector provided sterilizing immunity irrespective of the challenge dose. In ferrets, the limited number of animals used and an individual animal variation appears to have influenced the capability of the H9N2-H5 vaccined group to resist the challenge. One animal developed clinical disease and shed large amounts of virus, albeit onset of shedding was delayed by one day in this individual relative to the other unprotected ferrets. This animal remained in the same isolator with the 2 other animals in this group during 12 of the15 day-experiment. Although the active dissemination of virus by this ferret may have overwhelmed the protective efficacy of the H9N2-H5 vaccine, its cage-mates were completely protected from clinical disease and shed lower amounts of viruses relative to PBS and H9N2-GFP vector control groups. Even though ferrets used in this study were obtained from the same supplier and were age, and sex-matched, genetic and environmental elements may have been influenced the ability of this particular subject to respond to vaccination.

The mechanisms responsible for the lower anti-H9 immunity after immunization with the H9N2-H5 relative to the H9N2-GFP are unclear. However, these findings are in

agreement with findings reported by Nayak and colleagues (Nayak, Rout et al. 2009). These authors used a Newcastle disease virus (NDV)-vectored H5 vaccine and found that, even though the NDV-H5 replicated to similar levels to the NDV vector control *in vitro*, lower antibodies titers against NDV was observed in animals vaccinated with NDV-H5 as compared to recipients of the NDV vector control (Nayak, Rout et al. 2009).

Since its emergence in the human population in 1997, HPAIV H5N1 have been considered an imminent threat to public health. Besides H5N1, WHO currently recognizes the avian influenza H7 and H9 subtypes as viruses with pandemic potential. Furthermore, with the emergence of the swine-origin pandemic H1N1 in the spring of 2009, there is heightened concern that these highly pathogenic viruses may reassort with the pandemic virus to produce a highly pathogenic viruses. Should these viruses acquire the ability to spread sustainably among human, a prophylactic vaccine will be the mainstay in the global combat to these infections. A novel pandemic strain must be immunologically distinct from circulating viruses with respect to the HA to be able to spread in the naïve human population throughout the world. In naïve populations, LAIV had significantly better efficacy than inactivated vaccine, which would result in more rapid herd immunity and require fewer vaccine doses as compared to inactivated vaccines (Johnson, Feldman et al. 1985; Belshe, Mendelman et al. 1998). Our shuffled-based IAV vaccines might be an appropriate alternative to conventional inactivated and LAIV in case of a pandemic due to its safety profile and robust efficacy.

In summary, we demonstrated, for the first time, that influenza viruses are amenable to genome rearrangement and can satisfactorily accommodate large pieces of additional genetic material while preserving replication and immunogenicity. This vaccine does not require expensive high-containment manufacturing facilities and can be grown in eggs; so far the substrate of choice for producing IAV vaccines. Furthermore, development of this technology may pave the way to a novel class of influenza vectors that can provide immunity not only to influenza but also against other diseases.

# Chapter 8: Conclusions and future prospects

## *8.1. Conclusions*

The overall objectives of this dissertation work were to determine the molecular basis of virulence of the influenza A viruses (IAV) for the swine host and to develop live attenuated influenza vaccines and influenza-based vectors for their use in multiple species.

Here, we determined that the surface glycoproteins and the PB1-F2 protein are virulence determinants of IAV for pigs. We focused on swine because of the economical importance of this species for the livestock industry, its epidemiological relevance in IAV ecology, and the fact that swine influenza mirrors the human disease regarding the distribution of sialic acid receptors, cytokine production, and clinical-pathological outcomes after influenza infection. We consider these studies to be very timely considering the current scenario of IAV infection with triple reassortant viruses in both the human and swine population.

In the second part of this study, we developed temperature-sensitive live attenuated influenza vaccines that are safe and efficacious in pigs. These studies highlighted the safety of a live attenuated vaccine platform and its potential as a master donor strain for the generation of efficacious vaccines with applications in both human and veterinary medicine.

Lastly, we rearranged the genome of an avian H9N2 influenza virus to generate replication-competent influenza virus vectors that provide a robust system for expression and delivery of foreign genes. As a proof-of-principle, we expressed the hemagglutinin from a prototypical highly pathogenic avian influenza virus (HPAIV) H5N1 and shown that this vectored H5 vaccine retained its safety properties in avian and mammalian species, and induced excellent protection against aggressive HPAIV H5N1 challenges in mice and ferrets.

To our knowledge, the studies completed in this dissertation are the first to define molecular markers in the AIV genome that contributes to its virulence in pigs. In addition, the influenza-based vaccines and viral vectors engineered in this research are novel in the field of influenza virus research. These studies provided novel insights for understanding influenza virus pathogenesis and vaccinology and will contribute to the advancement of both research fields.

Based on the results presented in chapters 3 to 7, the general conclusions from this study are:

**1) Role of surface glycoproteins in the pathogenesis of triple reassortant H3N2 influenza viruses in swine (Chapter 3):**

a) We have successfully established a reverse genetics system for generation of triple reassortant H3N2 influenza A viruses (sw/99 and ty/04) from cloned cDNAs

b) We have developed a swine model of infection that reproduces clinical signs of disease under experimental condition.

c) Genetically related triple reassortant H3N2 IAV display different pathotypes in the swine host.

d) Surface glycoproteins are necessary and sufficient for the lung pathology induced by these viruses

e) The internal genes from triple reassortant H3N2 IAV play a major role in induction of the febrile response

f) Influenza virus virulence in swine is polygenic. Other genes in addition to HA and NA are required for reversion of the clinical disease phenotype.

**2) Role of PB1-F2 in the virulence of triple reassortant H3N2 and pandemic H1N1 influenza viruses in swine (Chapters 4 and 5):**

a) PB1-F2 in the context of the sw/99 strain impacts macrophage viability and viral yields in PAM, as well as IL-6 *in vivo*.

b) In the context of ty/04, PB1-F2 increases its replication in explants but not *in vivo*.

c) Knocking out PB1-F2 from ty/04 enhances pulmonary lesions in pigs, as well as the pulmonary levels of IL-8 and IFN-γ.

d) In the context of pH1N1, PB1-F2 increases polymerase activity and its replication in respiratory explants and in lungs of infected swine.

e) Restoring PB1-F2 in pH1N1 enhances microscopic pneumonia and the pulmonary levels of IFN-α and IL-1β.

f) In swine, PB1-F2 proteins derived from triple reassortant IAVs exhibit the following effects *in vivo*: no effect in the context of sw/99 strain; increases the virulence of pH1N1; and decreases the virulence of ty/04.

g) Overall, PB1-F2 has pleiotropic effects in the swine host, which are expressed in a strain-dependent manner.

**3) Modifications in the polymerase genes of a swine-like triple reassortant influenza virus to generate live attenuated vaccines for humans and livestock (Chapter 6):**

a) The double attenuating mutations first implemented for an H9N2 avian influenza strain could be successfully transferred to a heterologous nonattenuated swine-like triple reassortant influenza virus.

b) We have successfully developed a viral ribonucleoprotein (vRNP) reconstitution assay to study polymerase activity based on the secreted Gaussia luciferase

c) The live attenuated influenza vaccine (LAIV) based on ty/04 strain expresses desirable biological traits *in vitro.*

d) The ty/04-based LAIV rescued with H3N2 and pH1N1 surfaces retain their safety properties in vivo (mice and swine).

e) The ty/04-based LAIV induces excellent protection against aggressive pH1N1 challenges in both mice and swine.

f) The ty/04-based LAIV is cell-culture based, which makes it an attractive alternative for the possibility of vaccine shortage for livestock during an influenza pandemic.

g) The ty/04-based LAIV has the potential for the prevention of influenza across species

**4) Shuffling of the influenza genome to engineer vectors stably expressing foreign genes (Chapter 7):**

a) The vector allows for both cytosolic and ER bound translation.

b) The H5 hemagglutinin expressed from segment 8 is incorporated into the envelope of a shuffled H9N2 virus.

c) Shuffled vectors have impaired polymerase activity and viral replication *in vitro*.

d) At least 1.7kb can be expressed from an eight-segmented influenza virus vector.

e) Genome rearrangement was able to completely attenuate a ressortant virus containing 6 genes from a highly pathogenic H5N1 and the 2 modified segments from the shuffled vector (PB1-NEP/NS2 and NS1-GFP) in chickens.

f) The shuffled vector is highly attenuated *in vivo* and provides efficient protection against lethal H5N1 virus challenges in mammals.

*8.2. Future prospects*

Based on the findings from this dissertation research, the following studies are suggested:

**1) Role of surface glycoproteins in the pathogenesis of triple reassortant H3N2 influenza viruses in swine (Chapter 3):**

a) Test 7:1 reassortant with just HA gene to assess if the HA is sufficient for the lung pathology.

b) Test 5:3 reassortant (HA,NA,NS) to assess if these gene combination can revert the clinical disease phenotype.

c) Characterize other gene combinations to determine the molecular constellation that contributes to the high virulence of ty/04 relative to sw/99 in pigs.

d) Characterize the receptor binding profile of the sw/99 and ty/04 HA using glycan arrays and correlate that with the receptors in the pig.

e) Study the effects of glycosylation site at position 246 in both sw/99 and ty/04 with respect to virus infectivity, receptor binding, pathogenicity, and transmissibility. The mutation N246S arouse during amplification of the sw/99 in eggs. The presence of this mutation changes the reactivity of sw/99 in HA assays using either turkey or chicken RBCs. These mutations have already been introduced in both sw/99 and ty/04 and the viruses have been successfully recovered.

f) Study reassortment events and transmissibility of sw/99 and ty/04 with other IAV that exhibit pandemic potential such H2N2, H5N1, H7N1, and H9N2.

g) Characterize the transmissibility and pathogenesis of triple reassortant IAVs in ferrets and evaluate their pandemic potential upon adaptation and reassortment with other IAVs in both pigs and ferrets. Do the triple reassortants H3N2 IAVs have zoonotic or pandemic potential? If so, are these viruses better fit than seasonal H3N2 for transmission in the ferret model?

**2) Role of PB1-F2 in the virulence of triple reassortant H3N2 and pandemic H1N1 influenza viruses in swine (Chapters 4 and 5):**

a) Determine the molecular and cellular mechanisms responsible for PB1-F2 strain-specific effects in swine.
b) Test the PB1-F2 recombinant viruses generated herein in post-influenza secondary pneumonia in murine and swine models.
c) Use the PB1-F2 recombinant viruses rescued herein to evaluate the effects of PB1-F2 in other animal models such as chickens, turkeys, and ferrets.
d) Determine if the surface proteins contribute to the strain-specificity of PB1-F2 proteins derived from triple reassortant IAVs.
e) Determine if the PB1-F2 proteins studied here have a direct effect on cellular pathways involved in innate immune responses and cell death.
f) Test whether the PB1-F2 used herein have direct antibacterial effect and whether they have pro- or anti-inflammatory potential upon administration of these peptides to mice.

