# PANDEMIC PREPAREDNESS AGAINST HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology – Doctor of Philosophy

2014

## ABSTRACT

PANDEMIC PREPAREDNESS
AGAINST HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES

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Influenza is an infectious disease of birds and mammals (including humans) caused by influenza viruses. In humans the disease has two outcomes; seasonal influenza epidemics (3-5 million severe cases and 250,000-500,000 deaths worldwide each year) and human influenza pandemics (even greater public health and economic loss). Molecular characterization of pandemic viruses of 20$^{th}$ (1918 H1N1, 1957 H2N2, and 1968 H2N3) and 21$^{st}$ centuries (2009 H1N1pdm) have revealed that these pandemic viruses evolved from avian influenza viruses. Among avian influenza viruses, highly pathogenic H5N1 and H7 viruses pose the greatest pandemic potential. The risk posed by these viruses is encountered by pandemic preparedness strategies; constant surveillance of virus evolution, risk assessment and preparation of pre-pandemic vaccine seeds. I identified two major needs in the pandemic preparedness; the first was the evolution of novel PB1-F2 truncations in H5N1 viruses and the second was the poor immunogenicity of H7 viruses and vaccines. To fulfill the needs, I worked on two research projects which are compiled as two chapters of this dissertation.

Firstly, we studied evolution and prevalence of PB1-F2 truncations in mammalian versus avian influenza viruses along with their risk assessment. We for the first time found independent occurrence and evolution of PB1-F2 truncations in multiple clades of

H5N1 viruses. In our risk assessment, we found a virus expressing C-terminal (N-terminal truncated) PB1-F2 (A/chicken/Vietnam/NCVD-296/2009; VN/296) 1000 fold more virulent than another very similar virus (A/chicken/Vietnam/NCVD-281/2009; VN/281) expressing full length PB1-F2. In vitro studies with cloned PB1-F2 expressing proteins of 0, 24 and 90 amino acids did not show any significant difference on cell apoptosis and IFNβ expression. However protein expression from full length and deleted (ATG-ACG) mutants were obvious in transfected A549 cells with fractional mitochondrial localization. We propose the model that C-terminal fragment of PB1-F2 as in virus VN/296 is responsible for the higher virulence and the phenotype is countered by N-terminal fragment as present in VN/281.

Secondly, we evaluated and compared the immunogenicity of purified recombinant hemagglutinin (HA) proteins of two H7 and two seasonal influenza viruses. Our studies showed that H7 HAs are weaker immunogens than seasonal influenza virus HAs. This suggests the use of higher doses of HA in H7 vaccines or possible use adjuvants.

To summarize, the PB1-F2 risk assessment showed that virus expressing C-terminal fragment is 1000 times more virulent than another very similar virus having full length PB1-F2. These findings highly recommend including PB1-F2 truncations in pre-pandemic virus surveillance and risk assessment. The H7 HA immunogenicity study concludes that the poor immunogenicity of H7 viruses and vaccines is due to intrinsic properties of their HA protein. Further recommendations include using either higher doses of HA in H7 prepandemic vaccines or adjuvants to achieve accepted level of protection. These recommendations will strengthen the current pandemic preparedness studies.

I dedicate this work to my loving wife, Anita Kumari, and my parents, Bishan Singh and Lakshmi Devi. Without your love, support, patience and encouragement, this monumental accomplishment would not have been possible. You all have sacrificed a lot in order for this to come to fruition. In addition, I also dedicate this work to late Dr. Aleksander Lipatov (Sasha) who was a dear friend, great senior and colleague.

## ACKNOWLEDGMENTS

I have had the opportunity to work with some great people while pursuing my degree at Michigan State University. First, I would like to thank Dr. Ian A. York for giving me the opportunity to join his lab at MSU and later at CDC to pursue this degree. I feel very lucky to have you as my PhD supervisor. Any combination of words won't be able to explain how thankful I am for your guidance and mentorship over the years. From you, I have learned not only to be a good scientist, but also to be a good colleague, collaborator and supervisor. I hope we maintain a relationship in the future. I would also like to thank the members of my committee, Dr. Roger Maes, Dr. Henry Hunt, Dr. Sungjin Kim and Dr. Andrea Amalfitano for being easily available for my committee meetings, continuous review of my dissertation progress and your expert feedbacks.

I would like to specially thank CMIB Program Director, Dr. Vilma Yuzbasiyan-Gurkan for helping me out every time whether it's joining a lab, choosing a course, moving to CDC, organizing committee meetings, preliminary exam and final PhD exam. You have contributed significantly to my and other CMIB students' achievements. The journey would have been tougher if without your guidance and help.

A special thanks to Kristy, Pin and Amrita (CDC, Influenza Division) for your contribution to the dissertation. Without your support it could not be completed in a timely manner. I also thank my seniors and colleagues at CDC/ Influenza Division, Dr. Charles Todd, Dr. James Steven, Dr. Terry Tumpey, Dr. S. Sambhara, Dr. Priya Ranjan, Dr. Li-Mei Chen, Dr. Jaber Hossain, and Dr. Jessica Belser for guiding me through these years and generously sharing your expertise and reagents. My thanks also go to my group

members, Ned, Jason, Adrian and Irina, for your friendship and good times as well as scientific inputs and inspirations. In addition to our group members, I thank members of other teams of CDC-Influenza Division, Missy, Kortney, Xiangjie and Laura for your help and answering all my queries.

A big thanks to Dr. Jaqueline Katz and Dr. Ruben Donis for giving me the opportunity to do my PhD dissertation at CDC, Influenza Division. I will always be grateful for the opportunity, your support and performance tracking which led to the timely completion of this dissertation.

I thank CMIB PhD Program, Dept. of MMG and Grad School at Michigan State University (East Lansing, MI, USA) for assistantships and fellowships. I am grateful to Oak Ridge Institute for Science and Education (Oak Ridge, TN) for its fellowships during my dissertation research at CDC.

I would like to thank my MSU friends Yogesh, Sonika, Rahul, V, and Manish for your friendship, inspiration and wonderful times we spent together in Lansing. In addition, I thank all my friends for your wells wishes, support and fun-time.

Finally, I would like to thank my my parents, Bishan Singh and Lakshmi Devi, my brother Nijesh Kumar for their support during this endeavor. It has meant a lot and I am truly grateful. Above all, I must thank Anita, you are the best and I appreciate you and the sacrifices that you have made more than words can say!

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## KEY TO ABBREVIATIONS

| | |
|---|---|
| HA | Heamgglutinin |
| NA | Neuraminidase |
| IAV | Influenza A virus |
| AIV | Avian influenza virus |
| HPAIV | Highly pathogenic avian influenza virus |
| HP | Highly pathogenic |
| LV | Low pathogenic |
| LPAIV | Low pathogenic avian influenza virus |
| RNA | Ribonucleic acid |
| CDC | Centers for Disease Control and Preventions, Atlanta, GA USA |
| HA | Heamgglutination |
| HI | Heamgglutination Inhibition |
| MN | Microneutralization |
| ELISA | Enzyme linked immunosorbant assay |
| RDE | Receptor destroying enzyme |
| BSL | Biosafety level |
| OD | Optical density |
| OPD | ortho-phenylenediamine |
| BSA | Bovine serum albumin |
| RBC | Red blood cell |
| RBS | Receptor binding site |

| | |
|---|---|
| LD50 | Median lethal dose |
| qRT-PCR | Quantitative Reverse transcription- Polymerase Chain Reaction |
| GISAID | Global Initiative on Sharing Avian Influenza Data |
| EID50 | Median egg infectious dose |
| TCID50 | Median tissue culture infectious dose |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| PFU | Plaque forming unit |

# CHAPTER 1

## GLOBAL INTRODUCTION

### Influenza Disease:

Influenza is an infectious disease of birds and mammals which is caused by influenza viruses. Wild aquatic birds are the reservoir of influenza viruses since they harbor all the different types of influenza viruses and serve as sources for all influenza infection of domestic poultry and mammals, including humans. Wild birds typically manifest very mild or asymptomatic intestinal tract infection (Webster, Bean et al. 1992), while in domestic poultry and humans the disease manifestation may range from mild or sub-clinical respiratory tract infection (Swayne 2007) to severe respiratory disease with high lethality depending on the strain of influenza virus and the presence of underlying host factors. Clinical symptoms of human influenza infection can range from asymptomatic to serious sickness characterized by sudden onset of fever, myalgia, and respiratory symptoms. In humans, seasonal influenza epidemics occur each year which result in 3-5 million severe cases and 250,000-500,000 deaths worldwide (Influenza (Seasonal)). Occasionally influenza viruses of novel antigenic characteristics, which have attained efficient human to human transmission capability, cause human influenza pandemics. There were 3 influenza pandemics (and one pseudo-pandemic) in the 20$^{th}$ century and one in the 21$^{st}$ century.

## Influenza Viruses:

Influenza viruses were isolated from chickens at the beginning of the 20th century (A/Brescia/1902 [H7N7]), from pigs in the late 1920s, from humans in the early 1930s, from horses and domestic ducks in the 1950s, from terns in 1961, and from many waterfowl and shorebirds since 1974 (reviewed in (Webster, Bean et al. 1992)). These viruses are lipid enveloped and belong to the family *Orthomyxoviridae.* Influenza viruses are divided into three types (A, B and C) based on their matrix and nucleoprotein; only types A and B viruses are responsible for most of the human influenza infections. While type B viruses have only one subtype, influenza A viruses (IAVs) are further divided into multiple subtypes based on their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). IAVs can belong to any subtype based on the combination of 17 known HA and 10 NA genes (Li, Sun et al. 2012, Tong, Li et al. 2012, Zhu, Yang et al. 2012). Birds are the natural reservoir for most IAVs because they harbor viruses with most of the HA and NA combinations. Three subtypes (H1, H2, and H3) have caused pandemics in humans so far (Webster, Bean et al. 1992). However, a number of other subtypes, including H5, H6, H7, H9, and H10 have also infected humans (Webster, Bean et al. 1992, Li, Li et al. 2006, Zhu, Wang et al. 2010, Arzey, Kirkland et al. 2012).

## IAV Genes and Proteins:

A mature virion of influenza A virus is composed of the nucleocapsid made from M1 and the membrane envelope derived from host cell membrane. The virion envelope contains three viral proteins; HA, NA and M2 (figure 1.1).

The IAV genome is comprised of 8 gene segments of negative-sense single-stranded RNA (figure 1.1). Each segment contains a coding region that encodes one or more proteins, flanked by short 5' and 3' non-coding sequences. IAV encode 10 core proteins, and a variable number of “accessory” proteins that are variably present in different strains of viruses (table 1.1). Two segments encode surface envelope glycoproteins that function as viral antigens: haemagglutinin (HA; approximately 1700 nucleotides, segment 4), which is responsible for binding to sialic acid receptors and entry into host cells, and neuraminidase (NA; approximately 1400 nucleotides, segment 6), which is involved in release of budding virions from infected cells. Besides HA and NA, there are two additional structural proteins; one is nucleoprotein (NP; approximately 1500 nucleotides, segment 5), which binds to the viral RNA, and another is the matrix protein M1 (approximately 750 nucleotides, segment 7) which comprises the main component of the viral capsid. The seventh segment also encodes a second protein, M2 (291 nucleotides), which is an integral membrane protein that functions as an ion channel.

The IAV polymerase complex is composed of three polymerase proteins; basic polymerase 2 (PB2; about 2,275 nucleotides in coding region, segment 1), which controls the recognition of host-cell RNA; basic polymerase 1 (PB1; about 2,270 nucleotides in the coding region, segment 2), which is suggested to catalyze nucleotide addition and the acidic protein (PA; about 2,150 nucleotides, segment 3), which possesses an endonuclease and a cap-snatching activity. Segment 8 is the smallest segment of the viral genome, and encodes two proteins. The first is a non-structural protein, NS1 (690 nucleotides), with multiple functions that particularly target the host innate immune response. The second protein, encoded by an overlapping reading

frame, is the NS2 protein (363 nucleotides), and has a role in viral RNA transport (O'Neill, Talon et al. 1998). These proteins (HA, NA, PB1, PB2, PA, NP, NS1, NS2, M1, and M2) are the ten core proteins that are found in all IAV strains.

Gene segment 2, in addition to PB1, also encodes another protein from its +1 open reading frame (PB1-F2) which localizes to mitochondria and has been implicated in cell apoptosis. In 2009, an additional protein (N40) was discovered which is an N-terminally truncated version of PB1 protein and is translated from fifth AUG (codon 40) in PB1 gene (Wise, Foeglein et al. 2009). In 2012, another IAV protein (PA-X) was discovered which is synthesized from Segment 3 (PA) (Jagger, Wise et al. 2012). A wide range of other proteins, often comprised of N- or C-terminal truncations of longer core proteins, have been observed in strains of influenza, but frequently are evolutionarily unstable; many of these proteins have not been described in the peer-reviewed literature but have been mentioned in conference proceedings and informal discussions.

**Public Health Burden of Influenza:**

Influenza infections in human population result in annual epidemics with significant morbidity and mortality worldwide. Annual influenza epidemics account for 12% ($10 billion) of the total economic burden in direct medical costs, however the overall economic burden is much higher ($87.1 billion) (Molinari, Ortega-Sanchez et al. 2007). Influenza and pneumonia rank as the eighth leading cause of all deaths in US in 2011 (Centers for Disease and Prevention 2010, Hoyert and Xu 2012). Out of 2,512,873 deaths in 2011, 53,667 (2.14%) deaths were associated with influenza and pneumonia (Hoyert and Xu 2012).

Besides seasonal epidemics, influenza sporadically causes more devastating pandemics. Influenza A viruses have caused 3 pandemics in 20$^{th}$ century (1918, 1957, 1968) and one recent pandemic in 2009. The pandemics of 1918, 1957, and 1968 were due to either purely avian viruses or to reassortants of avian and mammalian viruses, and that of 2009 was due to a reassorted swine virus that included avian influenza components (figure 1.2). Therefore, the evolution of avian viruses poses a constant threat of human pandemics.

**Influenza Pandemics:**

The 1918 pandemic virus has been called "the mother of all influenza pandemic viruses" since all subsequent human pandemic viruses have included components of the 1918 strain (reviewed in (Elderfield and Barclay 2011)). Viruses derived from 1918 pandemic continued to circulate in humans as seasonal influenza viruses, usually causing annual epidemics with moderate morbidity and mortality. However, the nature of the segmented genome of the virus makes it capable of reassortment with circulating avian and animal viruses and thus gives the potential for the emergence of new virus (reviewed in (Elderfield and Barclay 2011)).

The 1957 ("Asian") pandemic virus was a new subtype (H2N2) that derived its HA, NA and PB1 genes by reassortment with avian H2N2 viruses, but its remaining five gene segments were derived from the 1918 H1N1 pandemic virus. Since the human population had not been exposed to the H2 HA, there was no population immunity to H2 and the virus spread rapidly in the susceptible population, resulting in a death toll of about 2 million people (reviewed in (Elderfield and Barclay 2011)).

Another such event happened in 1968 (“Hong Kong Flu”), when the circulating human H2N2 virus reassorted with an avian virus of H3 subtype and resulted in 1-2 million deaths. This virus acquired two genes (HA and PB1) from an avian H3 virus, and retained NA from the previously-circulating H2N2 virus and the rest of the 5 gene segments from 1918 virus (reviewed in (Elderfield and Barclay 2011)).

The pandemic viruses of 1957 and 1968 replaced the previously circulating viruses, which therefore became extinct in the wild. An unusual event occurred in 1977 (the “Russian Flu” pseudo-pandemic) when H1N1 virus re-emerged into the human population. This virus was later shown to be very similar to H1N1 strains circulating in the 1950s (Nakajima, Desselberger et al. 1978) . Since viruses circulating in the wild rapidly accumulate mutations, the most likely explanation for the re-emergence of this strain is that it was stored in a laboratory for about 20 years until it was accidentally released (Nakajima, Desselberger et al. 1978). Although people older than about 20 years were generally immune to this virus due to prior exposure, enough younger people were non-immune for the H1N1 virus to sustain global infection. Since then, the two subtypes H1N1 and H3N2 have continued circulating in the human population as seasonal viruses, causing annual epidemics.

The world witnessed the first influenza pandemic of the 21$^{st}$ century in 2009-2010. The pandemic started in April 2009, when a previously undescribed influenza virus of subtype H1N1 was isolated from people in Mexico and US (“H1N1pdm09”). Within a month of the initial identification, the virus had spread globally, infecting more than 8000 people and resulting in 74 deaths in 40 countries. According to CDC’s estimates,

between April 2009 and April 2010 H1N1pdm09 was responsible for about 61 million infections, 274,000 hospitalizations, and 12,470 deaths in the US (Ross, Mahmood et al. 2009), with about 201,200 deaths worldwide (Dawood, Iuliano et al. 2012).

Genetic analyses of the isolates suggested that the H1N1pdm09 virus derived its genes from 3 genetic lineages of viruses: Eurasian swine lineage (an avian virus that entered swine in 1979) provided NA and M; the classical swine H1N1 lineage (originally descended from the 1918 human influenza, which had further evolved in swine since shortly after 1918) provided HA, NS and NP; and the swine triple reassortant lineage (a complex reassortant virus of swine that included avian and human segments, which entered swine directly or through humans around 1998) provided PB1, PB2 and PA (Garten, Davis et al. 2009) (figure 1.2).

Like the 20$^{th}$ century pandemic viruses, therefore, the H1N1pdm09 virus's genes were originally derived from avian viruses. These genes entered the swine genetic lineage, evolved, and reassorted multiple times to make the pandemic virus. Thus, all the pandemic viruses since the 20$^{th}$ century were either direct avian-to-human transmission (1918), were reassortants of circulating human subtypes with avian viruses (1957 and 1968), or incorporated avian viruses that had been filtered through swine. *History therefore teaches that avian influenza viruses pose a great threat for public health.*

**Avian Influenza Viruses:**

In general, most emerging human infections are of zoonotic nature; wild animals are responsible for more than 70% of all emerging infections (Taylor, Latham et al. 2001). In

the case of influenza, wild waterfowl are the original reservoir for all influenza A viruses in poultry and mammals, including humans (Olsen, Munster et al. 2006). Novel strains or subtypes of influenza viruses from this reservoir may cross host species barrier and infect humans either directly by birds or via intermediate hosts such as swine. Subsequently, adaptation of this cross species viruses may lead to human influenza pandemics, as is already evident from history.

Avian influenza was first clearly described in 1878, in Northern Italy, as a severe disease of domestic poultry and wild birds. It was originally known as “fowl plague” and it was unclear if it was distinct from other avian diseases such as fowl cholera (reviewed in (Kaleta and Rülke 2009)). In 1950, however, the disease was confirmed to be caused by Influenza A virus (*reviewed in* (Krauss and Webster 2010)).

Avian influenza viruses (AIVs) are typically asymptomatic in their reservoir hosts, which are usually wild waterfowl. Some subtypes of virus regularly infect domestic poultry (chickens, ducks, and turkeys), and may be subclinical or cause clinical disease. Based on their pathogenicity in domestic poultry, AIVs are categorized into two types: highly pathogenic avian influenza viruses (HPAIV) and low pathogenicity avian influenza viruses (LPAIV) (reviewed in (Senne 2010). To date, all known HPAIVs have belonged to two HA types; H5 and H7 (reviewed in (Senne 2010)). A critical determinant of pathogenicity is the cleavage site of the HA: In HPAIV, this cleavage site includes a number of basic residues. This polybasic cleavage site can arise by mutation of the normal site, so that HP viruses evolve from LP viruses (Banks, Speidel et al. 2001).

The first evidence of human infection with avian origin virus was identified during the Asian influenza pandemic (H2N2, 1957) when an avian influenza virus isolated from a turkey (A/Turkey/Massachusetts/3740/65(H6N2)) reacted strongly with antisera to the 1957 pandemic strain (Pereira, Tumova et al. 1967). Subsequently, human infections with both LPAIV and HPAIV were detected. Human LPAIV infections have resulted from cross-species transmission of LPAIV H9N2, H7N2, H7N3, and H7N7 and H7N9 (Table 2, reviewed in ref (Reperant, Kuiken et al. 2012)), as well as very rare instances of H10N7 and H6N1 viruses (Li, Li et al. 2006, Zhu, Wang et al. 2010, Arzey, Kirkland et al. 2012). Most of these LPAIV infections of humans have caused very mild or subclinical infections, with conjunctivitis or mild respiratory symptoms most common. In humans, HPAIV infections have resulted from cross-species transmission of HPAIV H5N1, H7N3 and H7N7 (Table 1.2, reviewed in ref (Reperant, Kuiken et al. 2012)). Although infection of humans with the HPAIV H5N1 strain is frequently a very severe disease, virus pathogenicity in poultry is not a perfect predictor of human disease, since the LPAIV H7N9, which causes little disease in poultry, has a high mortality rate in humans, whereas HPAIV H7N3 viruses have not been associated with severe disease to date (Tweed, Skowronski et al. 2004, Centers for Disease and Prevention 2012, Lopez-Martinez, Balish et al. 2013).

As well as respiratory disease, atypical disease manifestations, such as gastrointestinal or neurological symptoms, have been reported following infection with HPAIV H5N1, while ocular infection was reported for HPAIV of the H7 subtype. In most of these cases, HPAIV are transmitted from birds to humans via the respiratory or ocular route, although some strains of HPAIV H5N1 may use both oral and respiratory routes of

infection in other mammals and possibly also in humans. So far, little human-to-human transmission of LPAIV or HPAIV has been reported.

H5N1 viruses are now endemic in many countries. H5N1 became a public health concern in 1997 when a poultry outbreak in Hong Kong resulted in 18 human infections with six fatalities (Yuen, Chan et al. 1998). Since then, 608 human cases with case fatality rate of 59% have been confirmed as of Aug. 10, 2012 (WHO 2012). H5N1 viruses are of particular pandemic concern because of their endemicity in many countries, constant evolution and high case fatality rate.

H7 influenza viruses have also caused outbreaks in domestic birds in all parts of the globe; USA (Suarez, Garcia et al. 1999, Spackman, Senne et al. 2003), Canada (Pasick, Handel et al. 2005, Berhane, Hisanaga et al. 2009), China (Li, Li et al. 2006), Pakistan (Abbas, Spackman et al. 2010), Chile (Spackman, McCracken et al. 2006), Italy (Banks, Speidel et al. 2001, Campitelli, Mogavero et al. 2004, Di Trani, Bedini et al. 2004, Campitelli, Di Martino et al. 2008), Germany (Rohm, Suss et al. 1996), Netherlands (Stegeman, Bouma et al. 2004) and Australia (Selleck, Arzey et al. 2003, Bulach, Halpin et al. 2010). H7 human infections have been rare events (reviewed in (Belser, Bridges et al. 2009)), with two exceptions in which large numbers of people were infected. In 2003, a H7N7 outbreak occurred in The Netherlands, which resulted in at least 89 human infections and one death (Koopmans, Wilbrink et al. 2004). The number of infected people is based on virus isolate confirmed cases; however the actual number is suggested to be much higher as evidenced by serological studies (Meijer, Bosman et al. 2006). During this outbreak, both bird-to-human and limited

human-to-human transmission of H7 viruses were reported (Koopmans, Wilbrink et al. 2004).

Since 2003, sporadic H7 human infections have been reported in USA, UK and Canada; until 2013, unlike H5N1 infections, H7 virus infections of humans mostly caused ocular symptoms and no mortality (reviewed in (Belser, Bridges et al. 2009)). H7 viruses, although less virulent than the H5N1 subtype, are still of significant pandemic concern because of their ability to infect humans and other mammals and the compatibility of H7 HA with multiple NAs. Unlike most other HAs which only occur with 1 or 2 NAs, H7 has been found with 5 different NAs, 3 among which (H7N2, H7N3, H7N7) have caused infection in humans (reviewed in (Belser, Bridges et al. 2009)). This suggests that H7 viruses have more potential, among circulating strains, to make new reassortant viruses and thus adapt to human population.

The risk of pandemics caused by avian influenza viruses is further emphasized by the 2013 Chinese influenza outbreak where a novel avian influenza subtype (H7N9) entered the human population for the first time, leading to 135 laboratory-confirmed human cases and 44 deaths (Campitelli, Di Martino et al. 2008). The outbreak started in February 2013 and within 2 months virus spread to multiple geographical locations in China and infected more than 100 people (Saikh, Martin et al. 1995). Following the closing of live bird markets by Chinese Government, the incidence subsided through the summer months. Like other avian influenza viruses, this virus also lacks efficient human to human transmission; however it is unique among human infections with H7 viruses because of its high case fatality rate (32.6%); unlike previous H7 viruses which are almost non-lethal in humans with mild symptoms. It is important to note that this virus is

a LPAIV that rarely makes birds sick, complicating surveillance of the virus's spread in poultry. Further, this incidence underscores the need for constant surveillance of avian influenza viruses and strengthening our pandemic preparedness strategies.

HPAIVs do not yet have the ability of efficient human-to-human transmission. Among the reasons for this inefficient human transmission is their preferential attachment to α2, 3-linked sialic acids which are rarely present in the upper regions of the human respiratory tract (Shinya, Ebina et al. 2006). Despite their poor human-to-human transmission capability, both H5N1 and H7 viruses appear to pose the biggest pandemic risk. For example, HPAIVs might reassort with a circulating human strain, thereby potentially integrating lethality (of H5N1) and transmissibility (of human strains). Thus, these viruses pose a constant threat of emergence of new viruses due to genetic shift, and pose a continuous pandemic threat to humans.

**Pandemic Preparedness:**

New influenza viruses are constantly emerging in animal reservoirs due to reassortment of gene segments and accumulation of point mutations. To counteract the constant threat posed by newly emerging viruses, we need to constantly strengthen our pandemic preparedness by animal and human surveillance, assessing the risk of new viruses, and making vaccine seed stocks in advance. New viruses having significant genotypic variations from the previously circulating strains are evaluated for their phenotype (such as virulence and transmission) in suitable animal models (particularly mice and ferrets). Simultaneously, the immunogenicity (the ability to mount protective antibody response) of these viruses are evaluated by hemagglutination inhibition assays

(HI), ELISA and microneutralization assays (MN). Of these, HI is best correlated with protection against seasonal influenza viruses, with a titer of 40 or more considered to be protective in humans (J.C. de Jong 2000). However, clear correlates of protection have not been determined for avian viruses (Keitel and Atmar 2009, Haaheim and Katz 2011, Katz, Hancock et al. 2011). Other factors, including geographic distribution, population immunity, and potential human exposure, are also considered as part of risk assessment (Trock, Burke et al. 2012).

I focused my dissertation research on the two most significant components of pandemic preparedness: Risk assessment and pre-pandemic vaccine planning.

*1. Determinants of Pathogenicity:*

Pathogenicity of influenza viruses is a multigene trait and is well reviewed in (Medina and Garcia-Sastre 2011) (table 1.3). Broadly, pathogenicity includes the ability of the virus to bind with appropriate host cell receptors (HA protein), inhibit the host immune reponse (NS1), replicate efficiently (polymerase genes) and transmit efficiently between indviduals (NA and multiple other gene products). Pathogenicity therefore depends on multiple pathogenic determinants, the number of which keep increasing with the evolution of new viruses and with new research.

Segment 2 of IAV genome captured our interest because of the fact that it was the only gene besides surface glycoproteins (HA and NA) which was derived from avian viruses in all the pandemic viruses. Furthermore, its role and mechanism in pandemic viruses is still not fully understood. We and others have hypothesized that PB1 might be important in viral virulence because of PB1-F2 (see below). All the 20$^{th}$ century pandemic viruses

expressed full length PB1-F2, however the 2009 H1N1pdm09 virus lacked fuctional protein. Furthermore mammalian viruses generally have a truncated version of PB1-F2 while avian viruses are mostly full length, suggesting a role in host specific transmission and pathogenic phenotypes. Analysis of this gene product became the first part of my dissertation research.

*2. Pandemic Vaccines:*

After the identification of new viruses which differ significantly in their antigenicity, virulence, and/or transmissibility in model animals or humans, there arises the need of making new vaccine seed stocks. Selection of a virus for vaccine seed stock depends on its ability to mount protective serum antibody titer and to show protection against a majority of related viruses. Vaccine seed stocks are produced by reassortment of the novel virus with a virus that is non-pathogenic to humans, A/Puerto Rico/8/34(H1N1), to produce a reassortant virus that is antigenically like the novel virus but that can be grown to high titers in eggs and manufactured safely. In addition to classical reassortment in eggs, vaccine stocks can be produced by reverse genetics (Hoffmann, Neumann et al. 2000), and multiple other vaccine systems are being investigated (Kreijtz, Osterhaus et al. 2009).

**Gaps in Pandemic Preparedness:**

*In our review of literature we identified two significant gaps in the pandemic preparedness of HPAIVs.* (i) Risk assessment of truncated PB1-F2 protein in H5N1 viruses. (ii) Poor immunogenicity of H7 viruses.

*1. H5N1 PB1-F2:*

PB1-F2 is a short (87–90 a.a.) IAV protein encoded by the +1 reading frame of PB1 (Chen, Calvo et al. 2001). It was discovered serendipitously in 2001 during a search for putative CD8 T cell epitopes encoded by alternative reading frames (Chen, Calvo et al. 2001). The potential importance of PB1 segment in mammalian pathogenicity and adaptation is based on the fact that it was the only gene segment (other than the glycoprotein genes, HA and NA) that was exchanged in the pandemic events of 1957 and 1968 (Kawaoka, Krauss et al. 1989). PB1's role in pathogenicity and transmission is still not fully understood but could be at least partially explained by the discovery of PB1-F2 [30], which was present as a full-length protein in the first 3 pandemics but not in the less-virulent 2009 pandemic.

PB1-F2 is relatively unstable and undergoes proteasomal degradation shortly after expression (Chen, Calvo et al. 2001). It localizes in mitochondria and, at least in some cell types, causes cell death (Chen, Calvo et al. 2001, Gibbs, Malide et al. 2003). Although it is not required for viral replication in eggs or in cultured cells, it has been associated with virulence in mice and ducks (Chen, Calvo et al. 2001, Zamarin, Ortigoza et al. 2006, McAuley, Hornung et al. 2007, McAuley, Chipuk et al. 2010, Schmolke, Manicassamy et al. 2011). Its potential importance was further demonstrated by a study in mice that resulted in attenuation of 1918 H1N1 pandemic virus after its polymerase segments (including PB1) were replaced with those of the modern H1N1 strain (Tumpey, Basler et al. 2005).Full length PB1-F2 inhibits IFN-β when compared with deleted PB1-F2, and the N66S mutation also increases IFN-β inhibitory effect (Conenello, Tisoncik et al. 2011, Dudek, Wixler et al. 2011, Varga, Ramos et al. 2011).

One recent study showed the opposite effect where PB1-F2 increased IFN-β expression in epithelial cells (A549 and BEAS-2B) while it had no effect in immune cells (U-937 and Jurkat) (Le Goffic, Bouguyon et al. 2010). Such conflicting studies suggest that the effect of PB1-F2 may depend heavily on its genomic context

This virulence-enhancing effect of PB1-F2 is virus strain specific, since it is particularly seen in the viruses which have a PB1 gene of recent avian origin (McAuley, Chipuk et al. 2010). This effect was not appreciable in mice infected with a highly virulent virus (A/WSN/33(H1N1)) but was obvious in low pathogenic viruses (McAuley, Hornung et al. 2007). This may be explained by masking of the PB1-F2 effect by the presence of multiple pathogenic determinants of IAVs. In one study with A/Viet Nam/1203/2004(H5N1) virus (HPAIV), deletion of PB1-F2 caused delayed onset of clinical symptoms and systemic spreading of virus (Schmolke, Manicassamy et al. 2011).

The mechanisms underlying the pathogenic effects of PB1-F2 are still not clear. It has been suggested to be due to its mitochondrial targeting and apoptosis activity (Chen, Calvo et al. 2001, Gibbs, Malide et al. 2003, Zamarin, Garcia-Sastre et al. 2005) but in one study, PR8 virus that lacked PB1-F2 had reduced replication and virus spreading independent of its pro-apoptotic features (Mazur, Anhlan et al. 2008). Whatever the mechanism might be, deletion of PB1-F2 has resulted in altered viral virulence in a virus strain- and host-specific manner.

Further, molecular analysis suggests a role of PB1-F2 in adaptation of AIVs to new hosts following interspecies transmission. PB1-F2 was found to be under positive

selection based on the analysis of non-synonymous and synonymous mutations (Obenauer, Denson et al. 2006, Smith, Naipospos et al. 2006). This inference was questioned because of overlapping of PB1-F2 frame with PB1 (so that non-synonymous PB1-F2 mutations might merely be due to synonymous mutations in PB1) (Holmes, Lipman et al. 2006). However, a more recent study also found PB1-F2 under positive selection pressure while considering PB1 frame (Suzuki 2006).

Several previous papers have noted that PB1-F2 of human influenza viruses has become truncated due to premature stop codons. For example, Zell et al. analyzed 2226 PB1 sequences and found that the proportion of truncated PB1-F2 was higher in human viruses (19%) than those of avian viruses (4%) (Table 1 and S1 in reference (Zell, Krumbholz et al. 2007)). That study included data available until June 15, 2006, and the number of available sequences has increased dramatically since then. We confirmed this analysis using over 15,000 public and non-public influenza virus sequences. However, in this analysis, we observed a new trend in avian influenza viruses. Whereas in previous analyses B1-F2 truncations only appeared as sporadic, transient events in avian influenza viruses, we found evidence for a truncation in PB1-F2 of Vietnam H5N1 viruses that persisted for long periods, and expanded geographically. Collectively the proposed role of truncated PB1-F2 in mammalian adaptation, its interference with host innate immune response, constant evolution of H5N1 viruses and novel persistence of PB1-F2 truncations in avian viruses, makes it one of the most important targets for pandemic preparedness studies.

To summarize, the dramatically higher prevalence of truncated PB1-F2 in human viruses when compared to avian viruses suggests that viral adaptation to mammals vs.

avian species may be enhanced by PB1-F2 truncation**.** To fill this gap in pandemic preparedness of H5N1 viruses, we proposed to test the pathogenicity of H5N1 viruses with full-length or truncated PB1-F2 in mice model and *in vitro* in mammalian and avian cells (**Chapter 2** of this dissertation).

*2. Poor Immunogenicity of H7 Viruses:*

The second and the most important step in pandemic preparedness is the development of pre-pandemic vaccine virus seed stocks. The pandemic vaccine candidates for H7, H5 and H9 viruses have continually been made, updated, and as deemed appropriate, tested in human clinical trials.

Avian influenza vaccines typically have not induced antibody titers comparable to the seasonal flu vaccines. Plans for potential deployment of H5 and H9 vaccines include the use of high HA doses, and administration of vaccine with adjuvants. These modifications have generally induced antibody titers closer to those of seasonal vaccines (reviewed in (Keitel and Atmar 2009)). However, even at high HA doses, in limited trials H7 vaccines have performed poorly. If H7 vaccines are ineffective due to poor immunogenicity, this would represent a significant gap in pandemic preparedness (Joseph, McAuliffe et al. 2008, Cox, Madhun et al. 2009).

Several pieces of evidence suggest that H7 viruses and vaccines are poorly immunogenic. First, human sero-surveillance studies rarely detect antibodies to H7 viruses (15, 16); even in the people who had close contact with H7-infected poultry (Russell, Gamblin et al. 2004, Jia, de Vlas et al. 2009). Second, only 2 out of 89 RT-PCR and virus isolation confirmed cases (in the 2003 Netherlands H7N7 outbreak) showed HI titers of 40, which for human seasonal influenzas is considered protective

(Meijer, Bosman et al. 2006). Third, a clinical trial using a monovalent H7N7 inactivated vaccine made by Sanofi Pasteur Inc yielded very poor antibody responses (Couch, Patel et al. 2012). In this trial, each group of 24 or 25 people were immunized with two doses of 7.5 μg, 15 μg (the usual dose of HA in seasonal vaccines), 45 μg and 90 μg (a common dose for H5N1 non-adjuvanted vaccines). Only 1 of 99 vaccinees showed HI titers of 40 or more (Couch, Patel et al. 2012). All these evidences suggest that H7 viruses and vaccines may be poorly immunogenic, but the reasons remain largely unknown.

Poor immunogenicity of H7 viruses as suggested by HI and MN assays (13-16) could be interpreted in different directions. First, it could be because of the absence of optimal immunological assays and the incompletely understood correlates of protection for these viruses. For example, H7 viruses may induce antibodies against the HA stalk region than its globular head; such antibodies would be poorly detected by HI assays, although MN assays would still detect anti-stalk antibodies that are neutralizing. Or, protection may come from anti-NA or M2 antibodies, or cell-mediated immunity may play an important role. By this interpretation, immunity induced by H7 vaccines may be fully protective, but poorly detected. However, since the only way to test this in humans may be to challenge people with virulent viruses, this interpretation is best kept hypothetical.

Secondly, the poor immunogenicity may truly be an intrinsic characteristic of H7 viruses and may reflect a true lack of protection following H7 immunization. This could also be because of many reasons. For example, HA epitopes may be hidden because of glycosylation sites; viruses may replicate poorly; or virus molecules may be poorly

detected by the by innate immune receptors etc. One potential reason for poor detection by the innate immune response may be reduced overall glycosylation, since avian influenza viruses tend to have fewer carbohydrates than do seasonal influenza viruses.

Based on our current knowledge of H7 viruses we cannot determine which of these is most correct, because of lack of studies on H7 viruses. However, it is clear that there is a significant need to study the immune response to H7 viruses and to standardize protective correlates for these viruses. To fill this gap, we proposed the hypothesis that "**H7-HA is inherently less immunogenic than seasonal influenza viruses".** To test our hypothesis, we evaluated and compared immune response of purified HAs of H7 and seasonal viruses using different serological tests (**Chapter 3** of this dissertation).

**Summary:**

In summary, H5N1 and H7 influenza viruses may pose a great pandemic threat. H5N1 pandemic risk is high due to its endemicity in many bird species in many countries, constant evolution in birds, and high case fatality rate in humans. PB1, particularly originating from avian species, seems to play an important role in pathogenicity of reassortants, and functional PB1-F2 may be implicated in viral fitness, pathogenicity, and species adaptation. Since PB1-F2 is truncated in a large fraction of mammalian viruses and present as a full length protein in avian species (Zell, Krumbholz et al. 2007), it may have some role in the adaptation of virus in mammalian species after avian-mammalian transmission. The pandemic potential of H7 viruses is high because of their abilities to infect humans, compatibility of H7 HA with multiple NAs, and their potential for reassortment and human adaptation. Pandemic preparedness for H7

viruses is hampered by the lack of highly effective vaccine seed stocks because of their poor immunogenicity. In this study, we propose to explore these gaps and strengthen pandemic preparedness for HPAIVs.

# APPENDICES

## APPENDIX 1A: Tables

**Table 1.1 Influenza A virus genes and proteins.**

| Gene ID | Segment | Protein name | Protein function |
|---|---|---|---|
| 1 | Polymerase B2 (PB2) | Polymerase B2 (PB2) | Internal protein, Virus Replication |
| 2 | Polymerase B1 (PB1) | Polymerase B1 (PB1)<br>PB1-F2 | Internal protein, Virus replication<br>Mitochondrial targeting, Apoptosis |
| 3 | Polymerase A (PA) | Polymerase A (PA) | Internal protein, Virus replication |
| 4 | Hemagglutinin (HA) | Hemagglutinin (HA) | Surface glycoprotein, viral attachment, Antigenic determinant |
| 5 | Nucleoprotein (NP) | Nucleoprotein (NP) | Nucleocapsid protein, RNA coating, nuclear targeting, RNA transcription |
| 6 | Neuraminidase (NA) | Neuraminidase (NA) | Surface glycoprotein, antigenic determinant, viral release from host cells |
| 7 | Matrix (M) | Matrix 1 (M1)<br>Matrix 2 (M2) | Membrane protein stability<br>Membrane protein, viral uncoating |
| 8 | Non-structural (NS) | Non-structural 1 (NS1)<br>Non-structural 2 (NS2) | Viral immune evasion<br>Regulation of virus life cycle, specially mRNA transcription and localization of viral ribonucleic proteins |

((*adapted from (Lamb and Krug 2001) with minor modifications*)).

**Table 1.2 Reported cases of avian influenza virus infections by viral isolation.**

| Host of origin | Year, Place | Patho-type* | Subtype | Associated activity |
|---|---|---|---|---|
| Wild birds, Swans | 2005, Azerbaijan | HP | H5N1 | De-feathering, meat processing |
| Chicken, domestic ducks | 1997-present, Asia and Africa | HP | H5N1 | Caregiving, Butchering, De-feathering |
| Chicken | 2007, UK | LP | H7N2 | Visiting bird market |
| Chicken | 2006, UK | LP | H7N3 | Caregiving |
| Chicken | 2004, Canada | HP | H7N3 | Caregiving, managing outbreak |
| Chicken | 2003, Netherlands | HP | H7N7 | Caregiving, managing outbreak |
| Chicken, turkeys | 2002-2003, Italy | LP | H7N3 | Managing outbreak |
| Turkeys | 2002, USA | LP | H7N2 | Managing outbreak |
| Domestic ducks | 1996, UK | LP | H7N7 | Caregiving |
| Unknown | 2007, Hong Kong | LP | H9N2 | Unknown |
| Unknown | 2003, USA | LP | H7N2 | Unknown |
| Unknown | 2003, Hong Kong | LP | H9N2 | Unknown |
| Unknown | 1999, Hong Kong | LP | H9N2 | Unknown |
| Unknown | 1998, China | LP | H9N2 | Unknown |

((*adapted from (Reperant, Kuiken et al. 2012) with minor modifications*)).

*HP- Highly pathogenic; LP- low pathogenic.

**Table 1.3 Molecular determinants of IAV pathogenicity.**

| Virulence marker and pathogenic determinant | Pandemic virus | | | | HPAI virus | | Contemporary seasonal virus | |
|---|---|---|---|---|---|---|---|---|
| | 1918 H1N1 | 1957 H2N2 | 1968 H3N2 | 2009 H1N1 | H5N1 | H7N7 | H1N1 | H3N2 |
| **HA** | | | | | | | | |
| Sialic acid linkage specificity | α-2,6 | α-2,6 | α-2,6 | α-2,6 and α-2,3 | α-2,3 | α-2,3 | α-2,6 | α-2,6 |
| Receptor binding specificity | D190 and D225 | Q226 and N186 | L226 and S228 | D190 and D225 | Q196, Q226, G228 | Q226 | D190 and D225 | L226 and S228 |
| Multibasic cleavage site | No | No | No | No | Yes | Yes | No | No |
| **PB1–F2** | | | | | | | | |
| S66 (associated with increased virulence), N66 or truncation | S66 | N66 | N66 | TR* | S66 or N66 | N66 | TR* | N66 |
| **PB2** | | | | | | | | |
| Adaptation to mammalian hosts | K627 | K627 | K627 | R591 | K627 or N701 | K627 or N701 | K627 | K627 |
| **NS1** | | | | | | | | |
| PDZ domain-binding motif | KSEV | RSKV | RSKV | TR* | ESEV or EPEV | ESEV | RSEV | RSKV |
| E91 associated with virulence | No | No | No | No | Yes | No | No | No |
| **M2** | | | | | | | | |
| Resistant to adamantanes (presence of N31) | No | No | No | Yes | Yes and no | No | No | Yes |
| **NA** | | | | | | | | |
| Resistant to oseltamavir (presence of Y275 & S294) | No | No | No | No | No | No | Yes | No |

*(adapted from (Medina and Garcia-Sastre 2011) with minor modifications).*

TR* means truncated proteins which do not express the mentioned region.