**3) Modifications in the polymerase genes of a swine-like triple reassortant influenza virus to generate live attenuated vaccines for humans and livestock (Chapter 6):**

a) Test the effects of the T6D mutation in the ty/04 PB1 att gene. This mutation interferes with PB1-PA interaction and is expected to attenuate further the ty/04 att vaccine. Ty/04 att viruses containing the T6D mutation has already been rescued.

b) Test the effects of introducing the H6 cleavage site (PQIET motif) into the HA cleavage site of the H3 (ty/04) and H1 (pH1N1) virus subtypes. These modifications have already been made and the viruses have been rescued.

c) Test the ty/04 LAIV against heterosubtypic challenges in pigs

d) Compare inactivated vaccines and ty/04 LAIV vaccines in a swine model of vaccine-induced enhanced pneumonia

e) Characterize, in depth, both the cellular and humoral immune responses induced by ty/04 LAIV in pigs.

f) Safety and efficacy studies of ty/04 LAIV in the field

g) Test the ty/04 vaccine in other species such as ferrets, turkeys and chickens.

h) Test the ty/04 *att* pH1N1 and H3N2 vaccines containing the elastase motif in the HA. These vaccines have already been made and their sequences were confirmed. These vaccines are expect to improve the safety profile of the original ty/04 LAIV.

i) Test the ty/04 *att* vaccines in which the pH1 and the H3 cleavage sites in the HA were replaced by the H6 cleavage site (PQIET motif) in the HA. These vaccines are expect to improve the safety profile of the original ty/04 LAIV.

j) Test alternative routes of immunization such as intradermal for delivery of ty/04 LAIV.

k) License the vaccine for field use in swine and turkey populations.

**4) Shuffling of the influenza genome to engineer vectors stably expressing foreign genes (Chapter 7):**

a. Use the shuffled vector to express molecular adjuvants in vaccine/challenge studies against influenza and other diseases.

b. Evaluate the safety and efficacy of the shuffled H9N2-H5 in the young and the elderly (mouse models).

c. Test shuffled H9N2 vectors expressing soluble H5 HA and headless H5 to evaluate whether these constructs improve the immunogenicity and the antigenic coverage of the H5 vaccine.

d. Use the GLuc-based vector developed herein to measure influenza-specific neutralizing antibodies in biological samples.

e. Study the types of cells infected by IAV and virus reassortment using the GFP and RFP reporters already generated.

f. Rearrange other genes in order to restore polymerase activity and virus growth. Then, perform pathogenesis studies using in vivo bioluminescence imaging systems.

g. Use the shuffled vectors for genome-wide RNA interference (RNAi) screens to identify host factors that are required for influenza replication.

a. Adapt the shuffled vector for high growth in Vero cells, which is an approved substrate to produce human vaccines.

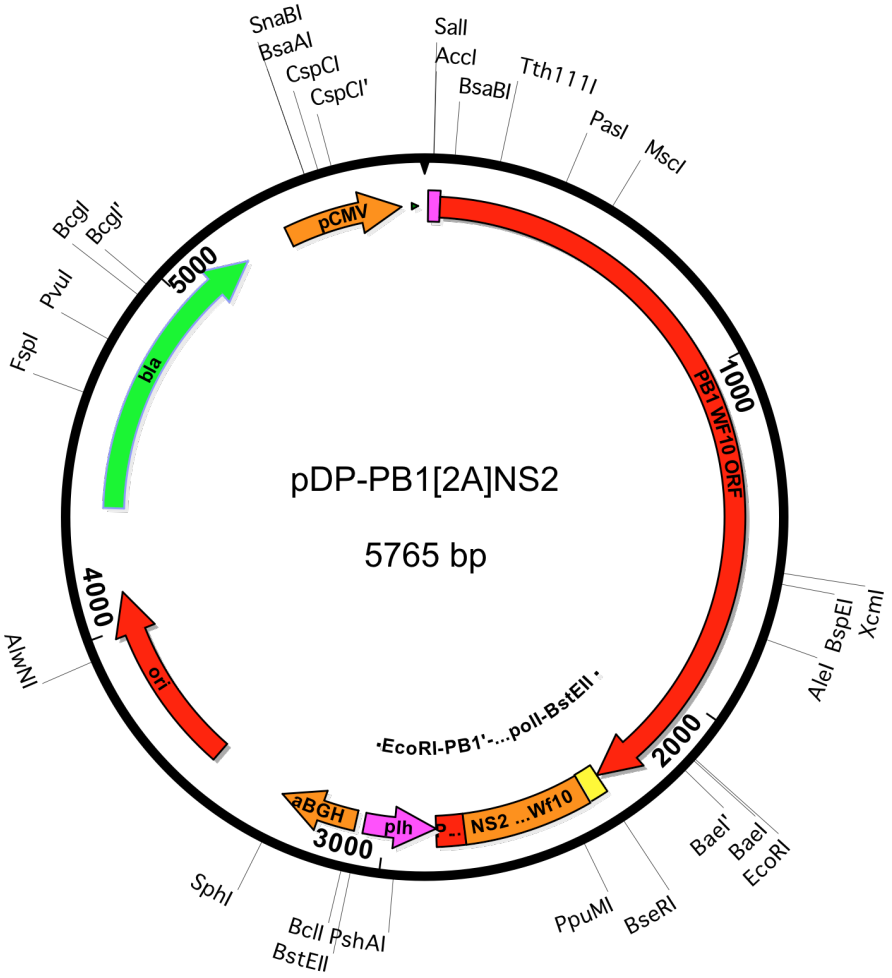
b. Use the vector against other pathogens and cancer.

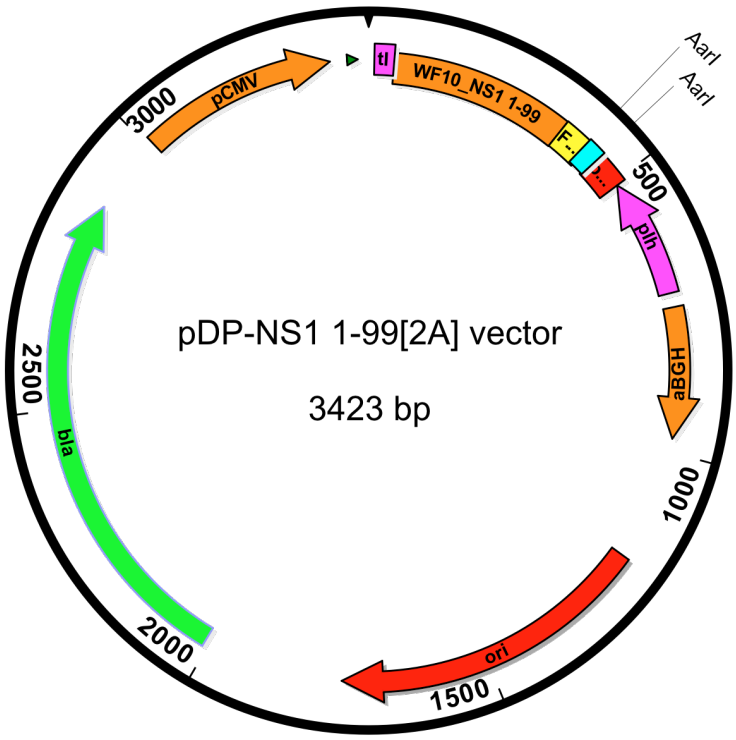
c. Product development and licensure for agriculture and human use.

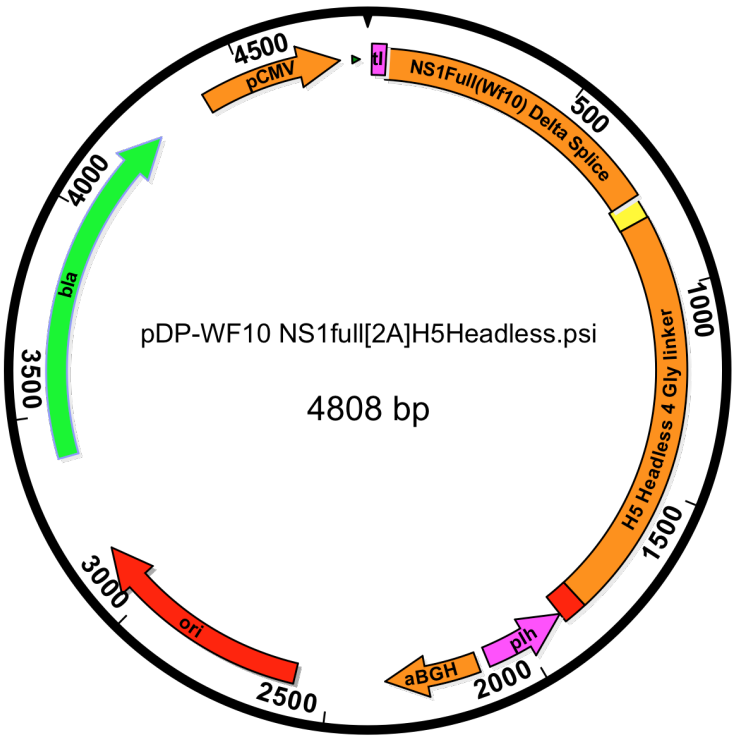
These studies will advance the scientific knowledge in the influenza field and will hopefully shed light for the rational development of vaccines and other intervention strategies not only against influenza, but also against other pathogens.

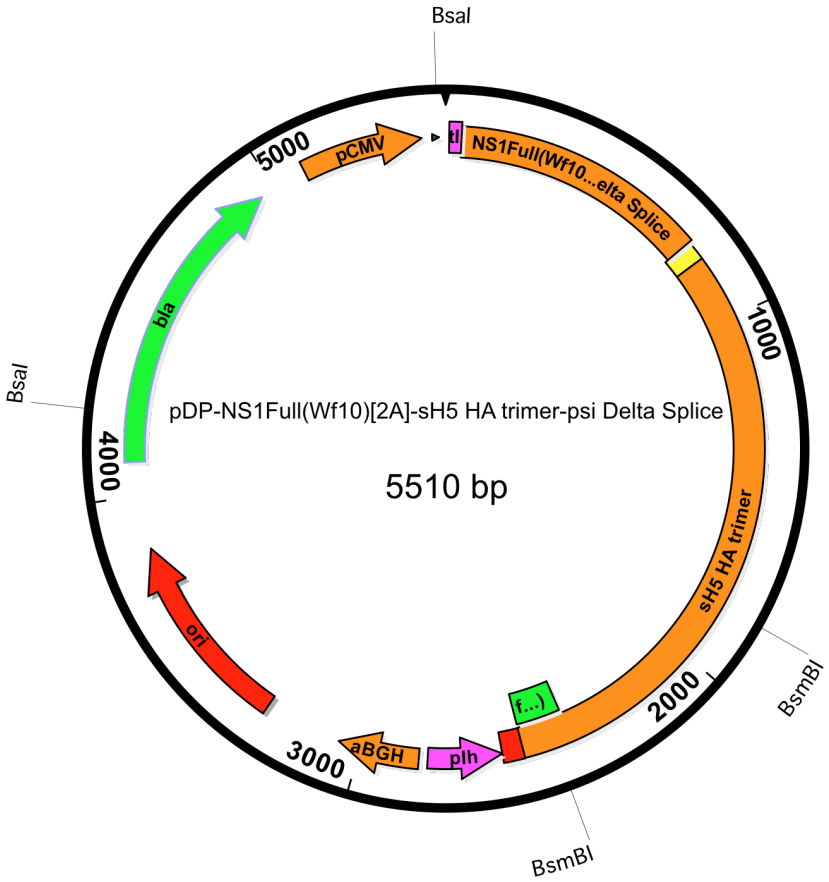
Appendices

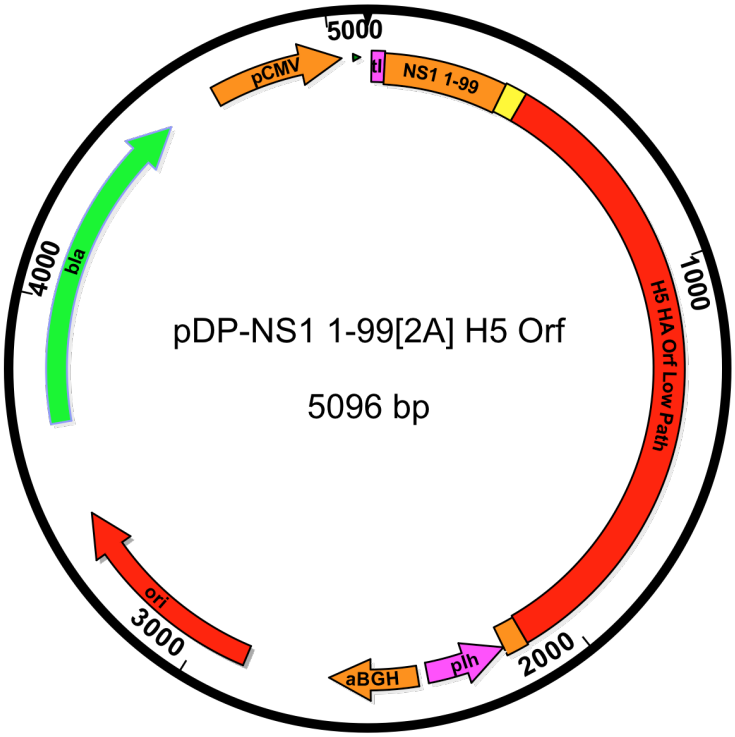
Some of the shuffled vectors developed in this study.




pDP-NS1 1-99[2A] vector
3423 bp
pCMV
tl
WF10_NS1 1-99
F2A
2A
pln
aBGH
ori
bla
AarI
AarI
500
1000
1500
2000
2500
3000


pDP-WF10 NS1full[2A]H5Headless.psi
4808 bp
pCMV
tl
NS1Full(Wf10) Delta Splice
H5 Headless 4 Gly linker
pIh
aBGH
ori
bla
500
1000
1500
2000
2500
3000
3500
4000
4500


BsaI
5000
pCMV
NS1Full(Wf10...elta Splice
1000
BsaI
bla
pDP-NS1Full(Wf10)[2A]-sH5 HA trimer-psi Delta Splice
5510 bp
4000
sH5 HA trimer
ori
2000
BsmBI
f...)
3000
aBGH
plh
BsmBI


5000
pCMV
NS1 1-99
1000
H5 HA Orf Low Path
2000
aBGH
pA
3000
ori
4000
bla
pDP-NS1 1-99[2A] H5 Orf
5096 bp

Bibliography


Al Hajjar, S. and K. McIntosh (2010). "The first influenza pandemic of the 21st century." Annals of Saudi medicine **30**(1): 1-10.

Al Hajjar, S. and K. McIntosh (2010). "The first influenza pandemic of the 21st century." Ann Saudi Med **30**(1): 1-10.

Ali, A., R. T. Avalos, et al. (2000). "Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein." Journal of virology **74**(18): 8709-8719.

Alymova, I. V., A. M. Green, et al. (2011). "Immunopathogenic and anti-bacterial effects of the H3N2 influenza A virus PB1-F2 map to amino acid residues 62, 75, 79, and 82." Journal of virology.

Ambrose, C. S., C. Luke, et al. (2008). "Current status of live attenuated influenza vaccine in the United States for seasonal and pandemic influenza." Influenza Other Respi Viruses **2**(6): 193-202.

Ambrose, C. S., T. Yi, et al. (2008). "Duration of protection provided by live attenuated influenza vaccine in children." Pediatr Infect Dis J **27**(8): 744-748.

Angata, T. and A. Varki (2002). "Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective." Chemical reviews **102**(2): 439-469.

Baigent, S. J. and J. W. McCauley (2003). "Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission." BioEssays : news and reviews in molecular, cellular and developmental biology **25**(7): 657-671.

Barbe, F., K. Atanasova, et al. (2010). "Cytokines and acute phase proteins associated with acute swine influenza infection in pigs." Vet J.

Barbe, F., K. Atanasova, et al. (2011). "Cytokines and acute phase proteins associated with acute swine influenza infection in pigs." Veterinary journal **187**(1): 48-53.

Barbe, F., X. Saelens, et al. (2010). "Role of IFN-alpha during the acute stage of a swine influenza virus infection." Res Vet Sci **88**(1): 172-178.

Barclay, W. S. and P. Palese (1995). "Influenza B viruses with site-specific mutations introduced into the HA gene." Journal of virology **69**(2): 1275-1279.

Barnard, D. L. (2009). "Animal models for the study of influenza pathogenesis and therapy." Antiviral research **82**(2): A110-122.

Barnard, D. L. (2009). "Animal models for the study of influenza pathogenesis and therapy." Antiviral Res **82**(2): A110-122.

Basler, C. F. and P. V. Aguilar (2008). "Progress in identifying virulence determinants of the 1918 H1N1 and the Southeast Asian H5N1 influenza A viruses." Antiviral research **79**(3): 166-178.

Basler, C. F., A. H. Reid, et al. (2001). "Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes." Proceedings of the National Academy of Sciences of the United States of America **98**(5): 2746-2751.

Baumgarth, N. and A. Kelso (1996). "In vivo blockade of gamma interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue." Journal of virology **70**(7): 4411-4418.

Bean, W. J., M. Schell, et al. (1992). "Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts." Journal of virology **66**(2): 1129-1138.

Belshe, R. B. (2004). "Current status of live attenuated influenza virus vaccine in the US." Virus research **103**(1-2): 177-185.

Belshe, R. B., C. S. Ambrose, et al. (2008). "Safety and efficacy of live attenuated influenza vaccine in children 2-7 years of age." Vaccine **26 Suppl 4**: D10-16.

Belshe, R. B., K. M. Edwards, et al. (2007). "Live attenuated versus inactivated influenza vaccine in infants and young children." The New England journal of medicine **356**(7): 685-696.

Belshe, R. B., P. M. Mendelman, et al. (1998). "The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children." The New England journal of medicine **338**(20): 1405-1412.

Block, S. L., R. Yogev, et al. (2008). "Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5-49 years of age." Vaccine **26**(38): 4940-4946.

Bourmakina, S. V. and A. Garcia-Sastre (2003). "Reverse genetics studies on the filamentous morphology of influenza A virus." The Journal of general virology **84**(Pt 3): 517-527.

Britton, A. P., K. R. Sojonky, et al. (2010). "Pandemic (H1N1) 2009 in skunks, Canada." Emerging infectious diseases **16**(6): 1043-1045.

Brockmeier, S. L., K. M. Lager, et al. (2009). "Adenovirus-mediated expression of interferon-alpha delays viral replication and reduces disease signs in swine challenged with porcine reproductive and respiratory syndrome virus." Viral immunology **22**(3): 173-180.

Brookes, S. M., R. M. Irvine, et al. (2009). "Influenza A (H1N1) infection in pigs." Vet Rec **164**(24): 760-761.

Brown, I. H., S. H. Done, et al. (1993). "Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains." The Veterinary record **132**(24): 598-602.

Brown, I. H., P. A. Harris, et al. (1998). "Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype." The Journal of general virology **79 ( Pt 12)**: 2947-2955.

Bruder, D., A. Srikiatkhachorn, et al. (2006). "Cellular immunity and lung injury in respiratory virus infection." Viral immunology **19**(2): 147-155.

Bryant, M. L., P. Brown, et al. (1999). "Comparison of the clearance of radiolabelled nose drops and nasal spray as mucosally delivered vaccine." Nucl Med Commun **20**(2): 171-174.

Castrucci, M. R. and Y. Kawaoka (1995). "Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein." Journal of virology **69**(5): 2725-2728.

Centanni, E. and E. Savonuzzi (1901). "La peste aviaria " Communicazione fatta all'Accademia delle Scienze mediche e naturali di ferrara.

Chambers, T. M., R. E. Holland, et al. (2001). "A new modified live equine influenza virus vaccine: phenotypic stability, restricted spread and efficacy against heterologous virus challenge." Equine Vet J **33**(7): 630-636.

Chan, M. C., R. W. Chan, et al. (2010). "Tropism and innate host responses of the 2009 pandemic H1N1 influenza virus in ex vivo and in vitro cultures of human conjunctiva and respiratory tract." The American journal of pathology **176**(4): 1828-1840.

Chan, W., H. Zhou, et al. (2008). "The cold adapted and temperature sensitive influenza A/Ann Arbor/6/60 virus, the master donor virus for live attenuated influenza vaccines, has multiple defects in replication at the restrictive temperature." Virology **380**(2): 304-311.

Chen, B. J., G. P. Leser, et al. (2008). "The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding." Journal of virology **82**(20): 10059-10070.

Chen, C. J., G. W. Chen, et al. (2010). "Differential localization and function of PB1-F2 derived from different strains of influenza A virus." Journal of virology **84**(19): 10051-10062.

Chen, G. L., E. W. Lamirande, et al. (2010). "Safety, immunogencity, and efficacy of a cold-adapted A/Ann Arbor/6/60 (H2N2) vaccine in mice and ferrets." Virology **398**(1): 109-114.

Chen, G. L. and K. Subbarao (2009). "Live attenuated vaccines for pandemic influenza." Curr Top Microbiol Immunol **333**: 109-132.

Chen, H., R. A. Bright, et al. (2007). "Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 influenza viruses in mice." Virus research **128**(1-2): 159-163.

Chen, W., P. A. Calvo, et al. (2001). "A novel influenza A virus mitochondrial protein that induces cell death." Nat Med **7**(12): 1306-1312.

Chen, Z., C. Santos, et al. (2009). "Evaluation of live attenuated influenza a virus h6 vaccines in mice and ferrets." Journal of virology **83**(1): 65-72.

Chen, Z., C. Santos, et al. (2009). "Evaluation of live attenuated influenza a virus h6 vaccines in mice and ferrets." J Virol **83**(1): 65-72.

Choi, Y. K., J. H. Lee, et al. (2004). "H3N2 influenza virus transmission from swine to turkeys, United States." Emerg Infect Dis **10**(12): 2156-2160.

Choi, Y. K., T. D. Nguyen, et al. (2005). "Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004." Journal of virology **79**(16): 10821-10825.

Chou, Y. Y., R. A. Albrecht, et al. (2011). "The M Segment of the 2009 New Pandemic H1N1 Influenza Virus Is Critical for Its High Transmission Efficiency in the Guinea Pig Model." Journal of virology **85**(21): 11235-11241.

Clouthier, S. C., T. Rector, et al. (2002). "Genomic organization of infectious salmon anaemia virus." The Journal of general virology **83**(Pt 2): 421-428.

Coleman, J. R. (2007). "The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages." Virol J **4**: 9.

Conenello, G. M. and P. Palese (2007). "Influenza A virus PB1-F2: a small protein with a big punch." Cell Host Microbe **2**(4): 207-209.
Conenello, G. M., D. Zamarin, et al. (2007). "A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence." PLoS Pathog **3**(10): 1414-1421.
Copeland, C. S., R. W. Doms, et al. (1986). "Assembly of influenza hemagglutinin trimers and its role in intracellular transport." The Journal of cell biology **103**(4): 1179-1191.
Cox, R. J., K. A. Brokstad, et al. (2004). "Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines." Scand J Immunol **59**(1): 1-15.
Crawford, P. C., E. J. Dubovi, et al. (2005). "Transmission of equine influenza virus to dogs." Science **310**(5747): 482-485.
Cross, K. J., L. M. Burleigh, et al. (2001). "Mechanisms of cell entry by influenza virus." Expert reviews in molecular medicine **3**(21): 1-18.
Da Silva, E. V., A. P. Da Rosa, et al. (2005). "Araguari virus, a new member of the family Orthomyxoviridae: serologic, ultrastructural, and molecular characterization." The American journal of tropical medicine and hygiene **73**(6): 1050-1058.
Dallegri, F. and L. Ottonello (1997). "Tissue injury in neutrophilic inflammation." Inflammation research : official journal of the European Histamine Research Society ... [et al.] **46**(10): 382-391.
Dawood, F. S., S. Jain, et al. (2009). "Emergence of a novel swine-origin influenza A (H1N1) virus in humans." N Engl J Med **360**(25): 2605-2615.
Dawood, F. S., S. Jain, et al. (2009). "Emergence of a novel swine-origin influenza A (H1N1) virus in humans." The New England journal of medicine **360**(25): 2605-2615.
de Felipe, P. (2004). "Skipping the co-expression problem: the new 2A "CHYSEL" technology." Genetic vaccines and therapy **2**(1): 13.
de Jong, J. C., D. J. Smith, et al. (2007). "Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe." Journal of virology **81**(8): 4315-4322.
de Jong, M. D., C. P. Simmons, et al. (2006). "Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia." Nature medicine **12**(10): 1203-1207.
De Vleeschauwer, A., K. Atanasova, et al. (2009). "Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs." PloS one **4**(8): e6662.
de Wit, E., V. J. Munster, et al. (2010). "Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host." Journal of virology **84**(3): 1597-1606.
de Wit, E., M. I. Spronken, et al. (2007). "A reverse-genetics system for Influenza A virus using T7 RNA polymerase." The Journal of general virology **88**(Pt 4): 1281-1287.
Desselberger, U., V. R. Racaniello, et al. (1980). "The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity." Gene **8**(3): 315-328.