## APPENDIX 1B: Figures

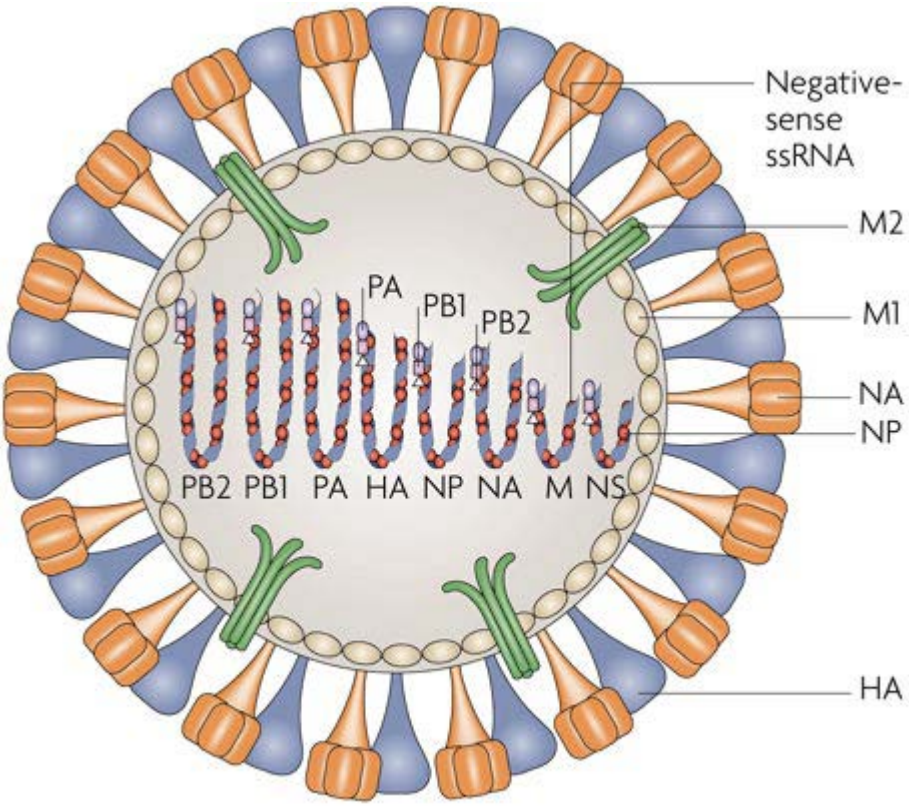


**Figure 1.1 Structure of influenza A virus** *(adapted from (Nelson and Holmes 2007))*. HA (Hemagglutinin) and NA (Neuraminidase) are the major glycoproteins on virus envelop. M1 protein makes the virus capsid which contains 8 gene segments, each bound to one molecule of each PA, PB1 and PB2 (polymerase complex) and multiple molecules of NP (nucleoprotein); the complex together is called RNP (ribonucleoprotein).

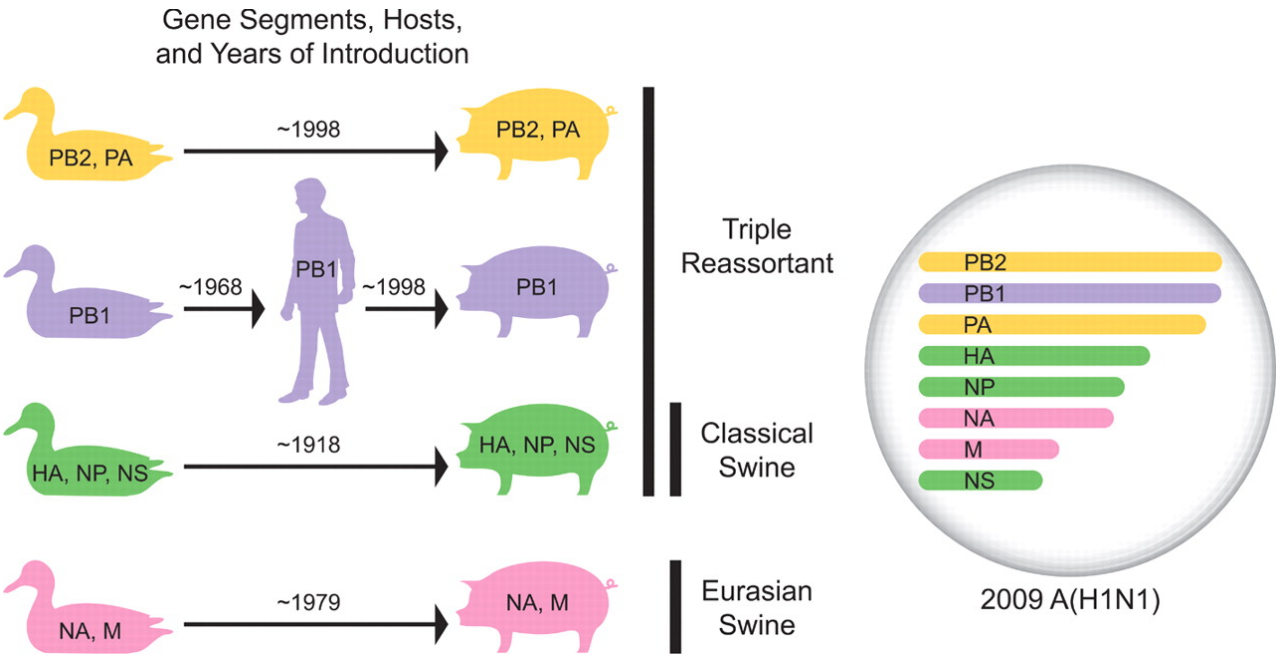


**Figure 1.2 Genetic components of 2009 H1N1pdm09 virus** *(Adapted from (Garten, Davis et al. 2009).* The figure shows interspecies transmission and evolution of gene segments of 2009 H1N1pdm09 virus.

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## CHAPTER 2

## PB1-F2 VARIANTS IN H5N1 AVIAN INFLUENZA VIRUSES

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**Abstract:**

Influenza A viruses (IAV) express the protein PB1-F2 from an alternate reading frame from the PB1 segment. Its roles are not well understood, but appear to involve modulation of host cell responses. A previous study by Zell et al. (J Gen Virol 88**:** 536-546 (2007)) showed that IAV of mammals frequently have premature stop codons that are expected to cause truncations of PB1-F2, whereas avian IAV usually express full-length PB1-F2 of 90 amino acids. Thousands of new IAV sequences have been added to databases since the previous study. With this expanded dataset, we also found that human and swine IAV frequently contain truncated PB1-F2, and that most avian IAV have full-length PB1-F2. However, in contrast to other avian IAV, since 2010 highly-pathogenic H5N1 viruses have had a high proportion of PB1-F2 truncations, due to several independent mutations that have persisted and expanded. A pair of closely related H5N1 viruses, differing mainly in the presence of PB1-F2, showed very different mouse lethality, with the virus containing defective PB1-F2 virus being 1000-fold more lethal in mice. Transient expression of full-length, truncated and deleted PB1-F2 in mammalian and avian cells did not show any effect on cell apoptosis or interferon expression. Full length PB1-F2 co-localized with mitochondria. We observed the

expression of an in-frame protein from downstream ATGs in PB1-F2 plasmids in which the PB1-F2 translation initiation codon was mutated as in some H5N1 viruses.

## Introduction:

Highly pathogenic avian influenza viruses (HPAIV) of H5N1 subtype are now endemic in many countries (OIE 2012). H5N1 became a public health concern in 1997 when a poultry outbreak in Hong Kong resulted in 18 human infections with six fatalities (Yuen, Chan et al. 1998). Since then, over 600 human cases with a case fatality rate of about 60% have been confirmed as of Oct. 8, 2013 (WHO 2013). Unlike seasonal influenza A viruses (IAV) that circulate among humans, HPAIVs are inefficient in human-to-human transmission. One of the key reason for inefficient human transmission of H5N1 viruses is the preference of their hemagglutinin (HA) for α 2, 3-linked sialic acids that are uncommon in the upper respiratory tract of humans (Shinya, Ebina et al. 2006).

Besides HA, other IAV genes have also been shown to contribute to viral virulence. However, the PB1 gene segment is of particular interest, since this was the only gene (other than the glycoprotein genes, HA and neuraminidase (NA)) reassorted in the pandemic events of 1957 and 1968 (Kawaoka, Krauss et al. 1989). Replacement of polymerase segments of 1918 H1N1 pandemic virus with those of the modern (pre 2009) H1N1 strain attenuated the former (Tumpey, Basler et al. 2005). One possible factor in PB1's role in pathogenicity and transmission of IAVs is the presence of a protein, PB1-F2, encoded by its +1 reading frame (Chen, Calvo et al. 2001), which was present in the first 3 pandemics but not in the less-virulent 2009 H1N1 pandemic.

PB1-F2 is a short (87–90 amino acids (aa)) IAV protein which was discovered serendipitously in 2001 during the search for CD8 T cell epitopes encoded by alternative reading frames (Chen, Calvo et al. 2001). It was found to localize in mitochondria and cause cell death (Chen, Calvo et al. 2001, Gibbs, Malide et al. 2003). It is dispensable for viral replication but has been associated with viral pathogenesis in mice and ducks (Chen, Calvo et al. 2001, Zamarin, Ortigoza et al. 2006, McAuley, Hornung et al. 2007, McAuley, Chipuk et al. 2010, Schmolke, Manicassamy et al. 2011), and truncation of PB1-F2 in the 2009 pandemic influenza virus (H1N1pdm09) is hypothesized to be one cause of its less severe pathology and lower case fatality rate (Ramakrishnan, Gramer et al. 2009). Its effect on viral phenotype is virus strain specific (Pena, Vincent et al. 2012, Pena, Vincent et al. 2012, Solbak, Sharma et al. 2013), particularly seen in the viruses which have a PB1 gene of recent avian origin (McAuley, Chipuk et al. 2010), perhaps because its effects are masked by other pathogenic determinants (McAuley, Hornung et al. 2007).

The mechanisms underlying its pathogenic effects are still not clear. It was suggested to be due to its mitochondrial targeting and apoptosis activity (Chen, Calvo et al. 2001, Gibbs, Malide et al. 2003, Zamarin, Garcia-Sastre et al. 2005) but in one study, PR8 virus that lacked PB1-F2 had reduced replication and virus spreading independent of its proapoptotic ability (Mazur, Anhlan et al. 2008). Full-length PB1-F2 inhibits early host cell responses including IFN-β (Conenello, Tisoncik et al. 2011, Dudek, Wixler et al. 2011, Varga, Ramos et al. 2011, Meunier and von Messling 2012, Varga, Grant et al. 2012, McAuley, Tate et al. 2013, Reis and McCauley 2013), although one recent study showed the opposite effect where PB1-F2 increased IFN-β expression in some, but not

all, cell types (Le Goffic, Bouguyon et al. 2010). PB1-F2 has also been linked to bacterial infections following IAV infection (Alymova, Green et al. 2011, Huber 2012, Weeks-Gorospe, Hurtig et al. 2012, Smith, Adler et al. 2013). However, most of these phenotypes have been difficult to generalize to all strains of virus and all hosts.

A striking feature of PB1-F2 in the global IAV population is the frequent presence of premature stop codons which render its protein product of varying lengths. For example, although full-length PB1-F2 is 90 amino acids long, almost all 2009 H1N1pdm viruses have PB1-F2 protein of 11 residues. Other common truncation produces protein that is 57 residues in length. In 2007, analysis of publicly-available PB1-F2 sequences indicated that a large proportion (19%) of mammalian IAVs had truncated PB1-F2, but such truncations were rare among avian IAVs (only 4%) (Zell, Krumbholz et al. 2007). This bias has led to the hypothesis suggesting a role of PB1-F2 truncations in adaptation of IAVs to mammalian hosts (Zell, Krumbholz et al. 2007).

Although PB1-F2 truncations have sporadically been observed in avian influenza viruses, until recently it has been unusual for groups of related avian influenza viruses to persistently carry this mutation. However, since 2009, an increasing number of H5N1 highly-pathogenic avian influenza virus (HPAIV) lineages have maintained PB1-F2 truncations over several years. Based on previous suggestions that these viruses may be particularly well adapted to mammalian replication, we tested these viruses for their ability to cause disease in mice and to alter cellular functions in mammalian and avian cells.

## Materials and Methods:

**Database analysis of PB1-F2 truncations**. We retrieved full-length PB1 sequences with collection dates prior to 2013 from GISAID and the NCBI Influenza databases (accessed May 22, 2013), and merged the sets based on virus strain name to avoid redundancy. We also included sequences for 169 H5N1 viruses sequenced at CDC (sequences available in GISAID). Sequences were selected by host species (human, swine, avian) and subtype (H5N1), and year of collection was included in the FASTA header of the downloads. In the case of H5N1, sequences prior to 1996 (the year of identification of the progenitor of current highly-pathogenic avian influenza (HPAI) H5N1 strains) and low pathogenicity H5N1 viruses were excluded. H5N1 sequences were excluded from the avian subset since those differed significantly from other avian subtypes in context of PB1-F2 truncations. PB1 sequences were aligned using MAFFT (Katoh, Misawa et al. 2002), and PB1-F2 sequences were translated from each PB1. The length of each PB1-F2 sequence was used to calculate the number of truncated versions present in each subset, per year of collection. In cases where no start ATG was present at the appropriate position the length of PB1-F2 was considered to be 0. In addition, we merged the set of H5N1 PB1 genes in which PB1-F2 was truncated with PB1 genes from the WHO/OIE/FAO H5N1 Evolution Working Group (annotated with the HA-based clade of the virus), aligned the genes using MUSCLE (Edgar 2004) and constructed a maximum likelihood tree with MEGA5 (Tamura, Peterson et al. 2011).

**Distribution of PB1-F2 truncations in H5N1 viruses.** For H5N1 viruses with PB1-F2 truncations, we obtained the corresponding hemagglutinin (HA) sequence from the appropriate database, annotating HA sequences with the length of the truncated PB1-

F2. We also included HA sequences from the report of WHO/OIE/FAO H5N1 Evolution Working Group in order to identify clades. We aligned HA sequences using MUSCLE and constructed a maximum likelihood tree with MEGA5.

**Identification of paired PB1-F2 full-length/truncation H5N1 viruses**. We analyzed the set of virus isolates available to us to identify a pair of similar viruses differing in PB1-F2 length. Viral RNAs were isolated by using QIAamp Viral RNA Mini Kit (#52904, QIAGEN Inc. – USA), reverse transcribed and amplified by AccessQuick™ RT-PCR System (#A1702, Promega Corporation, USA). Sequencing reactions were performed by using BigDye® Terminator v3.1 Cycle Sequencing Kit (#4337456, Life Technologies, Grand Island, NY) and run in Applied Biosystems 3730*xl* DNA Analyzer (Life Technologies, Grand Island, NY). RT-PCR and sequencing primers were selected from the primers routinely used at Molecular Virology and Vaccines Branch, CDC. For each H5N1 virus containing a PB1-F2 truncation, the amino acid sequence of each viral protein was compared to the most closely related virus containing full-length PB1-F2.

**Preparation of viral stocks.** Viruses A/chicken/Vietnam/NCVD-281/2009 (VN/281) (GISAID accession number EPI350009) and A/chicken/Vietnam/NCVD-296/2009 (VN/296) (GISAID accession number EPI350121) were grown in 10-11 days old embryonated chicken eggs incubated at 37$^{O}$C for 24 hours. Allantoic fluids from eggs, infected with the highest dilution of virus inoculum that gave a hemagglutination (HA) titer of 256 or more, were harvested, clarified by centrifugation and frozen at -80$^{O}$C as viral stocks.

**EID50.** Six dilutions ($10^{-5}$ to $10^{-10}$) were made from each virus. Five 9-11 day old embryonated hen eggs were inoculated per dilution and incubated at $37^{O}$C for 24 hours. Eggs were chilled overnight at $4^{O}$C to kill the embryos. Egg infections were detected by standard hemagglutination assay and EID50 was calculated by Reed and Meunch method (REED and MUENCH 1938).

**Plaque assay**. Confluent MDCK cells were infected with six dilutions ($10^{-5}$ to $10^{-10}$) of each virus, overlaid with 0.8% agarose medium and incubated at two different temperatures ($37^{O}$C and $40^{O}$C). 72 hours post infection, agarose medium were gently removed from wells and cell monolayers were stained with crystal violet-formalin mix. Plaques were counted in appropriate wells and EID50 values were calculated using the Reed and Muench method (REED and MUENCH 1938).

**Pathogenicity in mice.** All animal experiments were approved by IACUC. 6-8 weeks old female BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. 5 mice each were intranasally infected with one of 5 virus doses ($10^{O}$ to $10^{4}$ PFU in 100 µl volume) of each virus; 5 mice were mock infected with PBS. Mice were observed for weight loss and other clinical signs daily for a period of 16 days. Mice weighing less than 75% of their initial weight or showing nervous symptoms at any point during study were euthanized. Mouse LD50 values were calculated using the Reed and Muench formula (REED and MUENCH 1938).

**Cloning and mutagenesis of PB1-F2.** Wild type PB1-F2 sequences from A/chicken/Vietnam/NCVD-281/2009 (VN/281) and A/chicken/Vietnam/NCVD-296/2009 (VN/296) were RT-PCR amplified, using primers Kpn1PB1F76-94 and Xba1PB1R384-67 (summarized in Table 2.1) and directionally cloned into pTracer-CM2-GFP vector (Life Technologies, Grand Island, NY). Mutants of cloned PB1-F2 (summarized in table 2.2) were made by site-directed mutagenesis using Quick Change Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and mutagenesis primers (summarized in table 2.1 and 2.2). We also constructed PB-F2 constructs with a C-terminal HA tag so that the expression could be probed by using anti HA-tag antibodies. Cloned PB1-F2 plasmids were amplified in competent *E. coli* cells and were purified using Qiagen plasmid purification kit (QIAGEN Inc, Valencia, CA). The sequence of all plasmid clones was confirmed before use in experiments.

**Western blot:** PB1-F2 plasmid transfected cells were lysed using RIPA buffer (#R0278, Sigma-Aldrich, St. Louis, MO). Samples were prepared in Novex Tricin SDS Sample Buffer (#LC1676, Life Technologies, Grand Island, NY) and reduced with NuPAGE Sample Reducing Agent (#NP0009, Life Technologies, Grand Island, NY). Samples were run on Novex 10-20% Tricin Gels (#EC6625BOX, Life Technologies, Grand Island, NY) using Novex Tricin SDS Running Buffer (#LC1675, Life Technologies, Grand Island, NY). The gels were blotted to PVDF membrane and probed with mouse anti-HA tag antibody [HA.C5] (#ab18181, Abcam, Cambridge, MA). β-actin was probed as a loading control using mouse anti β-actin antibody (C4) (#sc-47778, SCBT Inc, Santa Cruz, CA). Goat anti-mouse IgG-HRP (#sc-2055, SCBT Inc, Santa Cruz, CA) was used as secondary conjugate. Blots were developed using SuperSignal West Pico

Chemiluminescent Substrate (#34080, Thermo Fisher Scientific Inc, Rockford, IL) and viewed in Kodak Image Station 4000R Pro (Carestream Health Inc, New Haven, CT).

**Confocal microscopy:** Transfected cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min, permeabilised with 0.5% Tween-20 in PBS for 10 min, washed with PBST (0.2% Tween-20), blocked with 2% BSA in PBST for 2 h, washed with PBST and incubated with primary antibody for HA and mitochondria MTCO2 (Abcam) 1:1000 dilution in 0.5% BSA in PBST overnight. Cells were then washed with PBST, incubated with secondary antibody (Alexa 594(mito)/ 350 (HA) solution in 0.5% BSA in PBST for 2 h and washed with PBST. After staining was complete, cover slips were mounted with mounting media (Invitrogen). Images were captured using Zeiss invert confocal microscope LSM 710 withX63 objective. Images were processed using Zen 2010 (Zeiss) and Adobe Photoshop.

**Quantitative RT-PCR for IFNβ expression:** Human A549 (#CCL-185, ATCC) and chicken DF1 cells (#CRL-12203, ATCC) , grown in 24-well cell culture plates were transfected with 1.0 µg of plasmid DNA using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI). Cells were lysed and lysate were directly used for qRT-PCR using CellsDirect One-Step qRTPCR Kit (Life Technologies, Grand Island, NY) and TaqMan gene expression assays for human and avian IFNβ (Life Technologies, Grand Island, NY). The Δ Δ Cq method in Bio-Rad CFX Manager 2.1 software was used to analyze the expression data. GAPDH expression was measured in each sample as a control.

**Flow cytometry for cell apoptosis:** A549 and DF1 cells were transfected as described in the last paragraph. After harvesting, cells were stained for apoptosis markers using

Violet Chromatin Condensation/Dead Cell Apoptosis Kit with Vybrant® DyeCycle™ Violet and SYTOX® AADvanced™ for Flow Cytometry (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Flow cytometry data were acquired within one hour of staining. Data were acquired on FACSCanto II flow cytometer (BD, Franklin Lakes, NJ), and analyzed using FlowJo software (Tree Star, Ashland, OR).

**Statistical analyses.** To compare weight loss, a linear mixed model with repeated measures was used, using a cutoff of $p<0.05$ for statistical significance. Kaplan-Meier survival curves were compared by the log-rank test. Effect on apoptosis and IFNβ expression were analyzed by Kruskal-Wallis test in GraphPad Prism software.

**Results:**

**Evolution of PB1-F2 truncations in avian and mammalian viruses**. In a previous study (Zell, Krumbholz et al. 2007), 2226 PB1 sequences having PB1-F2 ORF were retrieved from NCBI database and analyzed for PB1-F2 truncations. The study found that the proportion of truncated PB1-F2 was higher in human viruses (19%) than those of avian viruses (4%) (Table 1 and S1 in reference (Zell, Krumbholz et al. 2007)). The study included data available until June 15, 2006 and the number of available sequences has increased significantly since then. We retrieved 19666 full-length PB1 sequences from the combined GISAID, NCBI and CDC Influenza databases: 9285 human, 2003 H5N1, 6705 avian (excluding H5N1), and 1572 swine sequences. We identified 23 different truncations of PB1-F2, resulting in PB1-F2 length ranging from 0-87aa. A small number of viruses (53, 0.3% of total) had PB1-F2 that was longer than 90 amino acids at 101aa. 65.7% of viruses were full-length (87 or 90 aa). The prevalence

of PB1-F2 truncations (less than 79 amino acids in length (Zell, Krumbholz et al. 2007)) was 58% for human viruses, 7.3% for H5N1, 3.1% for avian viruses excluding H5N1, and 44.2% for swine viruses (table 2.3). Prevalence of PB1-F2 truncations by year of collection is shown in Figure 2.1. The most common truncations were of 11 and 57 amino acids (23.2% and 7.6% of total, respectively), with no other truncation exceeding 1% of total. The most prevalent truncation in swine viruses is 11 aa (as seen in the H1N1pdm virus) which has also replaced the previously most prevalent truncation (57aa) in human viruses after the 2009 pandemic. Among avian viruses, 57 and 79-residue versions were more prevalent before 2005, after which the 25aa variant became more prevalent.

A 25 aa truncation was first detected in an avian virus (H2N1) collected from Delaware, USA in 1988 (sequenced in 2006); but became persistent only after 2005. It has been detected sporadically in swine and human viruses since 2001 and 2007, respectively. Since its appearance its prevalence has been less than 3% in avian, human, and swine viruses separately and combined.

It is important to note that these percentages are skewed due to sampling bias. For example, human H1N1pdm viruses (almost all of which have truncated PB1-F2) were much more intensively sequenced in 2009 than 2010; similarly, genome sequencing of H5N1 differs greatly in different countries in which the virus is endemic, leading to differential clade representation in the databases. Nevertheless, these data show that the frequency of PB1-F2 truncations in H5N1 viruses has increased markedly in the past five years, increasing from 10 of 346 samples (2.9%) in 2007, to 49 of 98 samples (50%) in 2012, while other avian viruses consistently have full-length PB1-F2 and

mammalian viruses have a high proportion of truncated PB1-F2. This provides further support for the previous proposal (McAuley, Chipuk et al. 2010) that avian viruses may receive a significant fitness benefit from full-length PB1-F2, while for viruses of humans and swine either full-length PB1-F2 is dispensable, or a truncated version of PB1-F2 is actively beneficial. In the latter case, the implication is that avian viruses containing truncated PB1-F2 may be "pre-adapted" to mammalian infection.

**Evolution of PB1-F2 truncations in H5N1 viruses**. HPAIV H5N1 viruses have been categorized into multiple clades based on their HA sequences. We constructed phylogenetic trees, based on HA and full-length PB1 (Figure 2.2A and B), to analyze the distribution of PB1-F2 truncations in H5N1 clades. Until 2009, PB1-F2 truncations were rare (~2% of sequences, Figure 2.1) and sporadic, with few exceptions in which truncations persisted in a particular lineage for several months (e.g. clade 1 in Thailand from 2007-2008; see below). However, in 2009 and 2010, clade 2.3.4.1 viruses in Vietnam and China consistently had PB1-F2 truncated at 25 or 24 amino acids. These viruses included both poultry isolates and several human cases (A/Guizhou/1/2009, A Hunan/2/2009).

In 2010 and 2011, the 2.3.2.1 clade became dominant in Vietnam, and the 2.3.4.1 clade has not been isolated from Vietnam since 2010. However, in 2011/2012 a new PB1-F2 truncation of 25 amino acids arose in the clade 1.1a lineage in Vietnam. The earliest instance of a truncated PB1-F2 in this clade is from 2007 (a pair of viruses from Cambodia in the 1.1 lineage); however, since no further truncated PB1-F2 have been identified from Cambodian isolates, and the 25-residue truncation in Vietnam appears to be a separate lineage from the Cambodia isolates, these appear to have been sporadic

mutations. In 2008, A/duck/Vietnam/NCVD-118/2008 had a PB1-F2 truncation; this may represent the earliest version of the clade 1.1a mutation or may be a sporadic variant that did not give rise to successful descendants. Since PB1 in these viruses is phylogenetically distinct from that of the 2.3.4.1 lineage this mutation appears to have arisen independently. To date, these truncations have only been isolated from poultry.

A third PB1-F2 truncation, of 57 amino acids, arose in the clade 2.3.2.1b lineage in China and Vietnam in 2010 (first seen in A/duck/Vietnam/NCVD-885/2010) and this variant has persisted through 2012 in both countries, with the majority of 2.3.2.1b viruses containing truncated PB1-F2. Furthermore, in 2012 a reassortment event occurred between clade 2.3.2.1b and 2.3.2.1c lineages in Vietnam, with the former viruses donating 6 internal genes and the latter providing NA and the clade-determining HA. Since the internal genes in this lineage includes the 57-aa truncation of PB1-F2 that originally arose in 2.3.2.1a viruses, the vast majority of 2.3.2.1c viruses isolated in Vietnam in 2012 also contain this PB1-F2 variant. An earlier example of a 57-aa truncation of PB1-F2 arose in Thailand in 2007/2008 (Clade 1), although this variant has not been identified since 2008.

Finally, a number of less common PB1-F2 truncations have been isolated in HPAIV H5N1 since 2009, including 63, 34, 11, and 8 amino acids, and some variants that lack a start codon altogether. However, since none of these variants have been isolated in a subgroup of viruses for more than one year, we consider them to be sporadic mutations.

**Paired viruses for PB1-F2 studies.** We identified viruses A/chicken/Vietnam/NCVD-281/2009 (VN/281) (GISAID accession number EPI350009) and

A/chicken/Vietnam/NCVD-296/2009 (VN/296) (GISAID accession number EPI350121) as the most closely-matched pair of PB1-F2-disparate H5N1 viruses in our collection. Amino acid differences between viral proteins of this pair are summarized in Table 2.4. These viruses were isolated from chickens in 2009 in Vietnam and belong to clade 2.3.4.2. VN/281 contains a full length PB1-F2 of 90 amino acids, whereas virus VN/296 has a mutation in its translation initiation codon and hence is not expected to make full length PB1-F2 protein. However, it potentially could express N-terminally truncated versions of PB1-F2 from downstream in-frame ATGs at codon numbers 39, 46 and 51 (PB1-F2 ORF) as described previously (Zamarin, Ortigoza et al. 2006). Both the viruses VN/281 and VN/296 replicated to similar titers in eggs and cells, as measured by EID50 and plaque assay (Table 2.5.).

**Full length PB1-F2 (VN/281) is associated with reduced mice virulence.** To study the phenotype of selected pair of viruses, mice were infected with either virus VN/281 or VN/296. These viruses are very similar elsewhere in the genome outside PB1-F2. The virus pair differs only by 15 amino acids, none of which has been established as a pathogenicity determinant REF. Mice infected with both viruses were severely affected, showing weight loss and mortality (Figure 2.3 and Table 2.5). However, mice infected with virus VN/296 (mutated start codon PB1-F2) were more severely affected than those infected with VN/281 (full-length PB1-F2), showing marked weight loss and high mortality at lower doses. The LD50 of virus VN/281 was approximately 1000-fold higher than virus VN/296 ($10^{4.3}$ vs $10^{1.4}$ PFU) making the later more virulent.

**Full length H5N1 PB1-F2 localizes to mitochondria.** To find the mechanism underlying 1000 fold pathogenicity difference of the studied viruses, we explored phenotypes of cloned PB1-F2 plasmids *in vitro* in mammalian (A549) and chicken cells (DF1). We constructed PB1-F2 variants differing in length as outlined in Figure 2.4. We constructed 6 variants: 2 full-length versions (90 residues); 2 truncated at 24 residues; and 2 in which the coding sequence was intact but the start codon (1$^{st}$ ATG) was mutated to ACG. We confirmed the expression of the full length and truncated variants in A549 cells by western blotting (Figure 2.5). No band was obvious for ATG-ACG mutatnt. Both the full-length and the truncated versions of PB1-F2 were detected, however the truncated version was at lower levels than for full-length.

After confirming the expression of PB1-F2 from plasmid constructs, we explored the cellular localization of the protein in transfected cells using immunofluorescence. Confocal microscopy revealed that the full-length PB1-F2 proteins were expressed and were readily detected. The full length PB1-F2 from both the viruses localized with cell mitochondria in both A549 (data not shown) and DF1 cells (Figure 2.6). The truncated 25-amino acid version of PB1-F2 was not detectable under standard conditions; however, when the cells were treated with the proteasome inhibitor MG-132, some expression was detectable (Figure 2.6). Interestingly, presence of an HA-tagged protein was detectable by confocal microscopy in cells transfected with the ATG-mutated version of PB1-F2; this was detected in the absence of MG-132, but was more obvious in its presence. Since this presumably represents a protein in frame with the c-terminal HA, this protein expression probably originates from an in-frame ATG (residue number 39, 46, or 51; figure 2.4b) downstream of the authentic start site, leading to expression

of a truncated c-terminal fragment of the protein. The phenomenon has been described previously by Zamarin et al in 2006 (Zamarin, Ortigoza et al. 2006). Neither the 25 aa truncated version, nor the putative C-terminal fragment of PB1-F2, showed any association with mitochondria (Figure 2.6)

**PB1-F2 mutants had no effect on apoptosis.** Mitochondrial localization may lead to apoptosis and could be one explanation for the altered viral virulence. We transfected A549 and DF1 with PB1-F2 variants, and harvested cells at 12, 24, 36 and 48 hours post transfection, stained for apoptosis and cell death, and analyzed the cells by flow cytometry. Statistical analysis of the apoptosis data was done using the Kruskal-Wallis nonparametric test. No significant difference in percent of apoptotic or dead cells was found between different PB1-F2 mutants and between the studied cell lines (Figure 2.7). This is in accordance with previously published studied looking at the apoptosis phenotype of H5N1 PB1-F2 (Chen, Chen et al. 2010, McAuley, Chipuk et al. 2010). One of these studies evaluated this phenotype in all the 20$^{th}$ century pandemic viruses and H5N1 viruses and found the phenotype only in PR8 and 1918 H1N1 viruses but not in H5N1 and 1957 and 1968 pandemic viruses (McAuley, Chipuk et al. 2010).

**PB1-F2 truncations and IFNb expression.** A second possible mechanism for the differences in virulence could be the interference with innate immune system. PB1-F2 from PR8 virus inhibits type I interferons (Dudek, Wixler et al. 2011, Varga, Ramos et al. 2011, Varga, Grant et al. 2012); however, that from the WSN/33 virus increases IFNβ expression in epithelial cells (Le Goffic, Bouguyon et al. 2010). A549 cells were transfected with PB1-F2 plasmids and IFNb expression was measured at different time points using TaqMan assay qRT-PCR. We used empty plasmid vector as a negative

control and tri-phosphate RNA as a positive control for IFNb induction. The expression levels were normalized to GADPH and fold expression over empty vector transfection. As expected, the positive control (TP-RNA) consistently induced IFNb expression (data not shown). There was no significant difference in IFNb expression level with different PB1-F2 plasmids in A549 cells (Figure 2.8) and DF1 cells ($p>0.05$, Kruskall-Wallis Test, data not shown).

**Discussion:**

In 2007, Zell et al (Zell, Krumbholz et al. 2007) showed that the great majority of avian influenza viruses contain a full-length PB1-F2 protein, whereas mammalian viruses frequently have premature stop codons that truncate PB1-F2. Since the number of influenza virus sequences has increased 7-fold since 2007, we repeated the analysis and found a generally similar picture. In mammalian viruses (swine and human) PB1-F2 truncations are common (approximately 50% of sequences in the database). (A possible exception to this rule is in the equine and canine influenza viruses, in which PB1-F2 sequences are generally full length (not shown). However, a limited number of PB1 sequences are available for these virus strains, so they were not included in the present analysis. In contrast, in avian viruses over 95% of PB1-F2 sequences are full length. The exception to this is in the highly pathogenic H5N1 virus group, in which the frequency of PB1-F2 truncations in the databases has increased to 50% in 2012. It is important to note that some of this increase may be due to surveillance bias, since the published sequences of H5N1 are dominated by those from Vietnam and China where PB1-F2 truncations are increasingly common in the endemic clades.

We selected a pair of naturally-occurring viruses, VN/281 and VN/296, which differ only by 0.36% (15 amino acids out of 4440 in the proteome) aside from PB1-F2, and tested their replication in tissue culture and eggs, and virulence in mice. Although the viruses replicated identically in eggs and in MDCK cells, their 50% mouse lethal dose (MLD50) differed by 1000- fold. VN/281 (full length PB1-F2) was less pathogenic than VN/296 (deleted PB1-F2). This was unexpected since previous studies have linked PB1-F2 to virulence, so the absence of PB1-F2 was expected to reduce the virulence of the virus. On the other hand, the increased virulence of the PB1-F2-deleted virus is consistent with the observation that in some contexts PB1-F2 increases IFN expression and reduces viral replication (Le Goffic, Bouguyon et al. 2010, Le Goffic, Leymarie et al. 2011). The effect of PB1-F2 mutations on the H5N1 viruses has been rarely studied, since most of those studies were done on A/Puerto Rico/08/1934(H1N1) (PR8) where deletion of PB1-F2 decreases pathogenicity in mice. There are only two studies which looked at mice pathogenicity in context of full length and deleted PB1-F2 of H5N1 viruses in mice (Schmolke, Manicassamy et al. 2011, Leymarie, Jouvion et al. 2013) and neither of these found any significant effect. A slight reduction in lethality was observed after PB1-F2 deletion in a previous H5N1 study (Leymarie, Jouvion et al. 2013). In another H5N1 study, PB1-F2 deletion resulted in reduced pathogenicity in mice but not in ducks (Schmolke, Manicassamy et al. 2011). However, it has previously been shown that the effects of PB1-F2 are highly dependent on genomic context and the presence of other virulence factors (McAuley, Hornung et al. 2007, McAuley, Chipuk et al. 2010, Pena, Vincent et al. 2012, Pena, Vincent et al. 2012, Solbak, Sharma et al. 2013), and the viruses in the present study have evolved for many years separately

from the viruses (A/duck/Niger/2090/2006 and A/Vietnam/1203/2004) used in the former studies.

In studies with PR8 viruses, the pathogenic effect of PB1-F2 is linked with its mitochondrial localization, apoptosis in immune cells and regulation of innate immune response in epithelial cells. The only published study that has evaluated PB1-F2 of a H5N1 virus (A/HK/156/1997) in context of mitochondrial targeting and apoptosis found no effect on mitochondrial localization and apoptotic phenotype (Chen, Chen et al. 2010). However when PB1-F2 was mutated at Q69L and H75L, 50% of it localized to mitochondria (Chen, Chen et al. 2010). The virus pair used in this study has 69Q and 75R but localized efficiently to mitochondria.

To help understand the reason for the difference in pathogenicity associated with full-length vs. truncated PB1-F2, we assessed the effect of these variants on cellular function in human and avian cells. Based on previous reports on PB1-F2 effects we measured IFNβ response, and apoptosis induction. However, expression of PB1-F2 alone in human and chicken cells did not affect either apoptosis or IFN expression, suggesting that any effect on virulence may be acting through as-yet unknown functions.

It is intriguing that for the PB1-F2 plasmid lacking the initiating ATG, we observed expression of an HA-tagged protein by confocal microscopy. Since this was detected by staining for the HA tag at the C-terminus of the predicted PB1-F2 protein, the simplest explanation is that this protein is produced by initiation at an in-frame ATG (at position 39, 46, or 51) downstream of the authentic ATG, leading to an N-terminally

truncated version (or versions) of PB1-F2 that also partially localized to mitochondria. A similar observation has been described previously with PB1-F2 plasmid clones from PR8 and WSN/33 viruses (Zamarin, Ortigoza et al. 2006). This is in accordance with previous studies which have shown that the N-terminally truncated PB1-F2 and C-terminal peptides are sufficient and even more efficient in mitochondrial localization and its apoptotic phenotypes (Gibbs, Malide et al. 2003, Yamada, Chounan et al. 2004); however, those studies used a different virus, PR8. It is possible that the N-terminally truncated PB1-F2 is constitutively expressed in the presence of full length protein, but is expressed at a higher level when the standard ATG is not present. If so, this protein may also play a role in influenza virus virulence in vivo.

Studies using deleted PB1-F2 viruses and plasmids have used different strategies. These include mutation of multiple downstream ATGs along with the 1$^{st}$ start codon (Le Goffic, Bouguyon et al. 2010), or introduction of a stop after the last in-frame ATG (Zamarin, Ortigoza et al. 2006). These artificial strategies, which are not found in natural isolates, would prevent expression of the putative C-terminal fragment of PB1-F2. Other groups have both mutated the 1$^{st}$ start codon and inserted a stop codon at positions 11 (McAuley, Hornung et al. 2007, Mazur, Anhlan et al. 2008, McAuley, Chipuk et al. 2010, Meunier and von Messling 2012). The effect of these changes on expression of the C-terminal fragment of PB1-F2 is unknown. Alternatively, the lack of detection of a C-terminal fragment of PB1-F2 in these studies may be due to poor binding of antibodies for the truncated protein, the use of antibodies recognizing the N-terminus of PB1-F2, or the general difficulty of detecting very small proteins.

Our mouse observations are consistent with a recent study with A/USSR/90/77(H1N1), a study in which PB1-F2 was eliminated by start codon mutation and insertion of a stop codon at position 11 (Meunier and von Messling 2012), which may permit expression of the C-terminal fragment of PB1-F2. In this study, infection of ferret lung explants with the PB1-F2 deletion mutant led to increased pro-inflammatory cytokines, which in H5N1 infection may be associated with “cytokine storm” and increased virulence.

To summarize, historically rare PB1-F2 truncations are evolving among H5N1 HPAI viruses. Since H5N1 virus pose a significant public health threat, assessment of the risk of these truncations is very important for pandemic preparedness. To evaluate this, we used a pair of similar viruses with full length and N-terminally truncated PB1-F2 which differed by only 15 amino acids outside the PB1-F2 ORF. None among the 15 differing amino acid is a known pathogenicity determinant. We found that a H5N1 virus with a ATG-ACG mutation (VN/296) is 1000 fold more virulent than its paired virus (VN/281). In vitro apoptosis and IFNb experiments did not show any significant difference among different plasmids and between A549 and DF1 cells. Based on the previously published and above mentioned studies, we propose that in HPAIV H5N1, the N-terminal of PB1-F2 may reduce the pathogenic effects of the C-terminal fragment. Further immuno-pathogenesis studies will help clarify the mechanism of PB1-F2 pathogenicity.

# APPENDICES

## APPENDIX 2A: Tables

**Table 2.1 Oligonucleotides used for cloning and mutagenesis of PB1-F2.**

| Primer name | Primer Sequence | Used for |
|---|---|---|
| Kpn1PB1F76-94<br>Xba1PB1R384-67 | 5'- ATTA **GGTACC** GGAGACCCTCCATACAGCC -3'<br>5'- GAGG **TCTAGA** GTCTGTCGACCTTGGGT -3' | RT-PCR amplification |
| PB1C167T_C170T_F | 5'-TTCAGAAAAGGGGAAGTGGATAATAAACACAGAAACTGGAGCAC -3' | Mutagenesis |
| PB1C96T_F | 5'- AGACCCTCCATACAGCCATGGAACAGGGAC -3' | |
| PB1T96C _F | 5'- AGACCCTCCATACAGCCACGGAACAGGGAC-3' | |
| Kpn1PB1F68-90 | 5'-TAG ACG ACT AAT CTA TAG TAA CGC ATC GAG GA **G GTA CC** C TTA TAC TGG AGA CCC TCC ATA C -3' | RT-PCR amplification with C-terminal HA-tag |
| Xba1HAtagPB1R364-43 | 5'- GAGG **TCTAGA** *TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA* GTT TGT CCA CTC TTG TTT GTT G -3' | |
| PB1F151-185 (HA_C167T_C170T) | 5'- GAA AAG GGG AAG TGG A *TAC CCA TAC GAT GTT CCA GAT TAC GCT* **TAA TAA** ACA CAG AAA CTG G-3' | Mutagenesis with HA tag |

Numberings in oligo names represent PB1 nt number. Restrictions sites are indicated as bold and underlined. Mutated residues and stop codons are in red text. HA tag is purple and italicized.

**Table 2.2 Details of PB1-F2 mutants made in this study.**

| Sr. No. | Source | PB1-F2 | Mutagenesis Primer |
|---|---|---|---|
| 1 | VN/281/PB1-F2FU | Full | Kpn1PB1F76-94 and Xba1PB1R384-67 |
| 2 | VN/281/PB1-F2TR | Truncated | PB1C167T_C170T_F |
| 3 | VN/281/PB1-F2DL | Deleted* | PB1T96C _F |
| 4 | VN/296/ PB1-F2FU | Full | PB1C96T_F |
| 5 | VN/296/ PB1-F2TR | Truncated | PB1C167T_C170T_F |
| 6 | VN/296/ PB1-F2DL | Deleted* | Kpn1PB1F76-94 and Xba1PB1R384-67 |
| 7 | VN/281/PB1-F2FU-HA | Full | Kpn1PB1F68-90 and Xba1HAtagPB1R364-43 |
| 8 | VN/281/PB1-F2TR-HA | Truncated | PB1F151-185 (HA_C167T_C170T) |
| 9 | VN/281/PB1-F2DL-HA | Deleted* | PB1T96C _F |
| 10 | VN/296/ PB1-F2FU-HA | Full | PB1C96T_F |
| 11 | VN/296/ PB1-F2TR-HA | Truncated | PB1F151-185 (HA_C167T_C170T) |
| 12 | VN/296/ PB1-F2DL-HA | Deleted* | Kpn1PB1F68-90 and Xba1HAtagPB1R364-43 |

*The first ATG is mutated to ACG, leaving the downstream in-frame ATGs intact.

**Table 2.3 Prevalence of PB1-F2 truncations among all subtypes (human, swine and avian IAVs) versus H5N1.**

| | All | Human | Swine | Avian (except H5N1) | H5N1 |
|---|---|---|---|---|---|
| Total sequences | 19666 | 9268 | 1693 | 6703 | 2003 |
| No. (%) > 79aa | 13063 (66.4%) | 3843 (41.5%) | 880 (52.0%) | 6490 (96.8%) | 1850 (92.4%) |
| No. (%) ≤78aa | 6603 (33.6%) | 5425 (58.5%) | 813 (48.0%) | 213 (3.2%) | 152 (7.6%) |

**Table 2.4 Amino acid differences between viruses VN/281 and VN/296.**

| Protein | Size (aa) | aa diffs |
|---|---|---|
| HA | 567 | 1 |
| M1 | 252 | 0 |
| M2 | 97 | 2 |
| NA | 449 | 4 |
| NP | 498 | 1 |
| NS1 | 225[1] | 1 |
| NS2 | 121 | 0 |
| PA | 716 | 1 |
| PB1 | 756 | 3 |
| PB1-F2 | 90[2] | 3 |
| PB2 | 759 | 2 |
| Total | 4530 | 18 |
| Total (excluding PB1-F2) | 4440 | 15 |

[1] Both the viruses have a C-terminal truncation in NS1 that renders it 215aa long.
[2] PB1-F2 is 90aa in virus VN/281 and deleted in virus VN/296.