Dias, A., D. Bouvier, et al. (2009). "The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit." Nature **458**(7240): 914-918.
Draper, S. J. and J. L. Heeney (2010). "Viruses as vaccine vectors for infectious diseases and cancer." Nature reviews. Microbiology **8**(1): 62-73.
Ducatez, M. F., B. Hause, et al. (2011). "Multiple Reassortment between Pandemic (H1N1) 2009 and Endemic Influenza Viruses in Pigs, United States." Emerging infectious diseases **17**(9): 1624-1629.
Dudek, S. E., L. Wixler, et al. (2011). "The influenza virus PB1-F2 protein has interferon-antagonistic activity." Biological chemistry.
Dundon, W. G., P. De Benedictis, et al. (2010). "Serologic evidence of pandemic (H1N1) 2009 infection in dogs, Italy." Emerging infectious diseases **16**(12): 2019-2021.
Easterday, B. C. a. V. R., K. (1999). " Swine Influenza." Diseases of Swine **Iowa state University Press, Ames, Iowa, USA**: 277-290.
Ekiert, D. C., R. H. Friesen, et al. (2011). "A highly conserved neutralizing epitope on group 2 influenza A viruses." Science **333**(6044): 843-850.
Elleman, C. J. and W. S. Barclay (2004). "The M1 matrix protein controls the filamentous phenotype of influenza A virus." Virology **321**(1): 144-153.
Enami, M., W. Luytjes, et al. (1990). "Introduction of site-specific mutations into the genome of influenza virus." Proceedings of the National Academy of Sciences of the United States of America **87**(10): 3802-3805.
Enami, M. and P. Palese (1991). "High-efficiency formation of influenza virus transfectants." Journal of virology **65**(5): 2711-2713.
Enami, M., G. Sharma, et al. (1991). "An influenza virus containing nine different RNA segments." Virology **185**(1): 291-298.
Enserink, M. and J. Cohen (2009). "Virus of the year. The novel H1N1 influenza." Science **326**(5960): 1607.
Ferko, B., J. Stasakova, et al. (2001). "Hyperattenuated recombinant influenza A virus nonstructural-protein-encoding vectors induce human immunodeficiency virus type 1 Nef-specific systemic and mucosal immune responses in mice." Journal of virology **75**(19): 8899-8908.
Fernandez-Sesma, A., S. Marukian, et al. (2006). "Influenza virus evades innate and adaptive immunity via the NS1 protein." Journal of virology **80**(13): 6295-6304.
Fodor, E., L. Devenish, et al. (1999). "Rescue of influenza A virus from recombinant DNA." Journal of virology **73**(11): 9679-9682.
Fouchier, R. A., V. Munster, et al. (2005). "Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls." Journal of virology **79**(5): 2814-2822.
Fouchier, R. A., P. M. Schneeberger, et al. (2004). "Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome." Proceedings of the National Academy of Sciences of the United States of America **101**(5): 1356-1361.
Fujii, K., Y. Fujii, et al. (2005). "Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions." Journal of virology **79**(6): 3766-3774.

Fujii, K., M. Ozawa, et al. (2009). "Incorporation of influenza A virus genome segments does not absolutely require wild-type sequences." The Journal of general virology **90**(Pt 7): 1734-1740.

Fukuyama, S. and Y. Kawaoka (2011). "The pathogenesis of influenza virus infections: the contributions of virus and host factors." Current opinion in immunology **23**(4): 481-486.

Gagneux, P., M. Cheriyan, et al. (2003). "Human-specific regulation of alpha 2-6-linked sialic acids." The Journal of biological chemistry **278**(48): 48245-48250.

Gambaryan, A. S., A. I. Karasin, et al. (2005). "Receptor-binding properties of swine influenza viruses isolated and propagated in MDCK cells." Virus research **114**(1-2): 15-22.

Gao, Q., A. C. Lowen, et al. (2010). "A nine-segment influenza a virus carrying subtype H1 and H3 hemagglutinins." Journal of virology **84**(16): 8062-8071.

Gao, Q. and P. Palese (2009). "Rewiring the RNAs of influenza virus to prevent reassortment." Proceedings of the National Academy of Sciences of the United States of America **106**(37): 15891-15896.

Garcia-Sastre, A., T. Muster, et al. (1994). "Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus." Journal of virology **68**(10): 6254-6261.

Garten, R. J., C. T. Davis, et al. (2009). "Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans." Science **325**(5937): 197-201.

Garulli, B., Y. Kawaoka, et al. (2004). "Mucosal and systemic immune responses to a human immunodeficiency virus type 1 epitope induced upon vaginal infection with a recombinant influenza A virus." Journal of virology **78**(2): 1020-1025.

Gauger, P. C., A. L. Vincent, et al. (2011). "Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus." Vaccine **29**(15): 2712-2719.

Gibbs, A. J., J. S. Armstrong, et al. (2009). "From where did the 2009 'swine-origin' influenza A virus (H1N1) emerge?" Virology journal **6**: 207.

Gibbs, A. J., J. S. Armstrong, et al. (2009). "From where did the 2009 'swine-origin' influenza A virus (H1N1) emerge?" Virol J **6**: 207.

Gibbs, E. P. and T. C. Anderson (2010). "Equine and canine influenza: a review of current events." Animal health research reviews / Conference of Research Workers in Animal Diseases **11**(1): 43-51.

Gill, J. R., Z. M. Sheng, et al. (2010). "Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections." Arch Pathol Lab Med **134**(2): 235-243.

Golde, W. T., P. Gollobin, et al. (2005). "A rapid, simple, and humane method for submandibular bleeding of mice using a lancet." Lab Anim (NY) **34**(9): 39-43.

Gomez-Puertas, P., C. Albo, et al. (2000). "Influenza virus matrix protein is the major driving force in virus budding." Journal of virology **74**(24): 11538-11547.

Gong, J., W. Xu, et al. (2007). "Structure and functions of influenza virus neuraminidase." Current medicinal chemistry **14**(1): 113-122.

Govorkova, E. A., J. E. Rehg, et al. (2005). "Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004." Journal of virology **79**(4): 2191-2198.

Graef, K. M., F. T. Vreede, et al. (2010). "The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon." Journal of virology **84**(17): 8433-8445.

Graham, M. B., D. K. Dalton, et al. (1993). "Response to influenza infection in mice with a targeted disruption in the interferon gamma gene." The Journal of experimental medicine **178**(5): 1725-1732.

Gramer, M. R., J. H. Lee, et al. (2007). "Serologic and genetic characterization of North American H3N2 swine influenza A viruses." Canadian journal of veterinary research = Revue canadienne de recherche veterinaire **71**(3): 201-206.

Gray, G. C., T. McCarthy, et al. (2007). "Swine workers and swine influenza virus infections." Emerging infectious diseases **13**(12): 1871-1878.

Greninger, A. L., E. C. Chen, et al. (2010). "A metagenomic analysis of pandemic influenza A (2009 H1N1) infection in patients from North America." PloS one **5**(10): e13381.

Grivel, J. C. and L. Margolis (2009). "Use of human tissue explants to study human infectious agents." Nature protocols **4**(2): 256-269.

Guo, J., L. Yao, et al. (2010). "[Immunological evaluation of vector-expressed M2 and HA genes of H5N1 influenza virus in mice]." Sheng wu gong cheng xue bao = Chinese journal of biotechnology **26**(5): 649-656.

Hai, R., M. Schmolke, et al. "PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models." J Virol **84**(9): 4442-4450.

Halbur, P. G., P. S. Paul, et al. (1995). "Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus." Veterinary pathology **32**(6): 648-660.

Halbur, P. G., P. S. Paul, et al. (1995). "Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus." Vet Pathol **32**(6): 648-660.

Hale, B. G., R. E. Randall, et al. (2008). "The multifunctional NS1 protein of influenza A viruses." The Journal of general virology **89**(Pt 10): 2359-2376.

Harder, T. C. and T. W. Vahlenkamp (2010). "Influenza virus infections in dogs and cats." Veterinary immunology and immunopathology **134**(1-2): 54-60.

Hatta, M., P. Gao, et al. (2001). "Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses." Science **293**(5536): 1840-1842.

Hayden, F. G., R. Fritz, et al. (1998). "Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense." The Journal of clinical investigation **101**(3): 643-649.

He, J., D. Feng, et al. (2006). "Association of SARS susceptibility with single nucleic acid polymorphisms of OAS1 and MxA genes: a case-control study." BMC infectious diseases **6**: 106.

He, X. S., T. H. Holmes, et al. (2006). "Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines." J Virol **80**(23): 11756-11766.

Hensley, S. E., S. R. Das, et al. (2009). "Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift." Science **326**(5953): 734-736.

Hickman, D., M. J. Hossain, et al. (2008). "An avian live attenuated master backbone for potential use in epidemic and pandemic influenza vaccines." J Gen Virol **89**(Pt 11): 2682-2690.

Hirst, G. K. (1941). "The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected with Influenza Virus." Science **94**(2427): 22-23.

Hoffmann, E., G. Neumann, et al. (2000). "A DNA transfection system for generation of influenza A virus from eight plasmids." Proceedings of the National Academy of Sciences of the United States of America **97**(11): 6108-6113.

Hoffmann, E., G. Neumann, et al. (2000). "A DNA transfection system for generation of influenza A virus from eight plasmids." Proc. Natl. Acad. Sci. U S A. **97**(11): 6108-6113.

Hoffmann, E., J. Stech, et al. (2001). "Universal primer set for the full-length amplification of all influenza A viruses." Arch Virol **146**(12): 2275-2289.

Hoffmann, E., J. Stech, et al. (2001). "Universal primer set for the full-length amplification of all influenza A viruses." Arch Virol **146**(12): 2275-2289.

Hofshagen, M., B. Gjerset, et al. (2009). "Pandemic influenza A(H1N1)v: human to pig transmission in Norway?" Euro Surveill **14**(45).

Horimoto, T. and Y. Kawaoka (1994). "Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus." Journal of virology **68**(5): 3120-3128.

Horimoto, T. and Y. Kawaoka (2009). "Designing vaccines for pandemic influenza." Curr Top Microbiol Immunol **333**: 165-176.

Howard, W. A., S. C. Essen, et al. (2011). "Reassortant Pandemic (H1N1) 2009 Virus in Pigs, United Kingdom." Emerging infectious diseases **17**(6): 1049-1052.

Howden, K. J., E. J. Brockhoff, et al. (2009). "An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm." Can Vet J **50**(11): 1153-1161.

Howden, K. J., E. J. Brockhoff, et al. (2009). "An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm." The Canadian veterinary journal. La revue veterinaire canadienne **50**(11): 1153-1161.

Huang, I. C., W. Li, et al. (2008). "Influenza A virus neuraminidase limits viral superinfection." Journal of virology **82**(10): 4834-4843.

Hughes, M. T., M. Matrosovich, et al. (2000). "Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice." Journal of virology **74**(11): 5206-5212.

Hulse, D. J., R. G. Webster, et al. (2004). "Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens." Journal of virology **78**(18): 9954-9964.

Hutchinson, E. C., J. C. von Kirchbach, et al. (2010). "Genome packaging in influenza A virus." The Journal of general virology **91**(Pt 2): 313-328.

Ibricevic, A., A. Pekosz, et al. (2006). "Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells." Journal of virology **80**(15): 7469-7480.

Ito, T., J. N. Couceiro, et al. (1998). "Molecular basis for the generation in pigs of influenza A viruses with pandemic potential." Journal of virology **72**(9): 7367-7373.

Ito, T. and Y. Kawaoka (2000). "Host-range barrier of influenza A viruses." Veterinary microbiology **74**(1-2): 71-75.

Itoh, Y., K. Shinya, et al. (2009). "In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses." Nature **460**(7258): 1021-1025.

Iwatsuki-Horimoto, K., T. Horimoto, et al. (2006). "The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly." Journal of virology **80**(11): 5233-5240.

Jackson, D., M. J. Hossain, et al. (2008). "A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity." Proceedings of the National Academy of Sciences of the United States of America **105**(11): 4381-4386.

Jiao, P., G. Tian, et al. (2008). "A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice." Journal of virology **82**(3): 1146-1154.

Jin, H., G. P. Leser, et al. (1997). "Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape." The EMBO journal **16**(6): 1236-1247.

Jin, H., B. Lu, et al. (2003). "Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60." Virology **306**(1): 18-24.

Jin, H., S. Manetz, et al. (2007). "Toxicological evaluation of live attenuated, cold-adapted H5N1 vaccines in ferrets." Vaccine **25**(52): 8664-8672.

Johnson, P. R., Jr., S. Feldman, et al. (1985). "Comparison of long-term systemic and secretory antibody responses in children given live, attenuated, or inactivated influenza A vaccine." Journal of medical virology **17**(4): 325-335.

Joseph, T., J. McAuliffe, et al. (2008). "A live attenuated cold-adapted influenza A H7N3 virus vaccine provides protection against homologous and heterologous H7 viruses in mice and ferrets." Virology **378**(1): 123-132.

Karasin, A. I., S. Carman, et al. (2006). "Identification of human H1N2 and human-swine reassortant H1N2 and H1N1 influenza A viruses among pigs in Ontario, Canada (2003 to 2005)." J Clin Microbiol **44**(3): 1123-1126.

Karasin, A. I., C. W. Olsen, et al. (2000). "Genetic characterization of an H1N2 influenza virus isolated from a pig in Indiana." J Clin Microbiol **38**(6): 2453-2456.

Karasin, A. I., M. M. Schutten, et al. (2000). "Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: evidence for wholly human and reassortant virus genotypes." Virus Res **68**(1): 71-85.

Kawaoka, Y., C. W. Naeve, et al. (1984). "Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin?" Virology **139**(2): 303-316.
Kay, R. M., S. H. Done, et al. (1994). "Effect of sequential porcine reproductive and respiratory syndrome and swine influenza on the growth and performance of finishing pigs." Vet Rec **135**(9): 199-204.
Kida, H., T. Ito, et al. (1994). "Potential for transmission of avian influenza viruses to pigs." The Journal of general virology **75 ( Pt 9)**: 2183-2188.
Kim, H. M., Y. W. Lee, et al. (2008). "Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs." Journal of virology **82**(9): 4265-4274.
Kimble, B., G. R. Nieto, et al. (2010). "Characterization of influenza virus sialic acid receptors in minor poultry species." Virology journal **7**: 365.
Kimble, J. B., E. Sorrell, et al. (2011). "Compatibility of H9N2 avian influenza surface genes and 2009 pandemic H1N1 internal genes for transmission in the ferret model." Proceedings of the National Academy of Sciences of the United States of America **108**(29): 12084-12088.
Kittel, C., B. Ferko, et al. (2005). "Generation of an influenza A virus vector expressing biologically active human interleukin-2 from the NS gene segment." Journal of virology **79**(16): 10672-10677.
Kittel, C., S. Sereinig, et al. (2004). "Rescue of influenza virus expressing GFP from the NS1 reading frame." Virology **324**(1): 67-73.
Klimov, A. I., A. Y. Egorov, et al. (1995). "Genetic stability of cold-adapted A/Leningrad/134/47/57 (H2N2) influenza virus: sequence analysis of live cold-adapted reassortant vaccine strains before and after replication in children." J Gen Virol **76 ( Pt 6)**: 1521-1525.
Kobasa, D., A. Takada, et al. (2004). "Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus." Nature **431**(7009): 703-707.
Koh, Y. Y., E. Jung da, et al. (2007). "Bronchoalveolar cellularity and interleukin-8 levels in measles bronchiolitis obliterans." Chest **131**(5): 1454-1460.
Kothalawala, H., M. J. Toussaint, et al. (2006). "An overview of swine influenza." The Veterinary quarterly **28**(2): 46-53.
Krumbholz, A., A. Philipps, et al. (2011). "Current knowledge on PB1-F2 of influenza A viruses." Medical microbiology and immunology **200**(2): 69-75.
Kumar, S. R., L. Deflube, et al. (2011). "Genetic characterization of swine influenza viruses (H3N2) isolated from Minnesota in 2006-2007." Virus genes **43**(2): 161-176.
Kwon, T. Y., S. S. Lee, et al. (2011). "Genetic characterization of H7N2 influenza virus isolated from pigs." Veterinary microbiology **153**(3-4): 393-397.
Lamb, R. A. and M. Takeda (2001). "Death by influenza virus protein." Nature medicine **7**(12): 1286-1288.
Lamb, R. A., S. L. Zebedee, et al. (1985). "Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface." Cell **40**(3): 627-633.

Lange, E., D. Kalthoff, et al. (2009). "Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs." J Gen Virol **90**(Pt 9): 2119-2123.

Lazarowitz, S. G. and P. W. Choppin (1975). "Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide." Virology **68**(2): 440-454.

Le Goffic, R., E. Bouguyon, et al. (2010). "Influenza A virus protein PB1-F2 exacerbates IFN-beta expression of human respiratory epithelial cells." Journal of immunology **185**(8): 4812-4823.

Le Goffic, R., O. Leymarie, et al. (2011). "Transcriptomic Analysis of Host Immune and Cell Death Responses Associated with the Influenza A Virus PB1-F2 Protein." PLoS pathogens **7**(8): e1002202.

Lear, J. D. (2003). "Proton conduction through the M2 protein of the influenza A virus; a quantitative, mechanistic analysis of experimental data." FEBS letters **552**(1): 17-22.

Lee, J. H., P. N. Pascua, et al. (2009). "Isolation and genetic characterization of H5N2 influenza viruses from pigs in Korea." Journal of virology **83**(9): 4205-4215.

Lekcharoensuk, P., K. M. Lager, et al. (2006). "Novel swine influenza virus subtype H3N1, United States." Emerg Infect Dis **12**(5): 787-794.

Leser, G. P. and R. A. Lamb (2005). "Influenza virus assembly and budding in raft-derived microdomains: a quantitative analysis of the surface distribution of HA, NA and M2 proteins." Virology **342**(2): 215-227.

Li, S., J. Schulman, et al. (1993). "Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus." Journal of virology **67**(11): 6667-6673.

Li, Z., Y. Jiang, et al. (2006). "The NS1 gene contributes to the virulence of H5N1 avian influenza viruses." Journal of virology **80**(22): 11115-11123.

Li, Z. N., S. N. Mueller, et al. (2005). "Chimeric influenza virus hemagglutinin proteins containing large domains of the Bacillus anthracis protective antigen: protein characterization, incorporation into infectious influenza viruses, and antigenicity." Journal of virology **79**(15): 10003-10012.

Liang, Y., Y. Hong, et al. (2005). "cis-Acting packaging signals in the influenza virus PB1, PB2, and PA genomic RNA segments." Journal of virology **79**(16): 10348-10355.

Lipatov, A. S., S. Andreansky, et al. (2005). "Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses." The Journal of general virology **86**(Pt 4): 1121-1130.

Lipatov, A. S., Y. K. Kwon, et al. (2008). "Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses." PLoS pathogens **4**(7): e1000102.

Lorusso, A., K. S. Faaberg, et al. (2010). "One-step real-time RT-PCR for pandemic influenza A virus (H1N1) 2009 matrix gene detection in swine samples." Journal of virological methods **164**(1-2): 83-87.

Loving, C. L., S. L. Brockmeier, et al. (2010). "Influenza virus coinfection with Bordetella bronchiseptica enhances bacterial colonization and host responses exacerbating pulmonary lesions." Microbial pathogenesis **49**(5): 237-245.

Lu, B., H. Zhou, et al. (2005). "Improvement of influenza A/Fujian/411/02 (H3N2) virus growth in embryonated chicken eggs by balancing the hemagglutinin and neuraminidase activities, using reverse genetics." Journal of virology **79**(11): 6763-6771.

Lunn, D. P., S. Hussey, et al. (2001). "Safety, efficacy, and immunogenicity of a modified-live equine influenza virus vaccine in ponies after induction of exercise-induced immunosuppression." J Am Vet Med Assoc **218**(6): 900-906.

Luoh, S. M., M. W. McGregor, et al. (1992). "Hemagglutinin mutations related to antigenic variation in H1 swine influenza viruses." Journal of virology **66**(2): 1066-1073.

Luytjes, W., M. Krystal, et al. (1989). "Amplification, expression, and packaging of foreign gene by influenza virus." Cell **59**(6): 1107-1113.

Ma, K., A. M. Roy, et al. (2001). "Nuclear export of influenza virus ribonucleoproteins: identification of an export intermediate at the nuclear periphery." Virology **282**(2): 215-220.

Ma, W., M. Gramer, et al. (2006). "Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States." J Virol **80**(10): 5092-5096.

Ma, W., K. M. Lager, et al. (2009). "The role of swine in the generation of novel influenza viruses." Zoonoses and public health **56**(6-7): 326-337.

Ma, W., K. M. Lager, et al. (2009). "The Role of Swine in the Generation of Novel Influenza Viruses." Zoonoses Public Health.

Ma, W., A. L. Vincent, et al. (2007). "Identification of H2N3 influenza A viruses from swine in the United States." Proc Natl Acad Sci U S A **104**(52): 20949-20954.

Maes, L., Haesebrouck, F. and Pensaert, M (1984). "Experimental reproduction of clinical disease by intratracheal inoculation of fattening pigs with swine influenza virus isolates." Proc. Int. Congr. Pig Vet. Soc **8**: 60.

Mahmood, K., R. A. Bright, et al. (2008). "H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses." Vaccine **26**(42): 5393-5399.

Manicassamy, B., S. Manicassamy, et al. (2010). "Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus." Proceedings of the National Academy of Sciences of the United States of America **107**(25): 11531-11536.

Manicassamy, B., R. A. Medina, et al. (2010). "Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines." PLoS Pathog **6**(1): e1000745.

Marjuki, H., C. Scholtissek, et al. "Three amino acid changes in PB1-F2 of highly pathogenic H5N1 avian influenza virus affect pathogenicity in mallard ducks." Arch Virol **155**(6): 925-934.

Marjuki, H., C. Scholtissek, et al. (2010). "Three amino acid changes in PB1-F2 of highly pathogenic H5N1 avian influenza virus affect pathogenicity in mallard ducks." Archives of virology **155**(6): 925-934.