**Table 2.5 Growth characteristics and mouse virulence.**

| **Virus** | **PB1-F2** | **EID50/ml** | **PFU/ml** | **MLD50 (Log10 PFU)** |
|---|---|---|---|---|
| VN/281 | Full length | 3.2 X10^9 | 7.0 X10^8 | 4.3 |
| VN/296 | Deleted | 6.8X10^8 | 2.3 X10^8 | 1.4 |

## APPENDIX 2B: Figures

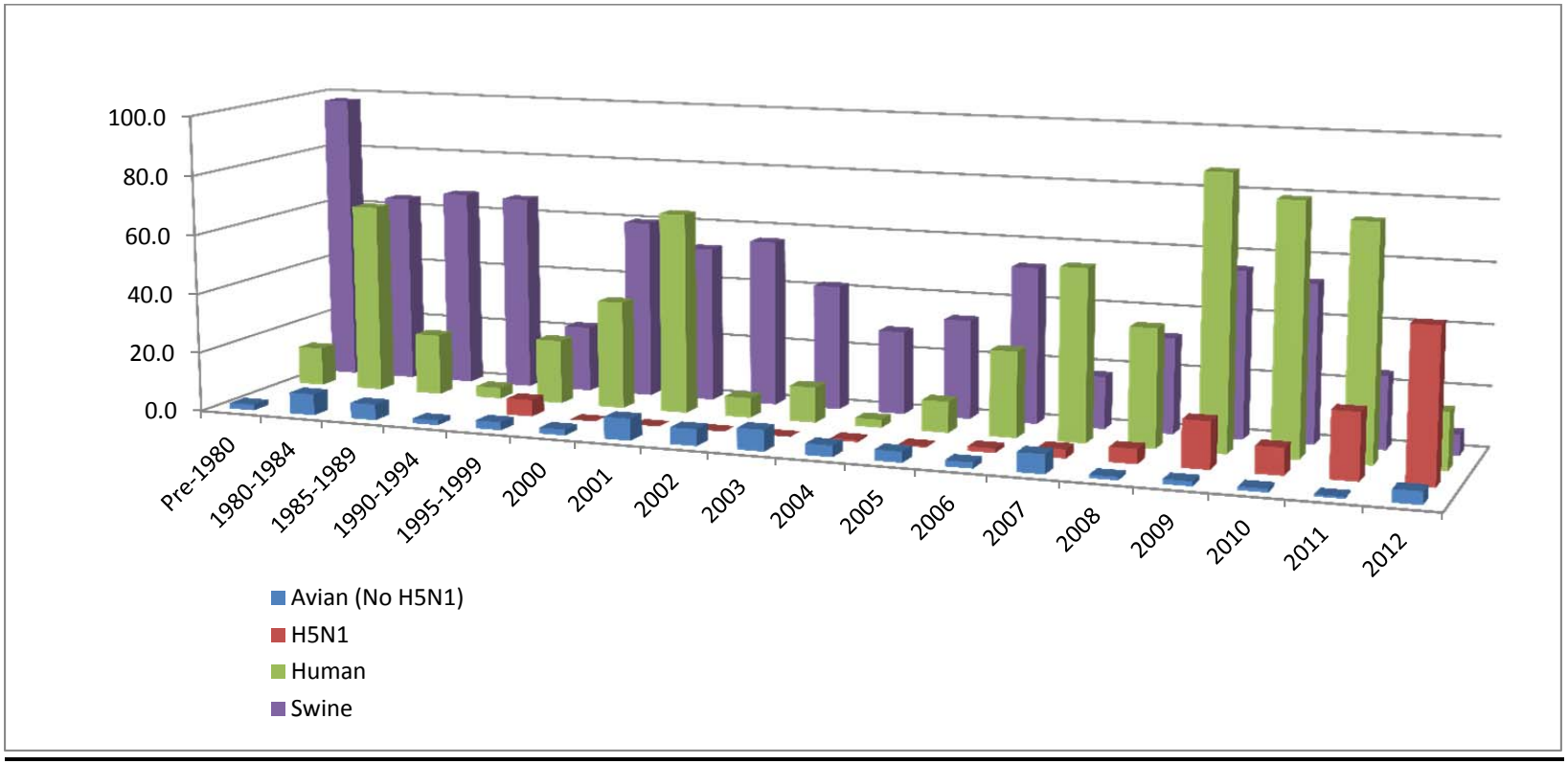



**Figure 2.1 PB1-F2 truncation prevalence.** PB1 sequences were retrieved from NCBI, GISAID and CDC databases. PB1 ORFs were aligned using MAFFT and looked for stop codons, hence length of the PB1-F2 protein. PB1-F2 proteins of length <79aa were marked truncated and represented as percent of total viruses (PB1-F2 sequences) by host and year of isolation.


**Figure 2.2 Evolutionary relationship of PB1-F2 truncations in H5N1 viruses.** Phylogenetic trees of HA gene (A) and PB1 gene (B) are plotted to show truncations in PB1-F2 viruses. Truncations of 24/25, and 57 aa are colored red and green, respectively. The blue font indicates virus strains having other truncations in PB1-F2 (63, 11, 8, and 0 amino acids). Clades were defined by taking representative sequences from the WHO/OIE/FAO H5N1 Classification Working Group. Viruses used in this study are indicated with green (VN/296, truncated PB1-F2) and red (VN/281, full-length PB1-F2) arrows.

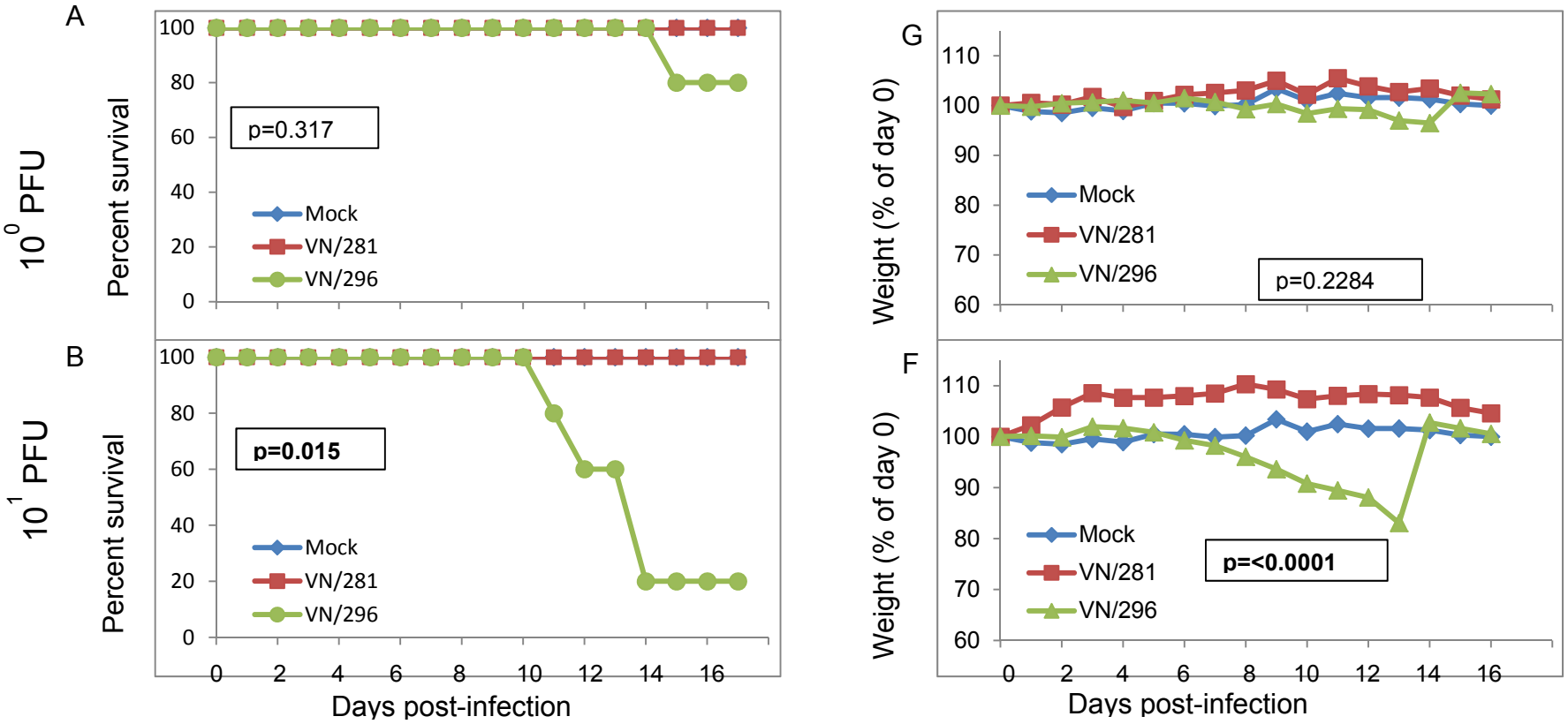
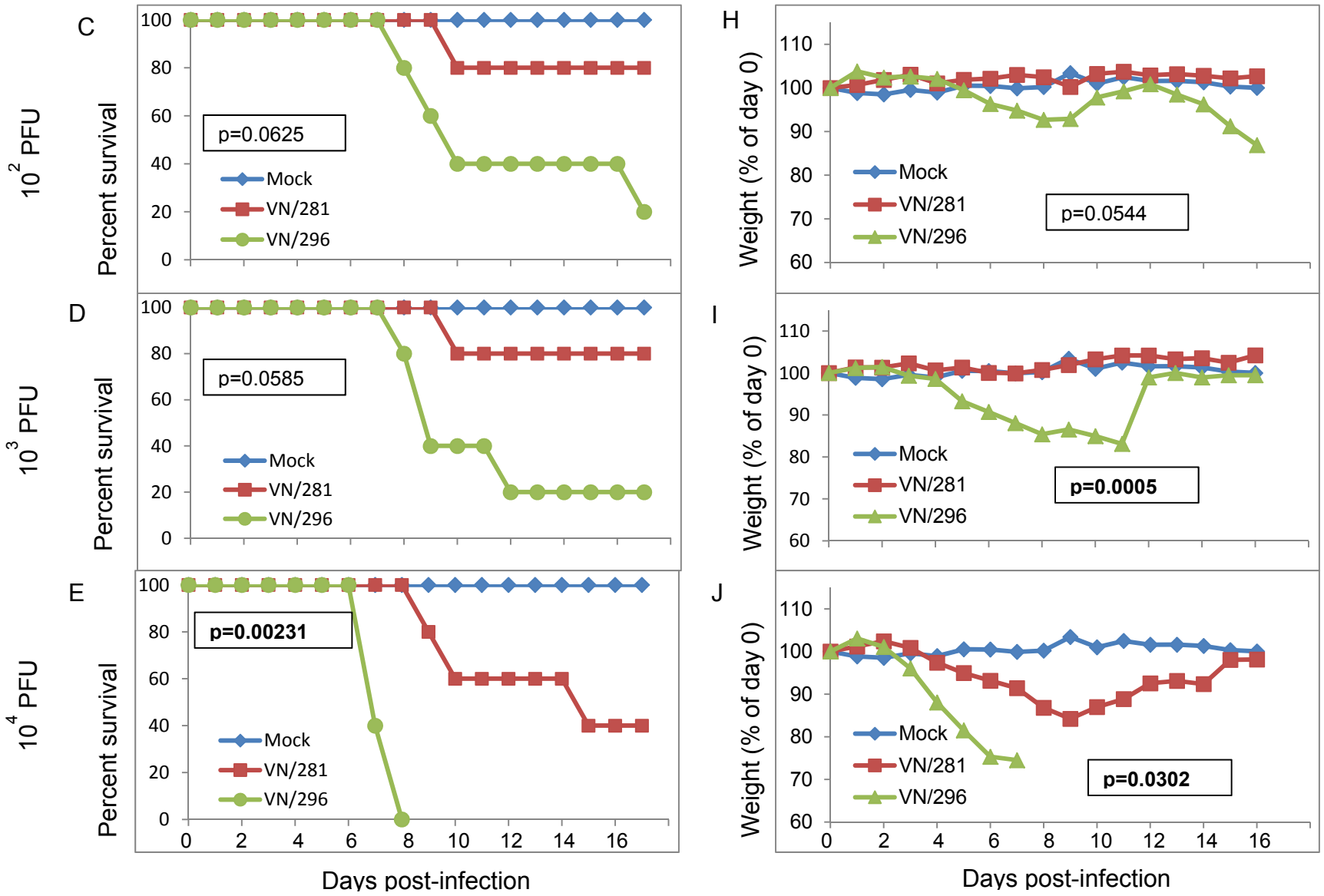



**Figure 2.3 Mice virulence of paired viruses with and without PB1-F2.** Five mice each were infected intranasally either with PBS, VN/281 (45) or VN/296 (57) and observed daily for 16 days. Survival data (A-E) and body weight loss data (F-J) of mice are shown here as percent of day "zero" values and are group averages of 5 mice. Statistical significances for survival and body weight loss between two viruses were calculated using a linear mixed model with repeated measures and Log Rank Test – Chi Square p-value, respectively.

**Figure 2.3 (cont'd)**



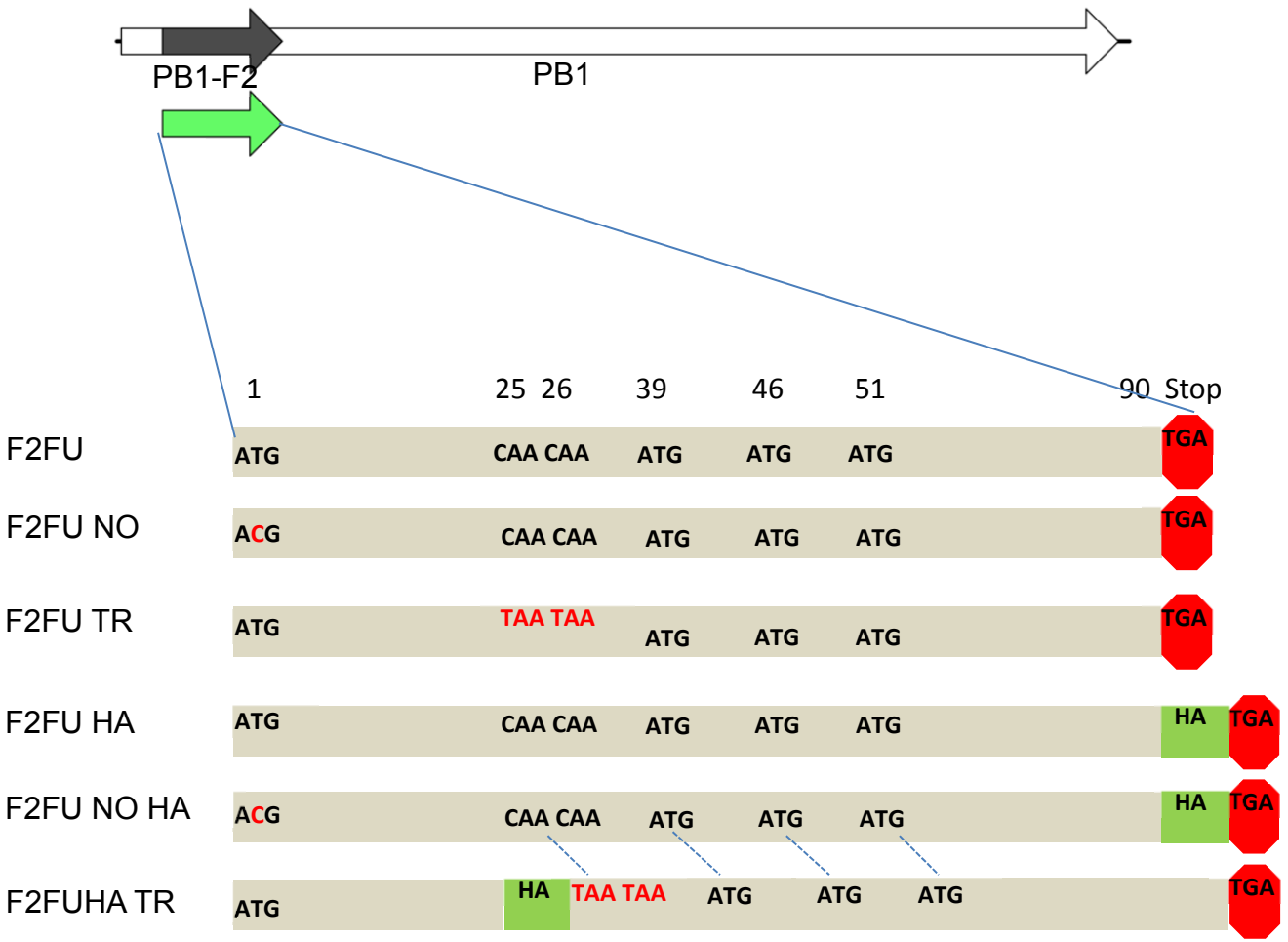



**Figure 2.4 Construction of PB1-F2 mutants' recombinant plasmid clones.** One full length (90aa), one truncated (24aa) and one deleted (0 aa) variants of PB1-F2 were made from each of VN/281 and VN/296 viruses. Similar mutants were also made with C-terminal HA tag. The mutated nucleotides and stop codons are shown in red.

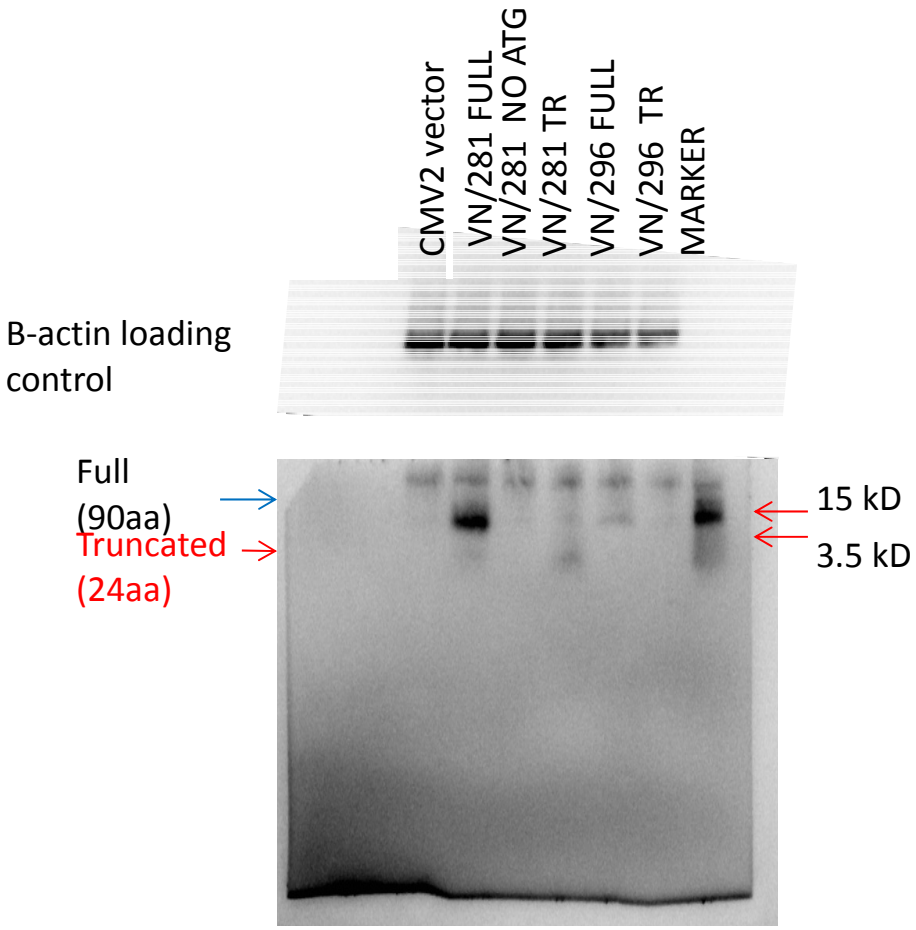



**Figure 2.5 Expression of PB1-F2 mutants in A549 cells**. Cells were transfected with either PB1-F2 plasmids having c-terminal HA tag or empty vector and examined for protein expression, 24 hour post transfection. Cell lysates were run on tris tricin gradient gel, blotted on PVDF membrane and probed with anti-HA antibody (abcam).