Marsh, G. A., J. M. Watson, et al. (2003). "An evaluation of the genetic stability and pathogenicity of the Russian cold-adapted influenza A donor strains A/Leningrad/134/17/57 and A/Leningrad/134/47/57 in ferrets." J Virol Methods **107**(1): 63-69.

Martina, B. E., P. van den Doel, et al. (2011). "A recombinant influenza A virus expressing domain III of West Nile virus induces protective immune responses against influenza and West Nile virus." PloS one **6**(4): e18995.

Martínez-Sobrido, L. and A. García-Sastre (2007). "Recombinant influenza virus vectors." Future Virology **2**(4): 401-416.

Masic, A., L. A. Babiuk, et al. (2009). "Reverse genetics-generated elastase-dependent swine influenza viruses are attenuated in pigs." J Gen Virol **90**(Pt 2): 375-385.

Masic, A., J. S. Booth, et al. (2009). "Elastase-dependent live attenuated swine influenza A viruses are immunogenic and confer protection against swine influenza A virus infection in pigs." Journal of virology **83**(19): 10198-10210.

Masic, A., J. S. Booth, et al. (2009). "Elastase-dependent live attenuated swine influenza A viruses are immunogenic and confer protection against swine influenza A virus infection in pigs." J Virol **83**(19): 10198-10210.

Mathieu, C., V. Moreno, et al. (2010). "Pandemic (H1N1) 2009 in breeding turkeys, Valparaiso, Chile." Emerging infectious diseases **16**(4): 709-711.

Matrosovich, M., A. Tuzikov, et al. (2000). "Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals." Journal of virology **74**(18): 8502-8512.

Matrosovich, M. N., T. Y. Matrosovich, et al. (2004). "Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium." Journal of virology **78**(22): 12665-12667.

Matsuoka, Y., E. W. Lamirande, et al. (2009). "The ferret model for influenza." Current protocols in microbiology **Chapter 15**: Unit 15G 12.

Matsuoka, Y., D. E. Swayne, et al. (2009). "Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice." Journal of virology **83**(9): 4704-4708.

Mazur, I., D. Anhlan, et al. (2008). "The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein." Cell Microbiol **10**(5): 1140-1152.

McAuley, J. L., J. E. Chipuk, et al. (2010). "PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology." PLoS pathogens **6**(7): e1001014.

McAuley, J. L., F. Hornung, et al. (2007). "Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia." Cell Host Microbe **2**(4): 240-249.

McAuley, J. L., F. Hornung, et al. (2007). "Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia." Cell host & microbe **2**(4): 240-249.

McAuley, J. L., K. Zhang, et al. (2010). "The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis." Journal of virology **84**(1): 558-564.

Melen, K., R. Fagerlund, et al. (2003). "Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein." The Journal of biological chemistry **278**(30): 28193-28200.

Mitnaul, L. J., M. N. Matrosovich, et al. (2000). "Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus." Journal of virology **74**(13): 6015-6020.

Moreno, A., L. Di Trani, et al. (2011). "Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus." Veterinary microbiology **149**(3-4): 472-477.

Murakami, S., T. Horimoto, et al. (2008). "Establishment of canine RNA polymerase I-driven reverse genetics for influenza A virus: its application for H5N1 vaccine production." Journal of virology **82**(3): 1605-1609.

Muraki, Y. and S. Hongo (2010). "The molecular virology and reverse genetics of influenza C virus." Japanese journal of infectious diseases **63**(3): 157-165.

Myers, K. P., C. W. Olsen, et al. (2007). "Cases of swine influenza in humans: a review of the literature." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **44**(8): 1084-1088.

Myers, K. P., C. W. Olsen, et al. (2006). "Are swine workers in the United States at increased risk of infection with zoonotic influenza virus?" Clin Infect Dis **42**(1): 14-20.

Naffakh, N., A. Tomoiu, et al. (2008). "Host restriction of avian influenza viruses at the level of the ribonucleoproteins." Annual review of microbiology **62**: 403-424.

Nagarajan, M. M., G. Simard, et al. (2010). "Single-step multiplex conventional and real-time reverse transcription polymerase chain reaction assays for simultaneous detection and subtype differentiation of Influenza A virus in swine." Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **22**(3): 402-408.

Nayak, B., S. Kumar, et al. (2010). "Contributions of the avian influenza virus HA, NA, and M2 surface proteins to the induction of neutralizing antibodies and protective immunity." Journal of virology **84**(5): 2408-2420.

Nayak, B., S. N. Rout, et al. (2009). "Immunization of chickens with Newcastle disease virus expressing H5 hemagglutinin protects against highly pathogenic H5N1 avian influenza viruses." PloS one **4**(8): e6509.

Nayak, D. P., E. K. Hui, et al. (2004). "Assembly and budding of influenza virus." Virus research **106**(2): 147-165.

Nelli, R. K., S. V. Kuchipudi, et al. (2010). "Comparative distribution of human and avian type sialic acid influenza receptors in the pig." BMC veterinary research **6**: 4.

Nelli, R. K., S. V. Kuchipudi, et al. (2010). "Comparative distribution of human and avian type sialic acid influenza receptors in the pig." BMC Vet Res **6**: 4.

Nermut, M. V. and H. Frank (1971). "Fine structure of influenza A2 (Singapore) as revealed by negative staining, freeze-drying and freeze-etching." The Journal of general virology **10**(1): 37-51.

Neumann, G., K. Fujii, et al. (2005). "An improved reverse genetics system for influenza A virus generation and its implications for vaccine production." Proceedings of the National Academy of Sciences of the United States of America **102**(46): 16825-16829.
Neumann, G. and G. Hobom (1995). "Mutational analysis of influenza virus promoter elements in vivo." The Journal of general virology **76 ( Pt 7)**: 1709-1717.
Neumann, G., T. Noda, et al. (2009). "Emergence and pandemic potential of swine-origin H1N1 influenza virus." Nature **459**(7249): 931-939.
Neumann, G., T. Watanabe, et al. (1999). "Generation of influenza A viruses entirely from cloned cDNAs." Proceedings of the National Academy of Sciences of the United States of America **96**(16): 9345-9350.
Neumann, G., A. Zobel, et al. (1994). "RNA polymerase I-mediated expression of influenza viral RNA molecules." Virology **202**(1): 477-479.
Newman, A. P., E. Reisdorf, et al. (2008). "Human case of swine influenza A (H1N1) triple reassortant virus infection, Wisconsin." Emerging infectious diseases **14**(9): 1470-1472.
Nfon, C., Y. Berhane, et al. (2011). "Molecular and Antigenic Characterization of Triple-Reassortant H3N2 Swine Influenza Viruses Isolated from Pigs, Turkey and Quail in Canada." Transboundary and emerging diseases **58**(5): 394-401.
Nicholson, T. L., D. Kukielka, et al. (2011). "Utility of a panviral microarray for detection of swine respiratory viruses in clinical samples." Journal of clinical microbiology **49**(4): 1542-1548.
Nidom, C. A., R. Takano, et al. (2010). "Influenza A (H5N1) viruses from pigs, Indonesia." Emerging infectious diseases **16**(10): 1515-1523.
Noble, S., M. S. McGregor, et al. (1993). "Antigenic and genetic conservation of the haemagglutinin in H1N1 swine influenza viruses." The Journal of general virology **74 ( Pt 6)**: 1197-1200.
Nofs, S., M. Abd-Eldaim, et al. (2009). "Influenza virus A (H1N1) in giant anteaters (Myrmecophaga tridactyla)." Emerging infectious diseases **15**(7): 1081-1083.
Nunes, S. F., P. R. Murcia, et al. (2010). "An ex vivo swine tracheal organ culture for the study of influenza infection." Influenza and other respiratory viruses **4**(1): 7-15.
O'Neill, R. E., J. Talon, et al. (1998). "The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins." The EMBO journal **17**(1): 288-296.
OIE. (2008, 2008). "Swine influenza." World Organization for Animal Health (OIE). Manual of diagnostic tests and vaccines for terrestrial animals 2008. Retrieved 10/10/11, from http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.0.
Olsen, C. W. (2002). "The emergence of novel swine influenza viruses in North America." Virus Res **85**(2): 199-210.
Olsen, C. W. (2002). "The emergence of novel swine influenza viruses in North America." Virus research **85**(2): 199-210.
Olsen, C. W., A. I. Karasin, et al. (2006). "Triple reassortant H3N2 influenza A viruses, Canada, 2005." Emerg Infect Dis **12**(7): 1132-1135.
Osterhaus, A. D., G. F. Rimmelzwaan, et al. (2000). "Influenza B virus in seals." Science **288**(5468): 1051-1053.

Ozawa, M., S. Basnet, et al. (2011). "Impact of amino acid mutations in PB2, PB1-F2, and NS1 on the replication and pathogenicity of pandemic (H1N1) 2009 influenza viruses." Journal of virology **85**(9): 4596-4601.

Ozawa, M., S. T. Victor, et al. (2011). "Replication-incompetent influenza A viruses that stably express a foreign gene." The Journal of general virology.

Paillot, R., D. Hannant, et al. (2006). "Vaccination against equine influenza: quid novi?" Vaccine **24**(19): 4047-4061.

Palese, P. and M. Shaw (2007). Orthomyxoviridae: the viruses and their replication. Fields Virology. Philadelphia, PA, Lippincott Williams & Wilkins. **2:** 1647-1689.

Pappas, C., P. V. Aguilar, et al. (2008). "Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus." Proceedings of the National Academy of Sciences of the United States of America **105**(8): 3064-3069.

Pena, L., A. L. Vincent, et al. "Modifications in the polymerase genes of a swine-like triple reassortant influenza virus to generate live attenuated vaccines against 2009 pandemic H1N1 viruses." J Virol.

Pena, L., A. L. Vincent, et al. (2011). "Modifications in the polymerase genes of a swine-like triple-reassortant influenza virus to generate live attenuated vaccines against 2009 pandemic H1N1 viruses." Journal of virology **85**(1): 456-469.

Percy, N., W. S. Barclay, et al. (1994). "Expression of a foreign protein by influenza A virus." Journal of virology **68**(7): 4486-4492.

Pereda, A., J. Cappuccio, et al. (2010). "Pandemic (H1N1) 2009 outbreak on pig farm, Argentina." Emerging infectious diseases **16**(2): 304-307.

Pereda, A., A. Rimondi, et al. (2011). "Evidence of reassortment of pandemic H1N1 influenza virus in swine in Argentina: are we facing the expansion of potential epicenters of influenza emergence?" Influenza and other respiratory viruses.

Perez-Padilla, R., D. de la Rosa-Zamboni, et al. (2009). "Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico." N Engl J Med **361**(7): 680-689.

Perez, D. R. and R. O. Donis (2001). "Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity." Journal of virology **75**(17): 8127-8136.

Perez, J. T., A. Varble, et al. (2010). "Influenza A virus-generated small RNAs regulate the switch from transcription to replication." Proceedings of the National Academy of Sciences of the United States of America **107**(25): 11525-11530.

Perroncito, E. (1878). "Epizoozia tifoide neigalliancei." Annali della Academia d'agricoltora di Torino **21**: 87-126.

Ping, J., S. K. Dankar, et al. (2010). "PB2 and hemagglutinin mutations are major determinants of host range and virulence in mouse-adapted influenza A virus." Journal of virology **84**(20): 10606-10618.

Pleschka, S., R. Jaskunas, et al. (1996). "A plasmid-based reverse genetics system for influenza A virus." Journal of virology **70**(6): 4188-4192.

Portela, A. and P. Digard (2002). "The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication." The Journal of general virology **83**(Pt 4): 723-734.

Presti, R. M., G. Zhao, et al. (2009). "Quaranfil, Johnston Atoll, and Lake Chad viruses are novel members of the family Orthomyxoviridae." Journal of virology **83**(22): 11599-11606.

Racaniello, V. R. and D. Baltimore (1981). "Cloned poliovirus complementary DNA is infectious in mammalian cells." Science **214**(4523): 916-919.

Ramakrishnan, M. A., P. Wang, et al. (2010). "Triple reassortant swine influenza A (H3N2) virus in waterfowl." Emerging infectious diseases **16**(4): 728-730.

Reading, P. C., J. L. Miller, et al. (2000). "Involvement of the mannose receptor in infection of macrophages by influenza virus." Journal of virology **74**(11): 5190-5197.

Reed, L. J. and H. Muench (1938). "A simple method for estimating 50 percent endpoints." Am J Hyg **37**: 493.

Rhorer, J., C. S. Ambrose, et al. (2009). "Efficacy of live attenuated influenza vaccine in children: A meta-analysis of nine randomized clinical trials." Vaccine **27**(7): 1101-1110.

Richt, J. A., K. M. Lager, et al. (2004). "Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses." Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **16**(5): 367-373.

Richt, J. A., K. M. Lager, et al. (2003). "Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States." J Clin Microbiol **41**(7): 3198-3205.

Richt, J. A., K. M. Lager, et al. (2003). "Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States." Journal of clinical microbiology **41**(7): 3198-3205.

Richt, J. A., P. Lekcharoensuk, et al. (2006). "Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine." J Virol **80**(22): 11009-11018.

Robb, N. C., M. Smith, et al. (2009). "NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome." The Journal of general virology **90**(Pt 6): 1398-1407.

Robertson, J. S. (1979). "5' and 3' terminal nucleotide sequences of the RNA genome segments of influenza virus." Nucleic acids research **6**(12): 3745-3757.

Robertson, J. S., M. Schubert, et al. (1981). "Polyadenylation sites for influenza virus mRNA." Journal of virology **38**(1): 157-163.

Robinson, J. L., B. E. Lee, et al. (2007). "Swine influenza (H3N2) infection in a child and possible community transmission, Canada." Emerging infectious diseases **13**(12): 1865-1870.

Rodgers, B. C. and C. A. Mims (1982). "Influenza virus replication in human alveolar macrophages." Journal of medical virology **9**(3): 177-184.

((Rossman, J. S. and R. A. Lamb (2011). "Influenza virus assembly and budding." Virology **411**(2): 229-236.
Rott, R., H. D. Klenk, et al. (1995). "Influenza viruses, cell enzymes, and pathogenicity." American journal of respiratory and critical care medicine **152**(4 Pt 2): S16-19.
Ruigrok, R. W., P. J. Andree, et al. (1984). "Characterization of three highly purified influenza virus strains by electron microscopy." The Journal of general virology **65 ( Pt 4)**: 799-802.
Ruigrok, R. W., E. A. Hewat, et al. (1992). "Low pH deforms the influenza virus envelope." The Journal of general virology **73 ( Pt 4)**: 995-998.
Salomon, R., J. Franks, et al. (2006). "The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04." The Journal of experimental medicine **203**(3): 689-697.
Schafer, W. (1955). "Vergleichende sero-immunologische untersuchungen uber die viren der influenza unf klassichen geflugelpest. ." Z Naturforsch **10B**: 81-91.
Schmitt, A. P. and R. A. Lamb (2005). "Influenza virus assembly and budding at the viral budozone." Advances in virus research **64**: 383-416.
Schmolke, M., B. Manicassamy, et al. (2011). "Differential Contribution of PB1-F2 to the Virulence of Highly Pathogenic H5N1 Influenza A Virus in Mammalian and Avian Species." PLoS pathogens **7**(8): e1002186.
Scholtissek, C. (1995). "Molecular evolution of influenza viruses." Virus genes **11**(2-3): 209-215.
Scholtissek, C., H. Burger, et al. (1983). "Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds." Virology **129**(2): 521-523.
Scholtissek, C., H. Burger, et al. (1985). "The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses." Virology **147**(2): 287-294.
Scholtissek, C., I. Koennecke, et al. (1978). "Host range recombinants of fowl plague (influenza A) virus." Virology **91**(1): 79-85.
Schotsaert, M., M. De Filette, et al. (2009). "Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments." Expert review of vaccines **8**(4): 499-508.
Schulman, J. L. and P. Palese (1977). "Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells." Journal of virology **24**(1): 170-176.
Seo, S. H., R. Webby, et al. (2004). "No apoptotic deaths and different levels of inductions of inflammatory cytokines in alveolar macrophages infected with influenza viruses." Virology **329**(2): 270-279.
Sereinig, S., M. Stukova, et al. (2006). "Influenza virus NS vectors expressing the mycobacterium tuberculosis ESAT-6 protein induce CD4+ Th1 immune response and protect animals against tuberculosis challenge." Clinical and vaccine immunology : CVI **13**(8): 898-904.
Shaw, M. L., K. L. Stone, et al. (2008). "Cellular proteins in influenza virus particles." PLoS pathogens **4**(6): e1000085.

Sheerar, M. G., B. C. Easterday, et al. (1989). "Antigenic conservation of H1N1 swine influenza viruses." The Journal of general virology **70 ( Pt 12)**: 3297-3303.

Shih, S. R. and R. M. Krug (1996). "Novel exploitation of a nuclear function by influenza virus: the cellular SF2/ASF splicing factor controls the amount of the essential viral M2 ion channel protein in infected cells." The EMBO journal **15**(19): 5415-5427.

Shih, S. R., M. E. Nemeroff, et al. (1995). "The choice of alternative 5' splice sites in influenza virus M1 mRNA is regulated by the viral polymerase complex." Proceedings of the National Academy of Sciences of the United States of America **92**(14): 6324-6328.

Shimizu, T., N. Takizawa, et al. (2011). "Crucial role of the influenza virus NS2 (NEP) C-terminal domain in M1 binding and nuclear export of vRNP." FEBS letters **585**(1): 41-46.

Shinde, V., C. B. Bridges, et al. (2009). "Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009." N Engl J Med **360**(25): 2616-2625.

Shinya, K., M. Ebina, et al. (2006). "Avian flu: influenza virus receptors in the human airway." Nature **440**(7083): 435-436.

Shinya, K., Y. Fujii, et al. (2004). "Characterization of a neuraminidase-deficient influenza a virus as a potential gene delivery vector and a live vaccine." Journal of virology **78**(6): 3083-3088.

Shope, R. E. (1931). "The Etiology of Swine Influenza." Science **73**(1886): 214-215.

Shope, R. E. (1931). "Swine Influenza : Iii. Filtration Experiments and Etiology." The Journal of experimental medicine **54**(3): 373-385.

Siebler, J., O. Haller, et al. (1996). "Thogoto and Dhori virus replication is blocked by inhibitors of cellular polymerase II activity but does not cause shutoff of host cell protein synthesis." Archives of virology **141**(8): 1587-1594.

Smith, A. M., F. R. Adler, et al. (2011). "Effect of 1918 PB1-F2 expression on influenza A virus infection kinetics." PLoS computational biology **7**(2): e1001081.

Smith, G. J., J. Bahl, et al. (2009). "Dating the emergence of pandemic influenza viruses." Proceedings of the National Academy of Sciences of the United States of America **106**(28): 11709-11712.

Smith, G. J., D. Vijaykrishna, et al. (2009). "Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic." Nature **459**(7250): 1122-1125.

Smith, W., Andrewes C. H., and Laidlaw P. P. (1933). "A virus obtained from influenza patients." Lancet **2**: 66-68.

Snyder, M. H., A. J. Buckler-White, et al. (1987). "The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys." Journal of virology **61**(9): 2857-2863.

Solorzano, A., R. J. Webby, et al. (2005). "Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs." J Virol **79**(12): 7535-7543.

Song, H., G. R. Nieto, et al. (2007). "A new generation of modified live-attenuated avian influenza viruses using a two-strategy combination as potential vaccine candidates." Journal of virology **81**(17): 9238-9248.
Song, H., G. R. Nieto, et al. (2007). "A new generation of modified live-attenuated avian influenza viruses using a two-strategy combination as potential vaccine candidates." J Virol **81**(17): 9238-9248.
Song, J., H. Feng, et al. (2011). "The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks." Journal of virology **85**(5): 2180-2188.
Song, J. M., N. Van Rooijen, et al. (2011). "Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus." Proceedings of the National Academy of Sciences of the United States of America **108**(2): 757-761.
Song, M. S., J. H. Lee, et al. (2010). "Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea." Journal of clinical microbiology **48**(9): 3204-3211.
Sorrell, E. M., G. C. Ramirez-Nieto, et al. (2007). "Genesis of pandemic influenza." Cytogenetic and genome research **117**(1-4): 394-402.
Sorrell, E. M., H. Song, et al. (2010). "A 27-amino-acid deletion in the neuraminidase stalk supports replication of an avian H2N2 influenza A virus in the respiratory tract of chickens." Journal of virology **84**(22): 11831-11840.
Soto-Abraham, M. V., J. Soriano-Rosas, et al. (2009). "Pathological changes associated with the 2009 H1N1 virus." N Engl J Med **361**(20): 2001-2003.
Sovinova, O., B. Tumova, et al. (1958). "Isolation of a virus causing respiratory disease in horses." Acta virologica **2**(1): 52-61.
Sponseller, B. A., E. Strait, et al. (2010). "Influenza A pandemic (H1N1) 2009 virus infection in domestic cat." Emerging infectious diseases **16**(3): 534-537.
Staeheli, P., R. Grob, et al. (1988). "Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation." Molecular and cellular biology **8**(10): 4518-4523.
Starick, E., E. Lange, et al. (2011). "Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany." The Journal of general virology **92**(Pt 5): 1184-1188.
Steel, J., A. C. Lowen, et al. (2010). "Influenza virus vaccine based on the conserved hemagglutinin stalk domain." mBio **1**(1).
Strobel, I., M. Krumbholz, et al. (2000). "Efficient expression of the tumor-associated antigen MAGE-3 in human dendritic cells, using an avian influenza virus vector." Human gene therapy **11**(16): 2207-2218.
Subbarao, E. K., Y. Kawaoka, et al. (1993). "Rescue of an influenza A virus wild-type PB2 gene and a mutant derivative bearing a site-specific temperature-sensitive and attenuating mutation." Journal of virology **67**(12): 7223-7228.
Subbarao, E. K., W. London, et al. (1993). "A single amino acid in the PB2 gene of influenza A virus is a determinant of host range." Journal of virology **67**(4): 1761-1764.
Subbarao, K. and T. Joseph (2007). "Scientific barriers to developing vaccines against avian influenza viruses." Nature reviews. Immunology **7**(4): 267-278.

Sugita, Y., T. Noda, et al. (2011). "Ultracentrifugation Deforms Unfixed Influenza A Virions." The Journal of general virology.

Suguitan, A. L., Jr., J. McAuliffe, et al. (2006). "Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets." PLoS medicine **3**(9): e360.

Suzuki, F., Y. Arase, et al. (2004). "Single nucleotide polymorphism of the MxA gene promoter influences the response to interferon monotherapy in patients with hepatitis C viral infection." Journal of viral hepatitis **11**(3): 271-276.

Suzuki, Y., T. Ito, et al. (2000). "Sialic acid species as a determinant of the host range of influenza A viruses." Journal of virology **74**(24): 11825-11831.

Swenson, S. L., L. G. Koster, et al. (2010). "Natural cases of 2009 pandemic H1N1 Influenza A virus in pet ferrets." Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **22**(5): 784-788.