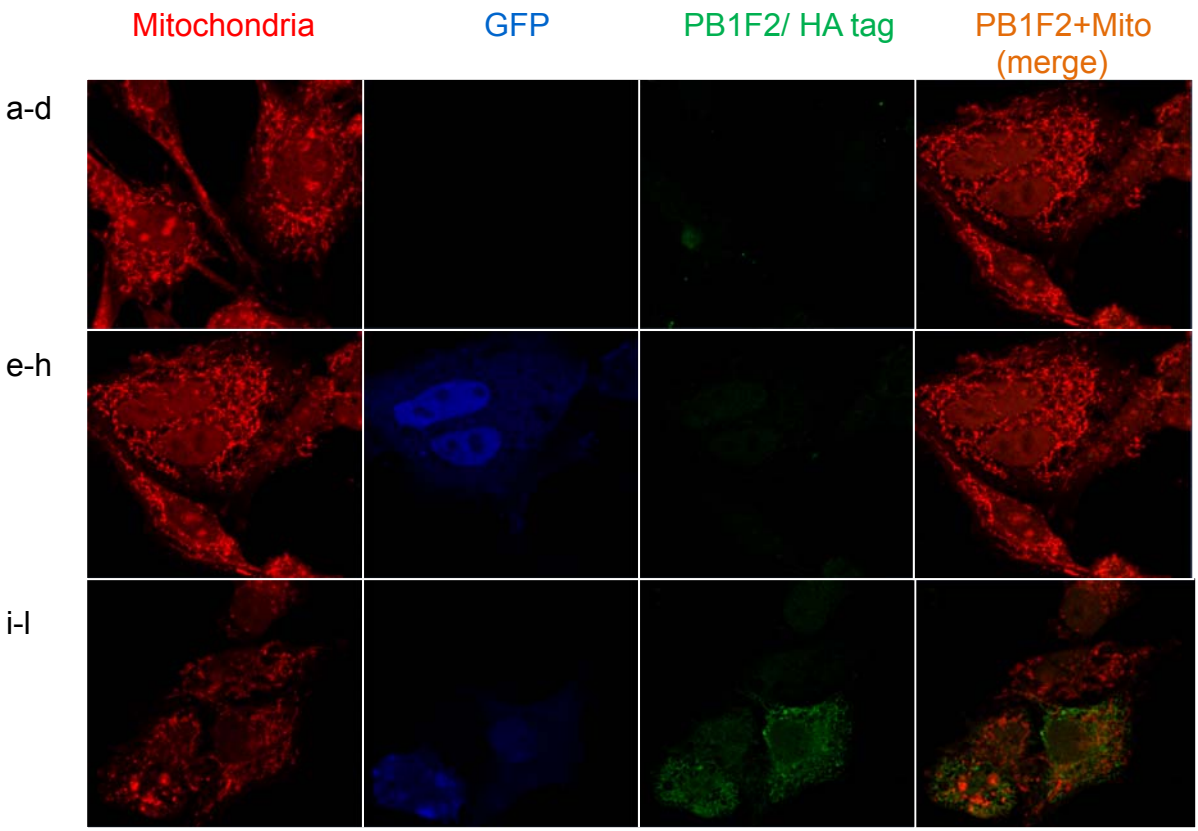
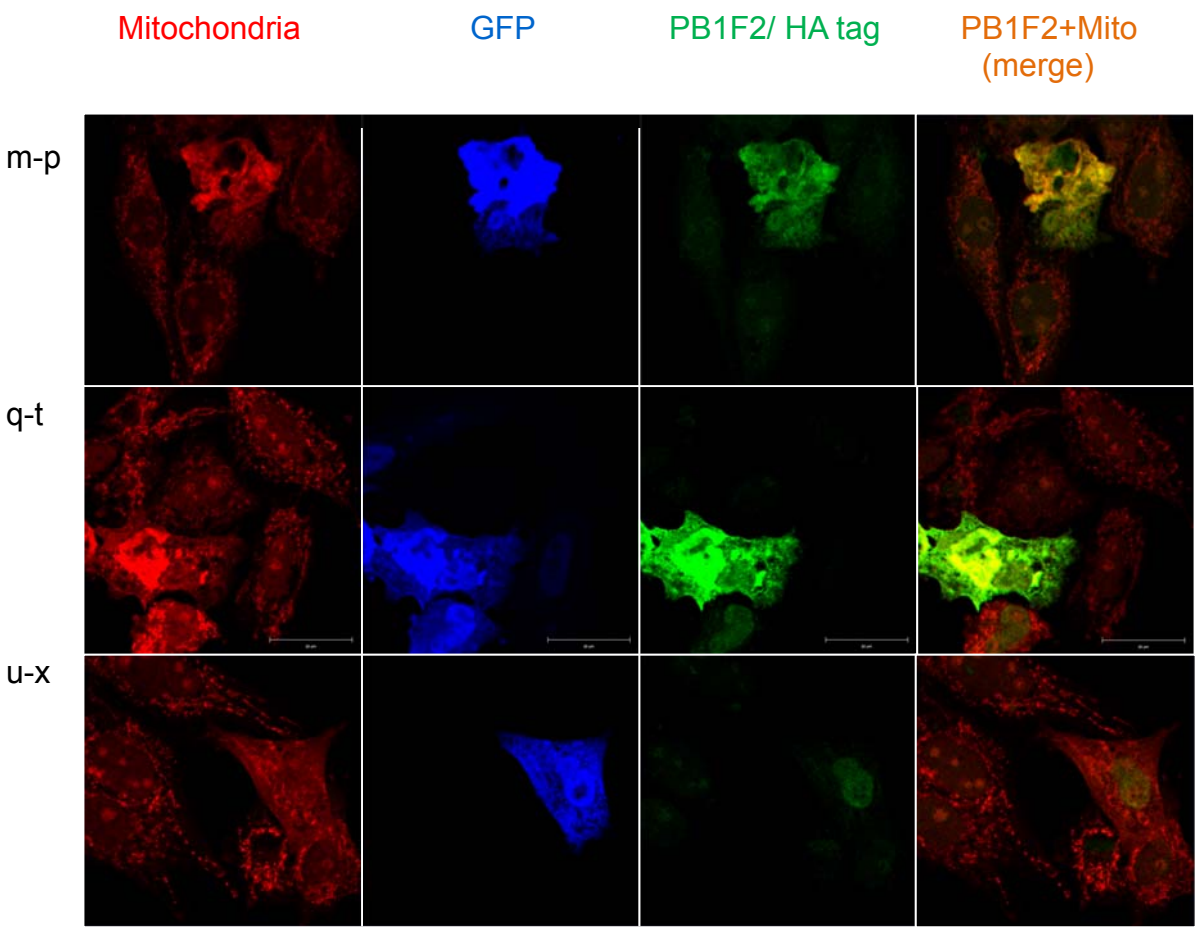



**Figure 2.6 PB1-F2 expression and mitochondrial localization**. DF1 (chicken) cells were grown on coverslips, transfected with either control plasmids or PB1-F2 plasmids from virus VN/296. 30 hr post transfection, MG132 (proteasomal inhibitor) were added to medium for 6 hr. 36 hr post transfection, cells were fixed with 4% paraformaldehyde and permeablized. PB1-F2 was probed by staining HA-tag (green), mitochondria were stained using MitoTracker (red) and examined under confocal microscope. a-d, untransfected; e-h, CMV2/GFP (empty cloning vector); i-l, ERAP1-HA clone as positive control for HA detection; m-p, q-t and u-x were transfected with full length, No-ATG and Truncated PB1-F2 mutant clones of virus VN/296, respectively.

**Figure 2.6 (cont'd)**



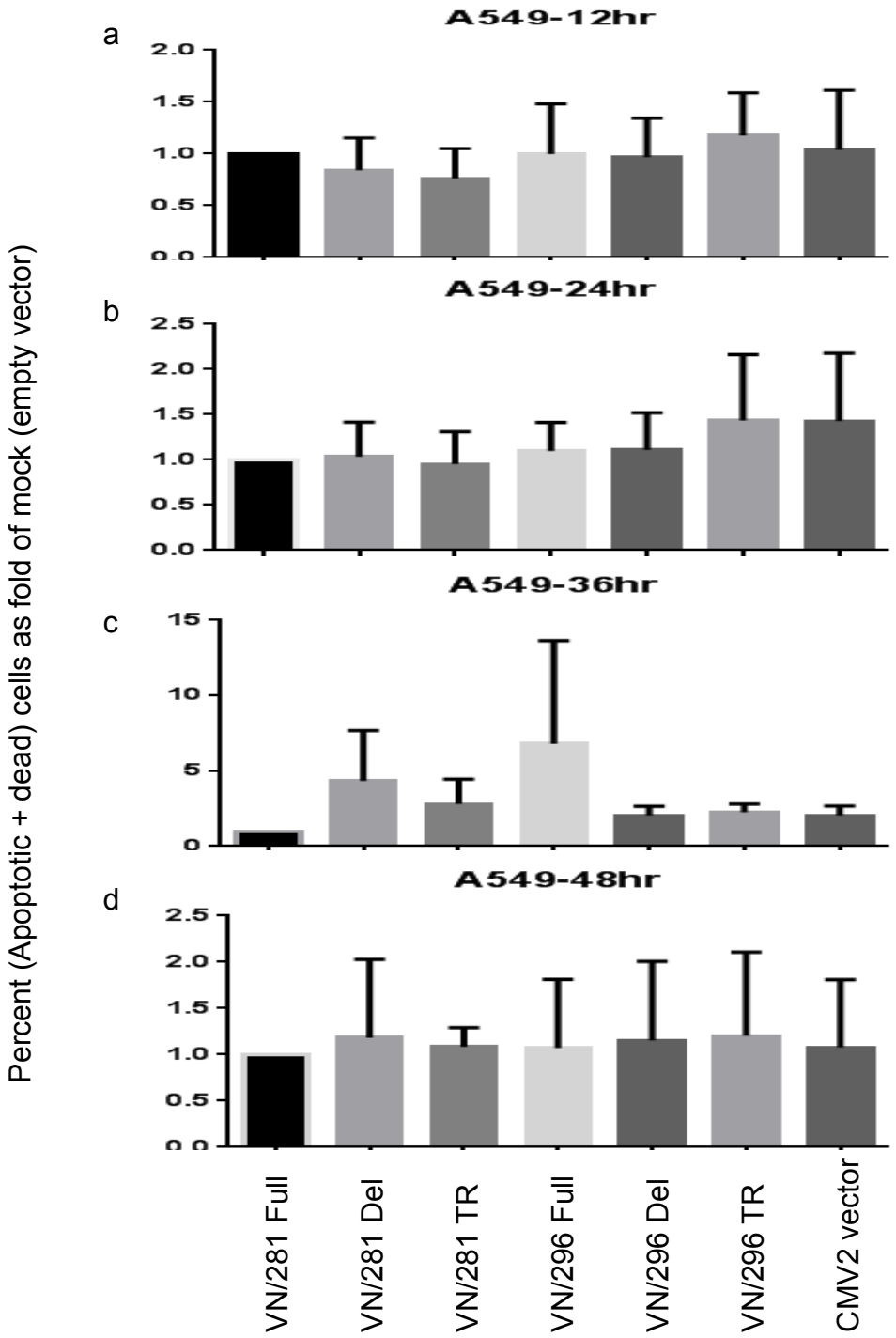



**Figure 2.7 PB1-F2 effect on apoptosis.** A549 cells were transfected with either empty vector or PB1-F2 mutant plasmids. Cells were harvested at 12 hr (a), 24hr (b), 36hr (c) and 48 hr (d) post transfection. Cells were stained apoptotic and necrotic dies. Data represented dead + apoptotic cells as fold change over CMV2 vector (control).
Note: scale different for different time points.

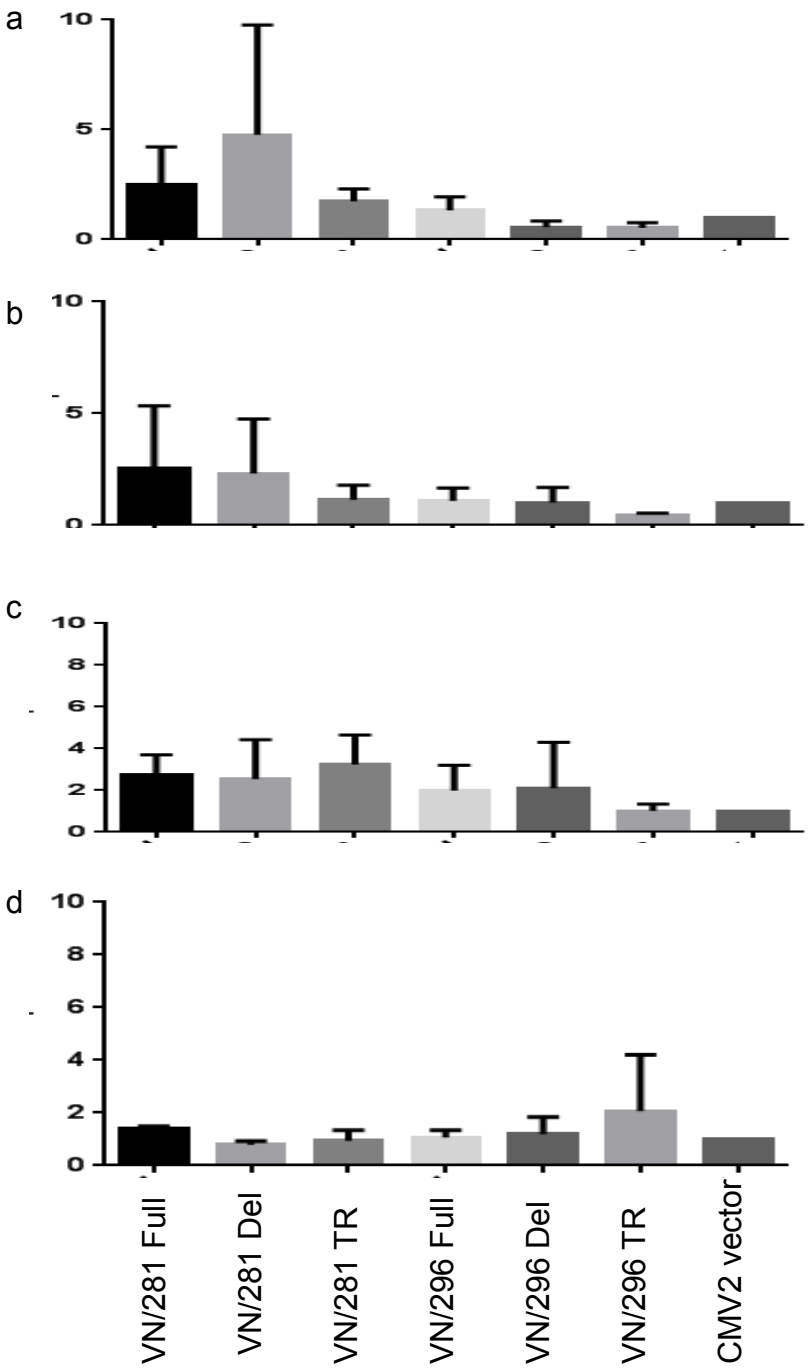



**Figure 2.8 PB1-F2 effect on IFNb expression.** A549 cells were transfected with either empty vector or PB1-F2 mutant plasmids. Cells were lysed at 12 hr (a), 24hr (b), 36hr (c) and 48 hr (d) post transfection. Expression was measured using TaqMan assay and qRT-PCR. IFNb expression is normalized to GAPDH (internal control) and presented as fold increase relative to CMV2-vector transfection.

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## JOINING CHAPTER

## PATHOGENICITY DETERMINACY TO PANDEMIC VACCINES

Avian influenza viruses are undergoing constant evolution in domestic poultry and occasionally infect other species including humans. All the 20$^{th}$ century and 21$^{st}$ century human influenza pandemic viruses have derived multiple gene(s) from avian viruses either directly or after further evolution in swine. While multiple avian viruses have sporadically infected humans, including H6N1 (Yuan, Zhang et al. 2013), H9N2 (Pawar, Tandale et al. 2012), and H10N7 (Arzey, Kirkland et al. 2012), viruses belonging to subtype H5 and H7 have been predicted to be major public health risks. Both these HA types include highly pathogenic viruses based on their phenotype in avian hosts. Although the highly pathogenic phenotype in domestic poultry doesn't necessarily translate to human infections, both of these subtypes also include viruses that have caused fatal infections in humans, and due to constant evolution and possible reassortment events, these subtypes pose the greatest human health threat. To counteract the risk, US Dept. of Human Health and Services, along with its national and international public health partners, constantly performs surveillance for new influenza infections and studies the evolution and emergence of new influenza virus isolates. Virus surveillance is followed by two major strategies of pandemic preparedness: Risk assessment (especially determining pathogenicity and transmission), and risk mitigation (especially preparing vaccines against potentially pandemic viruses).

In the first chapter of this dissertation, I focused on the risk assessment of HPAIV containing PB1-F2 mutations. I studied the evolution of H5N1 influenza viruses and

found a new trend in the emergence and evolution of PB1-F2 proteins. Historically rare in avian viruses, the truncated PB1-F2 has independently evolved in multiple different clades. In a mouse model, the virus VN/296 with a defective initiating ATG of PB1-F2 is 1000-fold more virulent in mice than a paired virus VN/281 expressing full length PB1-F2. I further explored the mechanism underlying the difference in virulence in vitro in human (A549) and chicken (DF1) cells. I studied the previously described phenotypes of PB1-F2 (apoptosis, IFNβ regulation and mitochondrial localization) with cloned plasmids in transient expression system. Most of these phenotypes have been found in studies with PR8 viruses while studies with H5N1 viruses are still lacking. As shown in previous studies with H5N1 viruses, I did not find significant difference in apoptosis of either of the studied cell lines. I also did not find any difference in the regulation of IFNβ expression. I demonstrated that full length PB1-F2 localizes to mitochondria, the first report showing the mitochondrial localization of H5N1 PB1-F2. I also demonstrated that the absence of the initiating ATG still permits expression of a protein from the PB1-F2 region, which I suggest is due to translation from a down-stream ATG to produce a C-terminal fragment of PB1-F2 that does not appear to localize to mitochondria. Based on my in vivo and in vitro data and previously published H5N1 studies, I propose a working model for the virulence difference I observed in mice. I propose that the full length PB1-F2 in VN/281 is playing a protective or neutralizing role against an exacerbated inflammatory response elicited by the C-terminal of PB1-F2 (present in VN/296). Further studies in particular with isogenic viruses made by reverse genetics will enhance the understanding and define the mechanism. Further research on the

mechanism was out of the reach of this PhD dissertation research due to the interim moratorium on research involving H5N1 gain of function mutations.

My second project (discussed in the following chapter) focused on the second major strategy of pandemic preparedness: Vaccines against potentially pandemic strains of influenza. A pre-pandemic vaccine is a vaccine which is made based on risk assessment of current and emerging influenza strains that could be used for protection against future pandemic events. Pre-pandemic vaccines against avian influenza viruses have been hindered by the poor immune response against these in humans. This is particularly true for vaccines against H7 viruses.

Protection against influenza viruses is mainly produced by anti-HA antibodies. Seasonal flu vaccines elicit protective antibody titers, for which the accepted serological correlate is an HI titer of 40 or more. However, human serosurveillance studies and results of H7 vaccine clinical trials have found very poor serum antibody titers as measured by HI. These findings suggested that the HA of H7 viruses may be poorly immunogenic. If so, pre-pandemic vaccines may not be effective when needed in the future.

In spite of widespread anecdotal concern about H7 immunogenicity, no previously published study had compared the immunogenicity of H7 and seasonal viruses. I therefore performed this study as a second project in pandemic preparedness. My specific aim was to evaluate and compare the immunogenicity of H7 HAs with that of seasonal viruses. The project is described in the following chapter of this dissertation.

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# CHAPTER 3

## HEMAGGLUTININ OF THE H7 SUBTYPE IS INTRINSICALLY POORLY IMMUNOGENIC

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**Abstract:**

Of the influenza viruses within the seventeen known influenza hemagglutinin (HA) subtypes, three have been responsible for pandemics in humans. Most subtypes continuously circulate in wild birds and domestic poultry, and some of these avian influenza viruses (AIVs) sporadically infect humans, with potential to cause a new pandemic. Vaccines are the main defense against these potentially pandemic AIVs. However, vaccines against AIVs often require high HA doses, or adjuvants, to achieve acceptable serological titers. Viruses of the H7 subtype, in particular, frequently have not induced strong antibody responses following infection or immunization. Here we compared the immunogenicity, in naïve mice, of purified recombinant HA from two H7 viruses (A/Netherlands/219/2003(H7N7) and A/New York/107/2003(H7N2)) to that of HA from seasonal viruses (A/California/07/2009(H1N1pdm09) and A/Perth16/2009(H3N2)). Mice produced antibodies to all HAs, but the response to seasonal viruses was greater than to H7s. The difference was relatively minor when measured by ELISA, yet greater when measured by hemagglutination inhibition assays, and more marked by microneutralization assays, by which H7s induced a very weak or no response. Antibodies induced by H7 were of significantly lower avidity than for H3 or H1pdm09. We conclude that H7 HAs may be intrinsically less immunogenic than HA from seasonal human influenza viruses.

**Introduction:**

Of the seventeen known subtypes of influenza virus hemagglutinin (HA), all but one commonly circulate in birds, in which they generally cause mild to no disease.

Reassortment and mutation events periodically produce influenza viruses capable of infecting mammals, and several influenza strains are now endemic in mammalian species including swine, horses, dogs, and humans. In humans, this process has resulted in several pandemics, during the 20$^{th}$ and 21$^{st}$ centuries, which have been associated with high mortality (Morens and Taubenberger 2010, Taubenberger and Kash 2010). Accordingly, public health services now closely monitor the sporadic infections of humans with avian influenza viruses, in the hope of predicting and preventing the next pandemic.

One avian influenza subtype with pandemic potential is the H7 lineage, in combination with several neuraminidase (NA) subtypes (reviewed in (Belser, Bridges et al. 2009)). These viruses have sporadically infected humans in multiple countries. In most cases, these infections have been mild, often causing only conjunctivitis (Belser, Bridges et al. 2009). Furthermore, on several occasions, H7 viruses have caused outbreaks that have infected larger contingents. In the Netherlands in 2003, at least 89 people were infected with an H7N7 influenza virus, and there was one fatality due to severe pneumonia (Fouchier, Schneeberger et al. 2004, Koopmans, Wilbrink et al. 2004). Recently, a human outbreak by a novel H7N9 avian influenza virus has led to the infection of 135 people in China, with frequent severe disease and a mortality rate of over 30% (Chen, Liang et al. 2013, Li, Zhou et al. 2013) as of August 12, 2013 (World Health Organization 2013).

Serological testing of people for evidence of infection with H7 viruses has proven difficult. Titers measured using hemagglutination inhibition (HI) and microneutralization

(MN) assays have generally been very low (Du Ry van Beest Holle, Meijer et al. 2005, Puzelli, Di Trani et al. 2005, Skowronski, Li et al. 2007, Kayali, Setterquist et al. 2008, Bos, Te Beest et al. 2010, Kayali, Ortiz et al. 2010, Chen, Liang et al. 2013, Li, Zhou et al. 2013) even in people known to have been infected with H7 viruses (Meijer, Bosman et al. 2006). This poor antibody response apparently extends to the vaccines against H7 that have been evaluated in pre-clinical and clinical studies (Joseph, McAuliffe et al. 2008, Cox, Madhun et al. 2009). For example, in a recent clinical trial of an H7N7 vaccine, only 2 of 99 vaccinees developed significant hemagglutination inhibition (HI) titers against H7 (HI titer ≥32), even though high doses of the protein (up to 90 μg per person) were used (Couch, Patel et al. 2012).

To our knowledge, the intrinsic immunogenicity of H7 HA has not been directly compared to that of seasonal viruses (such as H3N2 and H1N1pdm09) that are known to induce acceptable HI titers. We therefore used recombinant HA to compare immunogenicity of several HA subtypes, including two H7 subtypes, in naïve mice.

## Materials and Methods:

**Recombinant hemagglutinin.** The purified recombinant hemagglutinins (HA) and HA1 were expressed and purified from baculovirus vectors in insect cells, as described for HA from A/Netherlands/219/2003(H7N7) (NL219) (Yang, Carney et al. 2012); A/New York/107/2003(H7N3) (NY107) (Yang, Chen et al. 2010); A/California/7/2009(H1N1pdm09) (CA07) (Chen, Rivailler et al. 2011); and A/Perth16/2009(H3N2) (Perth16) (Stevens, Chen et al. 2010). GenBank accession

numbers for the HAs are ACS71642.1 (Perth16), ACP41953.1 (CA07), AAR02640.1 (NL219), and ACC55270.1 (NY107).

**Viruses.** Wild-type Perth16 and 2:6 A/Puerto Rico/1/1934 (PR8) reassortants of NL219, NY107, and A/Texas/5/2009(H1N1pdm09) (TX05, antigenically homologous to CA07) were used in hemagglutination inhibition (HI) and microneutralization (MN) assays. All the viruses used in this study were propagated in 10-11 days old embryonated chicken eggs and were stored as allantoic fluid.