Tada, T., K. Suzuki, et al. (2011). "NP body domain and PB2 contribute to increased virulence of H5N1 highly pathogenic avian influenza viruses in chickens." Journal of virology **85**(4): 1834-1846.

Takano, R., C. A. Nidom, et al. (2009). "A comparison of the pathogenicity of avian and swine H5N1 influenza viruses in Indonesia." Archives of virology **154**(4): 677-681.

Talaat, K. R., R. A. Karron, et al. (2009). "A live attenuated H7N3 influenza virus vaccine is well tolerated and immunogenic in a Phase I trial in healthy adults." Vaccine **27**(28): 3744-3753.

Talon, J., M. Salvatore, et al. (2000). "Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach." Proceedings of the National Academy of Sciences of the United States of America **97**(8): 4309-4314.

Tang, Y., C. W. Lee, et al. (2005). "Isolation and characterization of H3N2 influenza A virus from turkeys." Avian Dis **49**(2): 207-213.

Tang, Y., C. W. Lee, et al. (2005). "Isolation and characterization of H3N2 influenza A virus from turkeys." Avian diseases **49**(2): 207-213.

Tate, M. D., D. L. Pickett, et al. (2010). "Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice." Journal of virology **84**(15): 7569-7580.

Taubenberger, J. K. and J. C. Kash (2010). "Influenza virus evolution, host adaptation, and pandemic formation." Cell host & microbe **7**(6): 440-451.

Thompson, C. I., W. S. Barclay, et al. (2004). "Changes in in vitro susceptibility of influenza A H3N2 viruses to a neuraminidase inhibitor drug during evolution in the human host." The Journal of antimicrobial chemotherapy **53**(5): 759-765.

Townsend, H. G., S. J. Penner, et al. (2001). "Efficacy of a cold-adapted, intranasal, equine influenza vaccine: challenge trials." Equine Vet J **33**(7): 637-643.

Townsend, H. G., S. J. Penner, et al. (2001). "Efficacy of a cold-adapted, intranasal, equine influenza vaccine: challenge trials." Equine veterinary journal **33**(7): 637-643.

Treanor, J. J., J. D. Campbell, et al. (2006). "Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine." The New England journal of medicine **354**(13): 1343-1351.

Trebbien, R., L. E. Larsen, et al. (2011). "Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs." Virology journal **8**(1): 434.

Trifonov, V. and R. Rabadan (2009). "The Contribution of the PB1-F2 protein to the fitness of Influenza A viruses and its recent evolution in the 2009 Influenza A (H1N1) pandemic virus." PLoS Curr **1**: RRN1006.

Tscherne, D. M. and A. Garcia-Sastre (2011). "Virulence determinants of pandemic influenza viruses." The Journal of clinical investigation **121**(1): 6-13.

Tu, J., H. Zhou, et al. (2009). "Isolation and molecular characterization of equine H3N8 influenza viruses from pigs in China." Archives of virology **154**(5): 887-890.

Uetani, K., M. Hiroi, et al. (2008). "Influenza A virus abrogates IFN-gamma response in respiratory epithelial cells by disruption of the Jak/Stat pathway." European journal of immunology **38**(6): 1559-1573.

Upham, J. P., D. Pickett, et al. (2010). "Macrophage receptors for influenza A virus: role of the macrophage galactose-type lectin and mannose receptor in viral entry." Journal of virology **84**(8): 3730-3737.

Van Poucke, S. G., J. M. Nicholls, et al. (2010). "Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution." Virology journal **7**: 38.

Van Reeth, K. (2000). "Cytokines in the pathogenesis of influenza." Veterinary microbiology **74**(1-2): 109-116.

Van Reeth, K., I. H. Brown, et al. (2008). "Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002-2003." Influenza and other respiratory viruses **2**(3): 99-105.

Van Reeth, K., G. Labarque, et al. (1999). "Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity." Research in veterinary science **67**(1): 47-52.

Van Reeth, K., S. Van Gucht, et al. (2002). "Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs." Viral immunology **15**(4): 583-594.

Van Reeth, K., S. Van Gucht, et al. (2002). "In vivo studies on cytokine involvement during acute viral respiratory disease of swine: troublesome but rewarding." Vet Immunol Immunopathol **87**(3-4): 161-168.

van Riel, D., L. M. Leijten, et al. (2011). "Highly Pathogenic Avian Influenza Virus H5N1 Infects Alveolar Macrophages without Virus Production or Excessive TNF-Alpha Induction." PLoS pathogens **7**(6): e1002099.

Varga, Z. T., I. Ramos, et al. (2011). "The influenza virus protein PB1-F2 inhibits the induction of type i interferon at the level of the MAVS adaptor protein." PLoS pathogens **7**(6): e1002067.

Vijaykrishna, D., L. L. Poon, et al. (2010). "Reassortment of pandemic H1N1/2009 influenza A virus in swine." Science **328**(5985): 1529.
Vincent, A. L., J. R. Ciacci-Zanella, et al. (2010). "Efficacy of inactivated swine influenza virus vaccines against the 2009 A/H1N1 influenza virus in pigs." Vaccine **28**(15): 2782-2787.
Vincent, A. L., K. M. Lager, et al. (2010). "Experimental inoculation of pigs with pandemic H1N1 2009 virus and HI cross-reactivity with contemporary swine influenza virus antisera." Influenza Other Respi Viruses **4**(2): 53-60.
Vincent, A. L., K. M. Lager, et al. (2007). "Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine." Vet Microbiol.
Vincent, A. L., K. M. Lager, et al. (2008). "Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine." Vet Microbiol **126**(4): 310-323.
Vincent, A. L., W. Ma, et al. (2008). "Swine influenza viruses a North American perspective." Adv Virus Res **72**: 127-154.
Vincent, A. L., W. Ma, et al. (2008). "Swine influenza viruses a North American perspective." Advances in virus research **72**: 127-154.
Vincent, A. L., W. Ma, et al. (2007). "Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine." Vaccine **25**(47): 7999-8009.
Vincent, A. L., S. L. Swenson, et al. (2009). "Characterization of an influenza A virus isolated from pigs during an outbreak of respiratory disease in swine and people during a county fair in the United States." Veterinary microbiology **137**(1-2): 51-59.
Vincent, L. L., B. H. Janke, et al. (1997). "A monoclonal-antibody-based immunohistochemical method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues." Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc **9**(2): 191-195.
Waddell, G. H., M. B. Teigland, et al. (1963). "A New Influenza Virus Associated with Equine Respiratory Disease." Journal of the American Veterinary Medical Association **143**: 587-590.
Wan, H. and D. R. Perez (2006). "Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses." Virology **346**(2): 278-286.
Wang, P., P. Palese, et al. (1997). "The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal." Journal of virology **71**(3): 1850-1856.
Wang, Z. and G. M. Duke (2007). "Cloning of the canine RNA polymerase I promoter and establishment of reverse genetics for influenza A and B in MDCK cells." Virology journal **4**: 102.
Wasilenko, J. L., C. W. Lee, et al. (2008). "NP, PB1, and PB2 viral genes contribute to altered replication of H5N1 avian influenza viruses in chickens." Journal of virology **82**(9): 4544-4553.

Watanabe, T., S. Watanabe, et al. (2003). "Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes." Journal of virology **77**(19): 10575-10583.

Watanabe, T., S. Watanabe, et al. (2009). "Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets." Proceedings of the National Academy of Sciences of the United States of America **106**(2): 588-592.

Webby, R. J., K. Rossow, et al. (2004). "Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population." Virus Res **103**(1-2): 67-73.

Webster, R. G., W. J. Bean, et al. (1992). "Evolution and ecology of influenza A viruses." Microbiological reviews **56**(1): 152-179.

Weingartl, H. M., R. A. Albrecht, et al. (2009). "Experimental infection of pigs with the human 1918 pandemic influenza virus." J Virol **83**(9): 4287-4296.

WHO. (2009). "Influenza (Seasonal)." Fact sheet n°211 Retrieved 10/10/11, 2011, from http://www.who.int/mediacentre/factsheets/fs211/en/.

WHO. (2010). "Antigenic and genetic characteristics of influenza A(H5N1) and influenza A(H9N2) viruses and candidate vaccine viruses developed for potential use in human vaccines." Retrieved 10/10/2011, 2011, from http://www.who.int/csr/disease/avian_influenza/guidelines/h5n1virus/en/index.html.

WHO. (2011). "Manual for the laboratory diagnosis and virological surveillance of influenza " Retrieved 10/10/2011, 2011, from http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf.

Wise, H. M., A. Foeglein, et al. (2009). "A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA." Journal of virology **83**(16): 8021-8031.

Wolschek, M., E. Samm, et al. (2011). "Establishment of a chimeric, replication-deficient influenza A virus vector by modulation of splicing efficiency." Journal of virology **85**(5): 2469-2473.

Wrammert, J., D. Koutsonanos, et al. (2011). "Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection." The Journal of experimental medicine **208**(1): 181-193.

Wu, R., Y. Guan, et al. (2010). "A live bivalent influenza vaccine based on a H9N2 virus strain." Vaccine **28**(3): 673-680.

Yassine, H. M., M. Q. Al-Natour, et al. (2007). "Interspecies and intraspecies transmission of triple reassortant H3N2 influenza A viruses." Virol J **4**(1): 129.

Yassine, H. M., M. Q. Al-Natour, et al. (2007). "Interspecies and intraspecies transmission of triple reassortant H3N2 influenza A viruses." Virology journal **4**: 129.

Yassine, H. M., M. Khatri, et al. (2011). "Potential role of viral surface glycoproteins in the replication of H3N2 triple reassortant influenza A viruses in swine and turkeys." Veterinary microbiology **148**(2-4): 175-182.

Yasuda, J., S. Nakada, et al. (1993). "Molecular assembly of influenza virus: association of the NS2 protein with virion matrix." Virology **196**(1): 249-255.

Ye, J., E. M. Sorrell, et al. (2010). "Variations in the hemagglutinin of the 2009 H1N1 pandemic virus: potential for strains with altered virulence phenotype?" PLoS pathogens **6**(10): e1001145.

Yen, H. L., C. H. Liang, et al. (2011). "Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets." Proceedings of the National Academy of Sciences of the United States of America **108**(34): 14264-14269.

Yewdell, J. W., J. R. Bennink, et al. (1985). "Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes." Proceedings of the National Academy of Sciences of the United States of America **82**(6): 1785-1789.

Yin, S. C., X. M. Peng, et al. (2006). "[MxA gene-88 G/T polymorphism influences the outcomes of HBV infection]." Zhonghua gan zang bing za zhi = Zhonghua ganzangbing zazhi = Chinese journal of hepatology **14**(6): 418-421.

Zamarin, D., A. Garcia-Sastre, et al. (2005). "Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1." PLoS Pathog **1**(1): e4.

Zamarin, D., M. B. Ortigoza, et al. (2006). "Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice." J Virol **80**(16): 7976-7983.

Zell, R., A. Krumbholz, et al. (2007). "Prevalence of PB1-F2 of influenza A viruses." J Gen Virol **88**(Pt 2): 536-546.

Zhang, X., W. Kong, et al. (2009). "A one-plasmid system to generate influenza virus in cultured chicken cells for potential use in influenza vaccine." Journal of virology **83**(18): 9296-9303.

Zhou, N. N., D. A. Senne, et al. (1999). "Genetic reassortment of avian, swine, and human influenza A viruses in American pigs." J Virol **73**(10): 8851-8856.

Zhou, Y., M. Konig, et al. (1998). "Membrane-anchored incorporation of a foreign protein in recombinant influenza virions." Virology **246**(1): 83-94.

Zhu, H., B. Zhou, et al. (2011). "Novel reassortment of eurasian avian-like and pandemic/2009 influenza viruses in Swine: infectious potential for humans." Journal of virology **85**(20): 10432-10439.