**Immunization.** Female 6-8 week old BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. For immunization, groups of five mice were immunized intramuscularly (IM) 2 times, with 3 weeks between injections, with either 3 or 10 μg of purified recombinant HA in sterile PBS. Each immunization study included a control group of 5 mice who were injected with sterile PBS. These doses were chosen based on a pilot dose-response study (data not shown). Blood samples were collected on day 0 (before initial immunization), day 21 (before the boost), and on days 35, 49, and 63.

Animals were housed and handled under ABSL2 conditions. Live H7 viruses were handled under BSL3+ conditions (Chosewood, Wilson et al. 2009). Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

**Antibody assays.** All sera were initially incubated with receptor-destroying enzyme (RDE, from *Vibrio cholerae*, Denka Seiken, Tokyo, Japan), at the ratio of one volume of

serum and three volumes of RDE, overnight at 37$^{o}$C. RDE was inactivated at 56$^{o}$C for 30 minutes and then 6 volumes of PBS were added to each volume of serum, making the final serum dilution of 1:10. Serum antibody titers to TX05, Perth16, NL219, and NY107 were measured by HI, ELISA and MN. Each serum sample was tested at least in duplicate. Known-positive ferret sera for homologous viruses were used as positive controls.

**HI assay.** 96-well V-bottom plates were used for HA titration and HI assays. Freshly prepared 0.5% turkey red blood cells RBC (TRBC) or 1.0% horse RBC (HRBC) were used for HA titration and HI assay of seasonal and H7 sera samples, respectively, as described (World Health Organization 2011). Briefly, two-fold serial dilutions of sera (25 µl) were incubated with 4 HA units (25 µl) of homologous virus for 30 min at room temperature. Subsequently, 50 µl of RBCs were added for 30 min (TRBC, seasonal samples) or 1 hour (HRBC, H7 samples) and wells were read for inhibition of hemagglutination. HI titers were calculated as the reciprocal of the highest dilution of serum that inhibited 100 % of the hemagglutination of 4 HA units of homologous virus. As positive controls, we used serum from ferrets infected with wild-type CA07, Perth16, NL219 or NY107. The minimum detectable limit of this assay was an HI titer of 10.

**ELISA.** Individual wells of 96-well high binding plates (Costar, Corning, NY) were coated with purified recombinant full length ectodomain HA, or the HA1 domain, at a concentration of 1 µg/ml at 4$^{o}$C overnight. Recombinant full-length HAs used as antigen for ELISA were obtained through the Influenza Reagent Resource (IRR: Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of

Influenza, Centers for Disease Control and Prevention, Atlanta, GA) with the following catalog numbers: Perth16 HA, (FR-472), NL219 HA (FR-71), CA07 (FR-559), NY107 (FR-69). Wells were washed with PBS and then blocked with 0.1% bovine serum albumin in PBS for one hour at 37$^{O}$C. Blocking buffer was discarded and serially diluted sera samples were added to the plates and incubated for 1 hour at 37$^{O}$C. Plates were washed with phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST) and HRP-conjugated Goat Anti-Mouse IgG (Alpha Diagnostic, San Antonio, TX) was diluted and applied to each well. Plates were incubated for 1 hr at 37 $^{O}$C and were then washed with PBS. O-phenylenediamine (OPD) solution was added to each well as a peroxidase substrate and the reaction was stopped after 10 minutes by adding 50 µl 3N HCl. Absorbance was measured at 490 nm. Antibody titers are given as the reciprocal of the highest dilution which gave an $OD_{490}$ value greater than 2 times the average of the background wells.

**Avidity assay.** For avidity ELISA assays (el Zayyat, Khalifa et al. 1998, Bjorkman, Naslund et al. 1999, Rahbari, Keshavarz et al. 2012) we modified our ELISA protocol as follows. Plates and sera were prepared as described above, and serum samples were applied to the wells in duplicate. After one hour incubation, one replicate of samples was incubated with PBST for 5 minutes and the other replicate of samples were incubated in 4M urea (Sigma-Aldrich, St. Louis, MO) for 5 minutes. All samples were then washed twice with PBST, after which the rest of the ELISA protocol was completed as described above. The avidity index (AI) was calculated as previously described (Rahbari, Keshavarz et al. 2012) for the lowest dilution (1:2500) as AI= (U+/U-) x100,

where “U+” is the $OD_{490}$ for wells washed with urea and “U-” is the $OD_{490}$ for wells washed with PBST.

**Microneutralization assay.** MN assays were performed as described (World Health Organization 2011). RDE-treated sera samples were added to 96-well flat bottom microtiter plates (Costar) and twofold serially diluted with diluent (DMEM supplemented with 1% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM HEPES) for a final well volume of 50 µl. 100 tissue culture infectious dose 50 ($TCID_{50}$) of virus in 50 µl diluent was then added to all sample wells. As well as test samples, each plate contained 4 wells of cell control (cells without virus and serum), 4 wells of virus control (cells with virus and no serum), and back titration (cells with 2 fold dilution of virus). As positive controls, we used serum from ferrets infected with wild-type Perth16, CA07, NL219 or NY107. Plates were then incubated at $37^{o}$C for one hour followed by addition of $1.5 \times 10^{5}$ cells/ml in 100 µl to each well. Plates were incubated overnight (18-22 hrs) at $37^{o}$C. Plates were washed once with PBS, and then treated with cold fixative (80% acetone/20% PBS) for 8 minutes. An ELISA was then performed in order to determine antibody titers of serum samples as follows. Fixed plates were washed twice with PBST and 100 µl of anti-influenza A antibody (Millipore, Billerica, MA) diluted in blocking buffer (1% BSA-PBST) was added to each well. After 1 hour incubation, plates were then washed in PBST three times and 100 µl of horseradish peroxidase-conjugated goat anti-mouse IgG diluted in blocking buffer was added to each well. The plates were incubated at room temperature for 1 hour and then washed 4 times with PBST. 100 µl of freshly prepared OPD in citrate buffer was then added to each well and the reaction was

stopped by adding 50 µl 3N HCL after the color in the virus control wells reached a deep orange-yellow color. Absorbance was measured at 390 nm and the cutoff was determined by the following equation: X = [(median OD of VC wells) + (median OD of CC wells)]/2. All values equal to or less than X were considered positive for neutralization. Antibody titers provided are the reciprocal of the serum dilution for the corresponding well considered positive for neutralization.

**Statistical analyses.** Statistical analyses for serum HI, MN, and ELISA titers were performed using a linear mixed model with repeated measures, implemented in SAS, using a cutoff of $p \leq 0.05$ for significance. For avidity ELISAs, 1-way ANOVA was performed and significance was determined using a two-sided Student t test, with a cutoff of $p \leq 0.05$ for significance.

## Results:

**ELISA responses to purified HA.** ELISA responses were lowest to the HA from NY107 (Figure 3.1A, C, E). Perth16 HA induced the highest response, and this was statistically significant ($p<0.05$) for the 10 µg group (Figure 3.1C, E). However, NL219 responses did not differ significantly from the responses to the seasonal viruses for the 3 µg dose (Figure 3.1A, E), and was similar to H1pdm09 for the 10 µg dose. The same pattern was seen in considering only the maximum titer reached at any time point, with Perth16 being the highest and NY107 being the lowest. CA07 and NL219 titers were similar to each other, and in the 10 µg dose significantly lower than Perth16 (Figure 3.1E). Thus, although by ELISA NY107 was less immunogenic than the other three viruses tested, NL219 was similar to the seasonal virus CA07.

Similar results were obtained using HA1 as the antigen in ELISA (Figure 3.1B, D, F), although actual titers were significantly lower, as expected (since in this assay antibodies raised against the conserved stalk component of HA would not be detected). Perth16 HA induced the greatest HA1-specific response ($p<0.05$) while the response to CA07 was similar response to that for NL219 or NY107.

**Hemagglutination inhibition (HI) antibodies.** While ELISA measures the overall antibody response to HA, HI assays measure the antibodies that bind at or near the HA receptor binding site. Both the H7 HAs gave significantly lower HI titers than both the seasonal HAs at the 3 µg dose (Figure 3.2A). However, at the 10 µg dose, the two H7 viruses achieved similar titers to CA07, though all three of these HAs induced lower titers than did Perth16 (Figure 3.2B). A similar pattern was seen when considering only the GMT of the maximum titers induced by the HAs (Figure 3.2C).

**Virus-neutralizing antibodies.** Both the seasonal HAs gave MN titers that were generally equal to, or higher than, their HI titers (Figure 3.3A-C). In contrast, only two of 40 mice immunized with H7 HAs gave detectable MN titer during the whole study time. As positive controls we used sera from ferrets infected with wild-type NL219 or NY107. These sera had HI titers of 1280 and 320 for NL219 and NY107, respectively (Figure 3.3D) and MN titers were readily measured for these sera (Figure 3.3D) indicating that the MN assay is technically functional for H7 viruses.

Since the average HI GMT of H7-immunized mice were lower than that of seasonal HA-immunized mice, it was possible that MN titers were only proportionally reduced in H7-immunized mice. Since no H7-immunized mice achieved HI titers greater than 160, we

selected sera from individual animals, immunized with each HA, with HI titers of 80 or 160, and compared MN titers for each sample. We identified samples from 6 mice each for Perth16, CA07, NL219, and NY107. (Where the same animal had more than one sample with titers of 80 or 160, only one sample was chosen.) HI titers were similar for all HA, as expected, since these sera were specifically selected to be comparable (Figure 3.4A). As noted, none of the H7-immunized mice had detectable MN titers. However, all of the seasonal HAs induced easily-detectable MN responses, with MN titers being similar to or higher than the HI titers (Figure 3.4A). Thus, even when HI titers were similar, H7 HAs induced relatively low MN titers compared to H3 and H1pdm09.

**Avidity ELISAs**. To compare the avidity of the antibodies induced by each recombinant HA, we compared the effect of a 5 min PBST wash vs. a 5 min wash in 4 M urea on ELISA titers, as previously described (Bjorkman, Naslund et al. 1999). Again, we used the serum samples from mice with comparable HI titers of 80-160. Standard ELISA titers for these samples were also comparable (Figure 3.4B, “PBST”), although the titer for CA07 was slightly higher than for Perth16 ($p$=0.043). Adding the urea wash (Figure 3.4B, “Urea”) did not reduce the detected titer in serum samples from mice immunized with Perth16 HA, but caused moderate (approximately 2-fold) reduction in titers for CA07 and NL219 sera, and a 3.5-fold reduction in titer for the NY107 sera (Figure 3.4C). Following the urea wash, both NL219 and NY107 titers were significantly lower than either Perth16 or CA07 titers ($p < 0.05$), while CA07 and Perth16 titers were still comparable ($p$=0.363). The avidity index (AI) for the H7 HAs was significantly lower than for either seasonal HA (Figure 3.4C) ($p$ <0.05 vs. CA07; $p$ <0.001 vs. Perth16).

## Discussion:

Human serological responses to several strains of avian influenza viruses seem to be weaker than those to seasonal influenza strains (Beare and Webster 1991, Treanor, Wilkinson et al. 2001, Beigel, Voell et al. 2009, Brady, Treanor et al. 2009, Nicholson, Thompson et al. 2009, Talaat, Karron et al. 2011). In particular, people infected with H7 subtype influenza viruses rarely develop high antibody titers, as measured by HI or MN assays (Du Ry van Beest Holle, Meijer et al. 2005, Puzelli, Di Trani et al. 2005, Meijer, Bosman et al. 2006, Skowronski, Li et al. 2007, Kayali, Setterquist et al. 2008, Bos, Te Beest et al. 2010, Kayali, Ortiz et al. 2010, Chen, Liang et al. 2013, Li, Zhou et al. 2013). While this may reflect the nature of infection with H7 viruses, which commonly cause superficial infections such as conjunctivitis rather than respiratory or systemic disease, this was also seen with people immunized with H7 vaccines (Joseph, McAuliffe et al. 2008, Cox, Madhun et al. 2009, Couch, Patel et al. 2012), suggesting that humans may generally respond poorly to H7 subtypes of HA. However, in the absence of studies directly comparing the response to H7 to the response to human seasonal influenza viruses such as H1N1pdm09 and H3N2 strains, it has not been clear that H7 is exceptional. Furthermore, it is not clear if any difference in response might be related to intrinsic aspects of the HA, to pre-existing immunity in humans, or to some aspect of the virus or the vaccine. To distinguish between these possibilities, we immunized naïve mice with purified recombinant HA from two human seasonal viruses, H1N1pdm09 and H3N2, and two different H7 viruses, and compared the antibody response to each. The H7 HAs were from NL219 (H7N7), a highly pathogenic avian influenza virus (HPAIV) representing the Eurasian lineage of H7 (Fouchier, Schneeberger et al. 2004), and

NY107, a low-pathogenicity avian influenza virus (LPAIV) representing the North American lineage of H7 (Ostrowsky, Huang et al. 2012). Both viruses were isolated from infected humans.

ELISA assays measure a broad range of antibodies, including those that are protective as well as those that bind to HA but fail to protect. When measured by ELISA assays, the HA from NY107 was somewhat less immunogenic than either seasonal HA. However, the response to the HA from NL219 was similar to that to H1pdm09, though lower than that to Perth16. When measured by HI assays, both H7 HAs were considerably less immunogenic than either H3 or H1pdm09. HI assays measure the ability of antibodies to prevent RBC hemagglutination by influenza viruses, mainly due to blockade of the receptor-binding site (RBS) on the globular head of the HA (Katz, Hancock et al. 2011). Due to their correlation with clinical protection (Katz, Hancock et al. 2011), HI assays are widely used for determining antibody responses to influenza viruses. Our results here are consistent with clinical findings that HI titers following infection with H7 influenza viruses tend to be low.

The microneutralization assay (MN) is a third widely used assay for measuring antibody responses to influenza viruses. MN assays functionally measure the ability of antibodies to protect cells against infection, and as such are highly biologically relevant. By this assay, the difference between the responses to H7 HA, and HA from seasonal viruses was most dramatic. Even when limiting analysis to sera from H7-immunized mice where an HI response to H7 was clearly detectable, MN responses were absent or very low (Figure 3.4A). In contrast, all mice immunized with H3, and almost all those immunized with H1pdm, developed strong MN titers.

The H7 HAs, especially NL219, thus showed discordant results depending on the assay used to measure antibodies. Thus although by ELISA NL219 showed equivalent titers to CA07, the corresponding HI titers were comparatively low, and MN titers were almost undetectable in the same samples. If antibodies are being generated to H7 viruses (as shown in ELISA), why are they not detected in HI and MN assays? Two possibilities are that, although antibodies are being produced, they are either low affinity antibodies, or they target non-neutralizing regions of the HA, such as the stalk. We used HA1 as the antigen in ELISA assays to test whether the antibody response to H7s disproportionately targets the stalk region of HA. However, although titers against HA1 were significantly reduced for all HAs compared to those against the full-length HA, NL219 and the seasonal HAs all showed a 40-50% reduction in titer (Figure 3.1D). Titers for NY107 were very low for full-length HA and almost undetectable for HA1. Since the HA1-specific responses for NL219 and NY107 were similar to that for CA07 ($p > 0.05$), there is no evidence that the H7 and H1pdm09 HAs induced different levels of HA1-specific antibodies.

The antibodies produced by B cells increase in affinity through the course of an infection, as activated B cells undergo somatic hypermutation of their immunoglobulin variable regions (reviewed in (Teng and Papavasiliou 2007). This affinity maturation is enhanced by inflammation, including adjuvants (Khurana, Verma et al. 2011), as well as repeated antigen exposure (Teng and Papavasiliou 2007). Testing the avidity of the antisera suggested that H7, especially from NY107, did indeed induce low-affinity antibodies, since a brief urea wash reduced the bound antibody titer dramatically (Figure 3.4B, C). Antibodies induced by NL219 were also of relatively low affinity.

Interestingly, not only did Perth16 HA induce the highest titers of antibodies as measured in all assays, the avidity of these antibodies is also apparently very high, with titers being unaffected by the urea wash (avidity index = 100.3%).

The molecular mechanism(s) for the difference in immunogenicity is unknown. In humans infected with avian influenza, weak immunogenicity may reflect the lack of prior exposure to avian HA, compared to the repeated exposure to seasonal HA that may act as priming immunization. Another possibility is that some feature of the vaccine or virus, other than HA itself, may influence the response. However, in these experiments, the use of naïve mice and purified recombinant HA suggests that intrinsic features of the HA itself may affect immunogenicity.

One possible explanation is that the amount of glycosylation on the HA may influence its interaction with the immune system (Crouch, Hartshorn et al. 2000, Reading, Tate et al. 2007, Reading, Pickett et al. 2009, Wang, Chen et al. 2009, Tate, Brooks et al. 2011, Tate, Job et al. 2011, Wanzeck, Boyd et al. 2011, Medina, Stertz et al. 2013). Although H3N2 viruses are believed to have evolved increasing glycosylation over time in order to prevent immune recognition of antigenic sites (Cherry, Lipman et al. 2009, Das, Puigbo et al. 2010, Suzuki 2011, Kobayashi and Suzuki 2012), the protection is mainly against memory responses rather than primary induction of antibodies. That is, added glycosylation reduces recognition of the virus by pre-formed antibodies raised against previous versions of the virus, whereas increasing glycosylation of HA increases the immunogenicity, and reduces the pathogenicity, of H3N2 (Vigerust, Ulett et al. 2007, Tate, Job et al. 2011, Wanzeck, Boyd et al. 2011) and other influenza viruses (Medina, Stertz et al. 2013, Sun, Jayaraman et al. 2013). The findings here are consistent with

those findings, since the number of potential glycosylation sites on the HAs in these experiments parallels their immunogenicity, with Perth16 (the most immunogenic HA) having 11 potential carbohydrate addition sites, and CA07, NL219, and NY107 having 7, 6, and 5 sites, respectively.

It is important to note that these results are based on immunization with purified recombinant protein. Purified proteins are intrinsically less immunogenic than the same protein in a vaccine, which not only presents the proteins in multimeric form but also provide adjuvant factors, such as viral nucleic acids (Jeisy-Scott, Kim et al. 2012). Similarly, viral infection provides strong inflammatory signals that might be able to overcome low intrinsic immunogenicity. For example, even though immunization of mice with recombinant H7 from NL219 and NY107 induced weak or no MN titers, positive-control serum from ferrets infected with these viruses had easily detectable MN antibodies (Figure 3.3D). It is interesting (and consistent with findings for humans infected with H7 viruses) that for both cases the MN titer is lower than the HI titer, while for seasonal viruses the reverse is typically true (compare Figures 3.2 and 3.3). Nevertheless, our findings suggest that, with all other factors being equal, H7 HAs may be less immunogenic than seasonal H3 or H1pdm09 HA, and H7 vaccines may require higher doses or adjuvant, or may require introduced mutations in HA, in order to achieve acceptable titers.

**Acknowledgements:**

We thank James Stevens, Paul Carney, and Hua Yang for purified recombinant hemagglutinins. Amanda Balish and Jessica Belser for positive ferret sera, Li-mei Chen and Jaber Hossain for PR8 ressortant viruses.

# APPENDIX

## APPENDIX: Figures

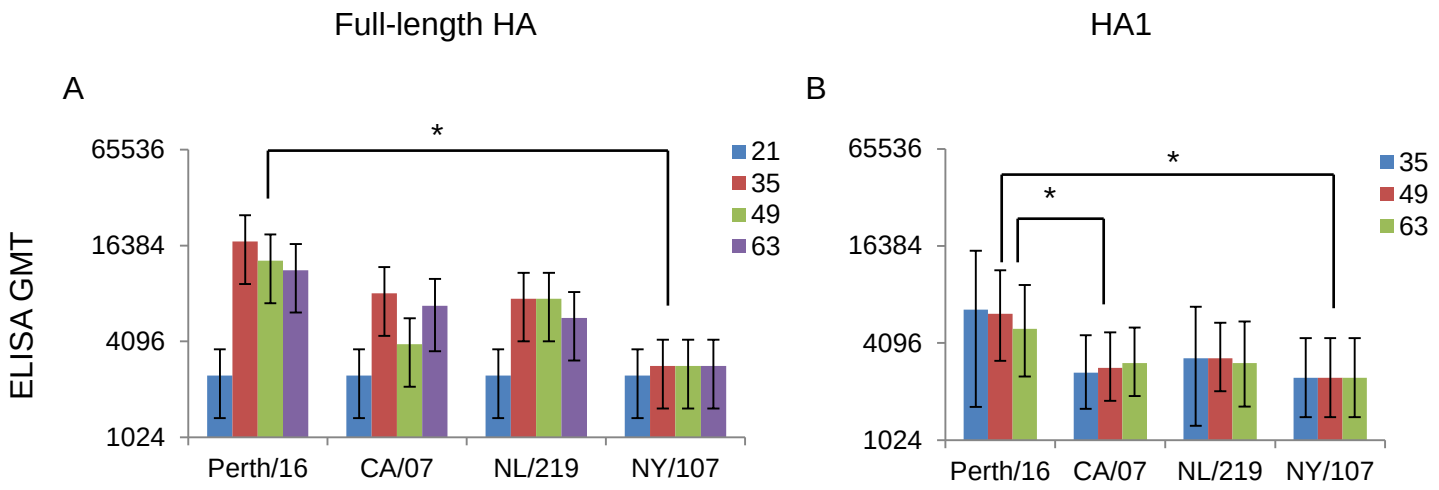
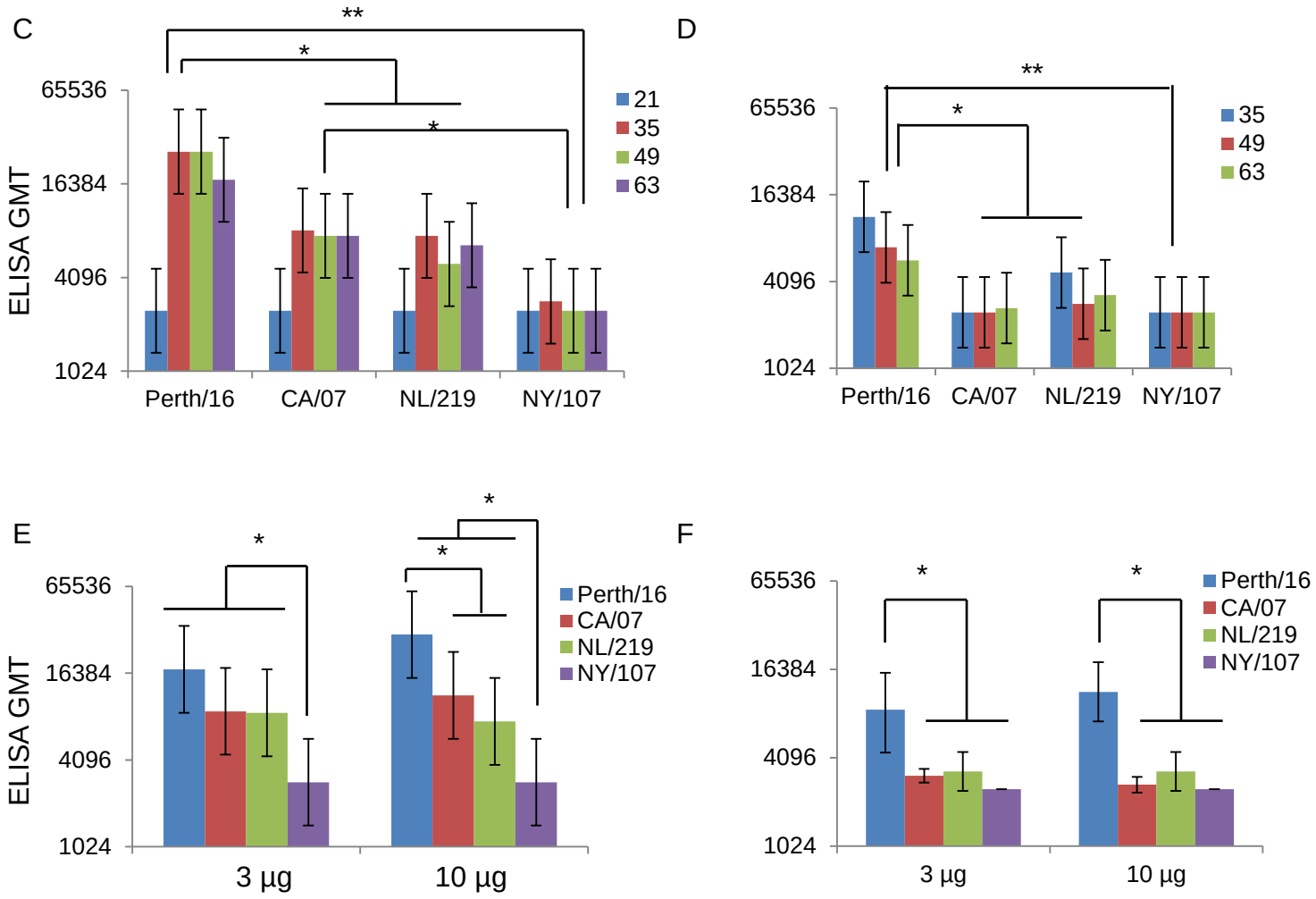



**Figure 3.1 ELISA responses to recombinant hemagglutinins.** Mice were immunized with (A, B) 3 µg or (C, D) 10 µg of purified recombinant HA from Perth/16 (H3), CA/07 (H1pdm), NL/219 (H7, Eurasian lineage), or NY/107 (H7, North American lineage) on two occasions 21 days apart. On days 0, 21, 35, 49, and 63, serum anti-HA titer was tested by an ELISA assay, using purified recombinant homologous full-length HA (A,C, E) or for days 35, 49, and 63, HA1 (B, D, F) as antigen. (E,F) Same experiment as A-D, showing the highest titer achieved by each mouse at any time point. Values are shown as geometric mean titers (GMT). Error bars represent 95% confidence intervals. Differences between groups (A-D) or peak GMT (E,F) are indicated by * ($0.0001<p<0.05$) or ** ($p<0.0001$). One representative experiment of at least 3 replicate experiments.

**Figure 3.1 (cont'd)**



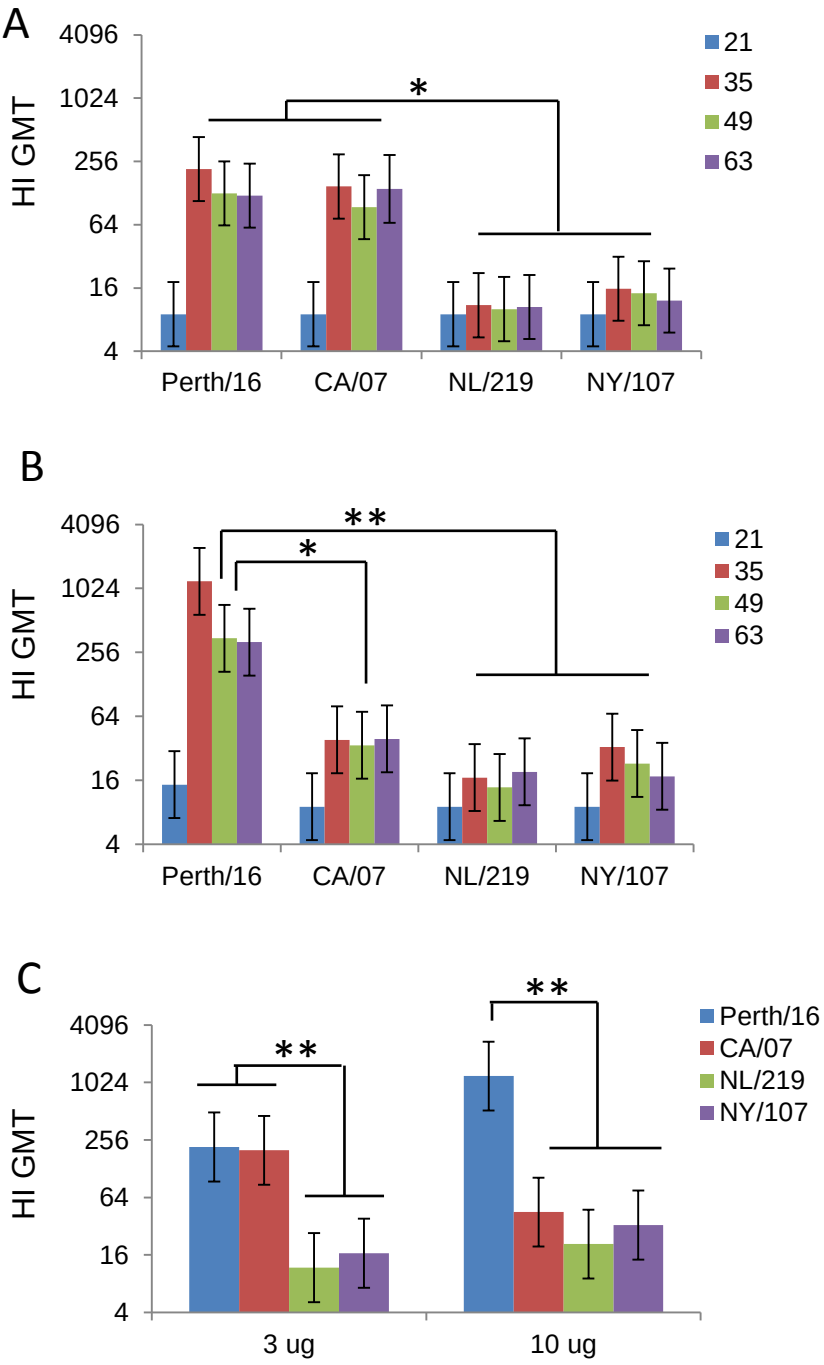



**Figure 3.2 HI responses to recombinant hemagglutinins**. Mice were immunized with (**A**) 3 µg or (**B**) 10 µg of purified recombinant HA from Perth/16, CA/07, NL/219, or NY/107 on two occasions 21 days apart. On days 0, 21, 35, 49, and 63, serum anti-HA titer was tested by an HI assay, using homologous live virus as antigen. (**C**) Same experiment as A and B, showing the highest titer achieved by each mouse at any time point. Values are shown as GMT. Error bars represent 95% confidence intervals. Differences between groups (**A, B**) or peak GMT (**C**) are indicated by * ($0.0001<p<0.05$) or ** ($p<0.0001$). One representative experiment of at least 3 replicates.

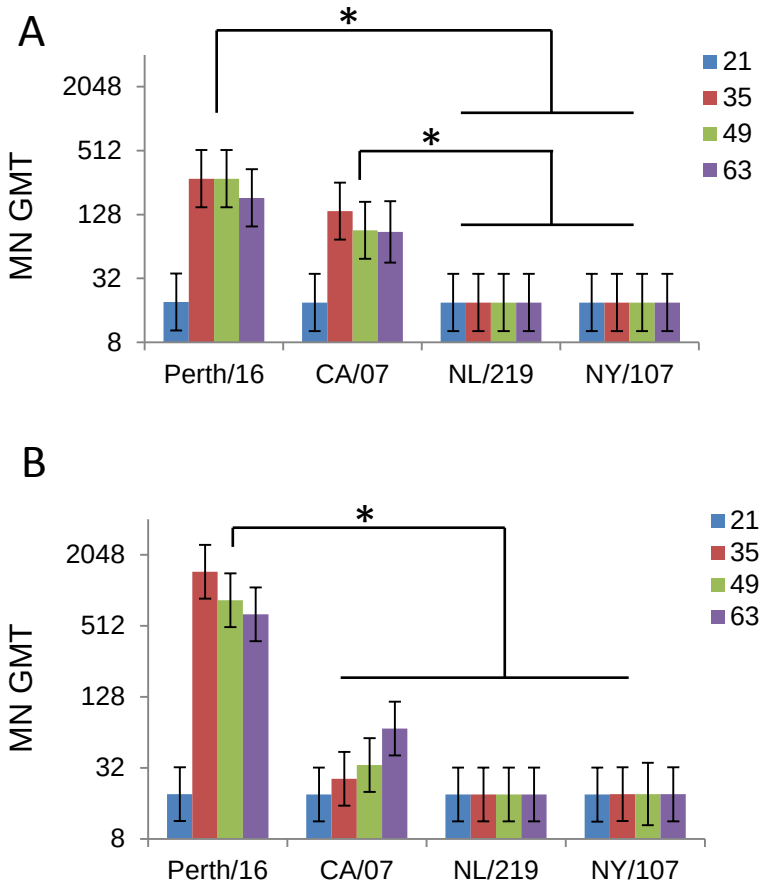
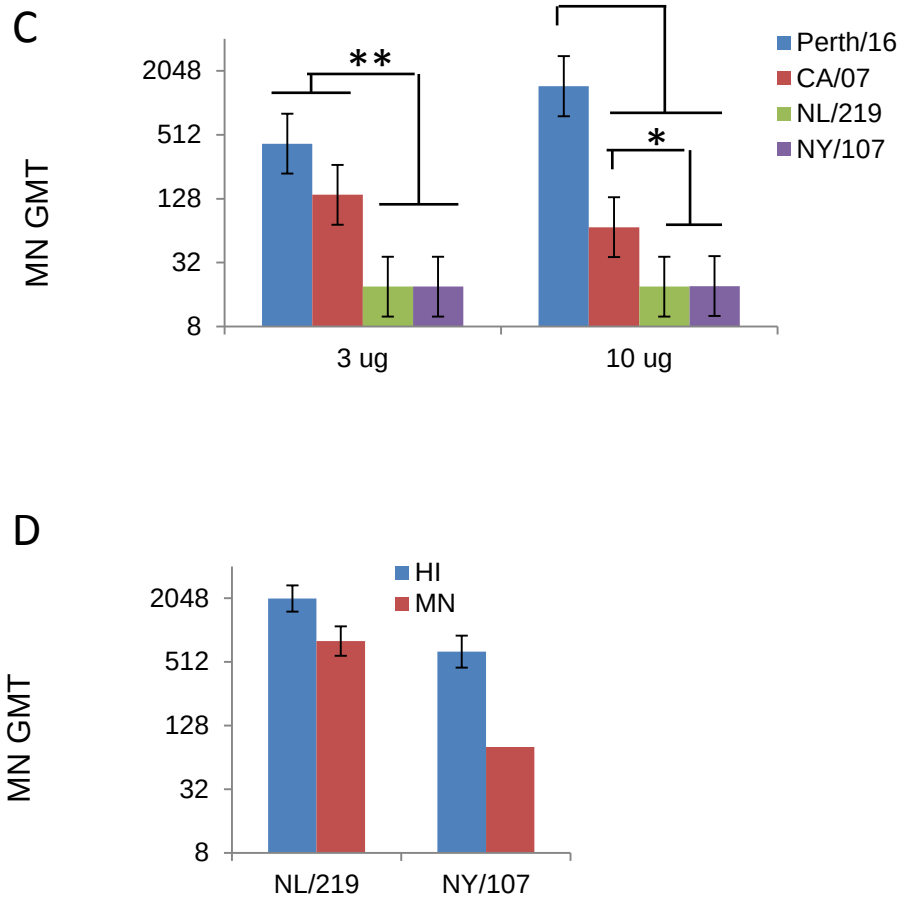



**Figure 3.3 MN responses to recombinant hemagglutinins**. Mice were immunized with (**A**) 3 µg or (**B**) 10 µg of purified recombinant HA from Perth/16, CA/07, NL/219, or NY/107 on two occasions 21 days apart. On days 0, 21, 35, 49, and 63, serum anti-HA titer was tested by a MN assay, using homologous live virus as antigen. (**C**) Same experiment as A and B, showing the highest titer achieved by each mouse at any time point. (**D**) MN and HI values for positive control ferret sera, from ferrets infected with live NL/219 or NY/107. Values are shown as GMT. Error bars represent 95% confidence intervals. Differences between groups (**A, B**) or peak GMT (**C**) are indicated by * ($0.0001<p<0.05$) or ** ($p<0.0001$). One representative experiment of at least 3 replicates.

**Figure 3.3 (cont'd)**



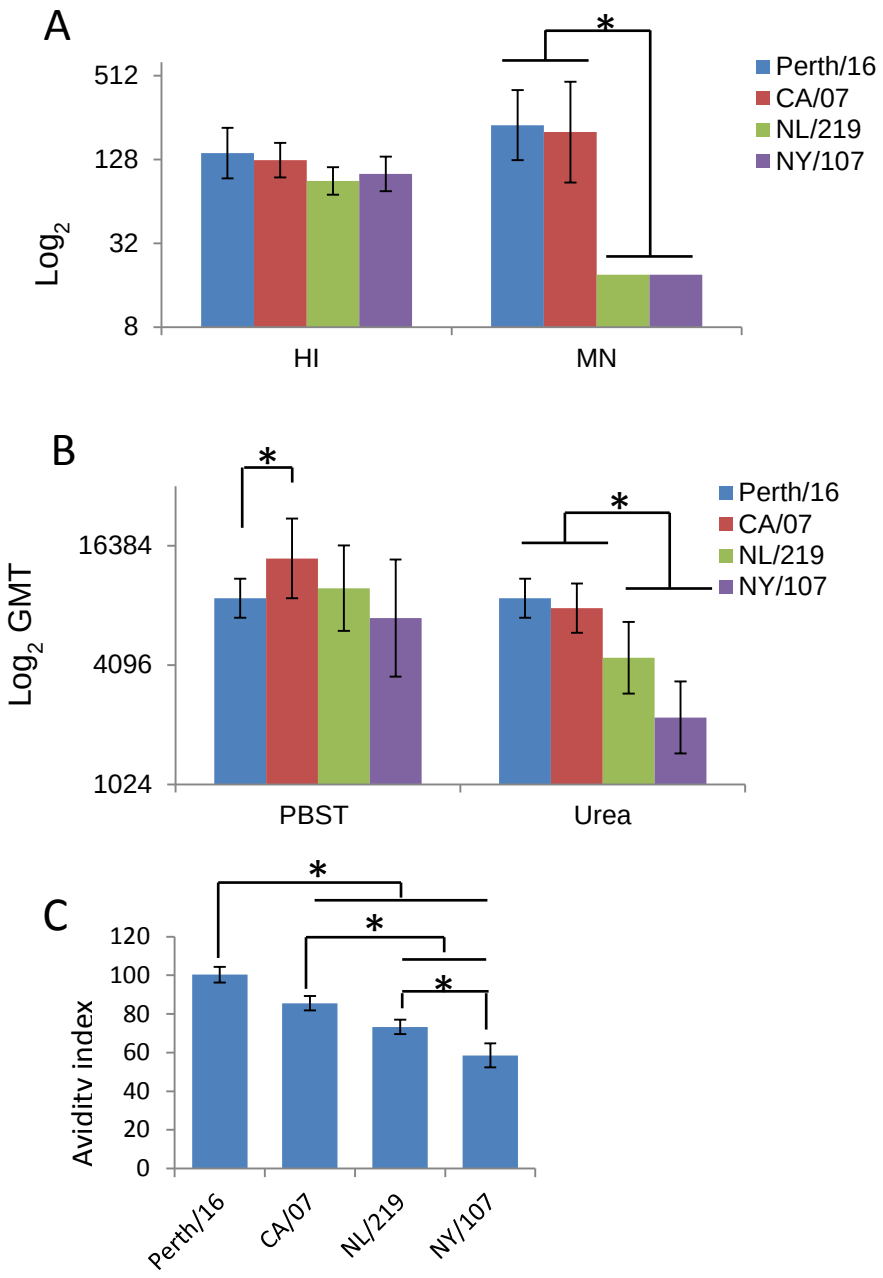



**Figure 3.4 HI and MN titers and avidity ELISA for mice with HI titers between 80 and 160.** Serum samples from mice with HI titers between 80 and 160 were selected (n=6 mice per group), and avidity ELISAs were performed as described in the manuscript. (**A**) HI and MN titers for each group. (**B**) To test avidity of antibodies, ELISAs were performed according to the standard procedure with the addition of a 5 min PBST incubation ("PBST", left grouping) or with a 5-minute incubation in 4M urea, followed by two washes with PBST ("Urea", right grouping). Values are shown as GMT. (**C**) The avidity index (AI) was calculated for each sample. Values are shown as average AI (percent). Error bars represent 95% confidence intervals. Differences between groups are indicated by * ($0.0001<p<0.05$) or ** ($p<0.0001$).

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# CHAPTER 4

## OVERALL DISCUSSION AND SUMMARY

The Influenza Division at the CDC is tasked with influenza pandemic preparedness. The history of the 20th and 21st centuries teach us that influenza pandemics appear at unpredictable intervals, and arise from avian influenza viruses, either directly or filtered through swine. However, a vast number of avian influenza viruses exist, and resources are finite, so a main goal of the CDC is to identify avian influenza viruses that are particular concerns for pandemics. A formal risk assessment process exists for this, but this risk assessment depends heavily on research and evaluation of specific viral gene products, to determine their potential contribution to adaptation to mammalian infection and transmission. After risk assessment is performed, vaccines against pre-pandemic influenza viruses are generated. However, the utility of these vaccines depends on their immunogenicity. In my dissertation research, I described my contribution to this process at the CDC. We identified two significant gaps in pandemic preparedness against HPAIVs: One in risk assessment, the other in vaccine effectiveness.

For risk assessment, I analyzed the significance of historically rare but emerging PB1-F2 truncations in H5N1 viruses. For vaccine effectiveness, I evaluated the immunogenicity of H7 hemagglutinins (HA) in comparison with those of seasonal viruses. We performed a comprehensive analysis of prevalence, phylogeny, and evolutionary dynamics of PB1-F2 truncations in all the available influenza sequences of human, swine, and avian isolates. Our database analyses results are largely in accordance with the previously published studies which have found that the large

fraction of mammalian influenza viruses have truncated PB1-F2 while the prevalence of such truncation are rare in avian influenza viruses. However, critically, we demonstrated that PB1-F2 truncations are increasingly common in the H5N1 subtype, which is of particular pandemic concern. We here report for the first time, the emergence and evolution of PB1-F2 truncations independently in multiple different clades of H5N1 viruses. In the previously published study (2006) only 0.3% (1 out of a total of 328) H5N1 viruses had truncated PB1-F2. Our database analyses found that 152 out of a total of 2002 (7.6%) H5N1 viruses had truncated PB1-F2, a 25 fold increase from the previously published study.

To achieve our first aim of risk assessment, we studied the pathogenicity of H5N1 PB1-F2 truncations. We found that an H5N1 virus lacking expression of PB1-F2, due to mutation of the initiating ATG, has much higher virulence in mice than a virus with full-length PB1-F2. Since these different PB1-F2 did not show in vitro differences in apoptosis or modulation of IFN (functions previously ascribed to PB1-F2) we propose that PB1-F2 deletion may lead to altered virulence through as-yet unknown functions, perhaps related to expression of a C-terminal fragment of PB1-F2 from downstream ATG. We suggest that PB1-F2 truncations and ATG mutations be monitored as part of ongoing avian influenza virus surveillance and risk assessment, and recommend that the ATG deletion in particular be considered a potential risk factor for extreme virulence in mammals.

In our second project under the overall aim of pandemic preparedness, we evaluated the immunogenicity of H7 hemagglutinins in comparison with seasonal viruses. From previously published serosurveillance studies and H7 vaccine clinical trials, it is known

that the H7 viruses and vaccines are poorly immunogenic. However, no one has evaluated the immunogenicity of H7 viruses and compared it in parallel with seasonal viruses. Furthermore, it is not clear if the differences in response is due to intrinsic aspects of the HA, to pre-existing immunity in humans, or to some aspect of the virus or the vaccine. We found that H7 viruses, of both the Eurasian and North American lineages, are significantly less immunogenic than human seasonal virus HAs from the H3N2 and H1N1pdm09 subtypes. This finding raises important considerations about production of pre-pandemic vaccines against H7-subtype viruses. In particular, it seems likely that to achieve adequate protective immunity vaccination will require large amounts of H7 HA, or the presence of an adjuvant. This means that US government influenza vaccine stocks must either be much larger than previously anticipated, or that vaccine strategies must be revised to account for the potentially lower amounts of vaccine available.

These findings also point toward possible solutions for the concern. If the H7 HA is intrinsically less immunogenic, then making different virus seed stocks is unlikely to solve the problem; rather it is critical to identify the molecular causes of poor immunogenicity and to identify ways of overcoming them. One possibility is that, although antibodies are being produced, they are either low affinity antibodies, or they target non-neutralizing regions of the HA, such as the stalk. Further experiments can identify specific antigenic sites in H7 HAs, and in combination with new or different serological assays may be used to identify protective titers more efficiently. As well, it may be possible to genetically or chemically modify the H7 HA in order to overcome the lack of intrinsic immunogenicity. For example, if the lack of glycosylation of avian

influenza HA results in poor immunogenicity, then new glycosylation sites may be engineered into vaccine stocks to increase immunogenicity.

In summary, my dissertation research has helped advance the global pandemic preparedness for avian influenza virus. Future work will extend these advances by adding to the formal risk assessment process, and by enhancing pre-pandemic vaccine stock generation.