ABSTRACT

Title of Document : ZOONOTIC TRANSMISSION OF INFLUENZA H9 SUBTYPE THROUGH REASSORTMENT

James Brian Kimble, Doctor of Philosophy, 2013

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

Influenza A virus causes disease across a broad host range including avian and mammalian species. Most influenza viruses are found in wild aquatic birds, are of low consequence and refrain from zoonotic transmission. However, some strains occasionally cross the species barrier, into domestic birds and a plethora of mammalian species, most notably swine and humans. Many of these infections are dead ends and quickly disappear from the species, but occasionally, a stable lineage is established and becomes endemic in an animal population. Avian Influenza virus (AIV) H9N2 was predominantly found in wild ducks and shore birds across the globe with occasional infections in turkeys until the late 1980's, at which point the virus became established in Eurasian poultry populations. In the late 1990's the virus again jumped hosts, first into swine, and then into humans. Across many regions, these viruses appear to be gaining human-like virus characteristics. Here, the influenza receptor distribution in a range of poultry species has been characterized and shown that many of the birds were able to bind human-like binding viruses. While no large-scale H9N2 human infections have occurred, the threat is there. The most likely route for this to occur is through reassortment with human viruses. The

2009 human pandemic H1N1 (pH1N1) is a likely candidate as it is found in multiple species and seems to readily reassort. The two viruses were shown to be compatible for reassortment and H9:pH1N1 viruses would readily infect and transmit in both ferrets (a human model animal) and swine. Finally, a novel method of modeling reassortment *in vivo* was developed, which simultaneously tests the breadth of possible reassortant and utilizes natural host selective pressure to select the most-fit progeny. Furthermore, the characterization of these viruses in ferrets showed they readily infect, efficiently transmit, and exhibit mild to moderate pathological consequences. Taken together, these findings broaden our understanding of natural observations, characterize the potential for zoonosis, highlight the dangers H9 viruses may pose to humans, and give scientists a new tool to deepen our understanding of reassortment.

Zoonotic Transmission of Influenza H9 subtype through Reassortment

By

James Brian Kimble

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2013

Advisory Committee:

Professor Daniel Perez, Chair

Professor Siba Samal

Professor Jim Culver

Professor Xiaoping Zhu

Professor Alison McBride

Acknowledgements

I would like to start by thanking my advisor, Dr. Daniel Perez. I came to you as a naïve first year PhD student, and through your guidance, expertise, teaching, anger, consoling, levity, prodding, coercing, demanding, and questioning I have been formed into a competent scientist. The waters may not have always been smooth, but the sailing was always interesting and the destination is within sight. Thank you.

I would also like to thank Dr. Siba Samal, Dr. Jim Culver, Dr. Xiaoping Zhu, and Dr. Alison McBride for their time, effort and advice as my PhD advisory committee.

I would like to thank the people at the USDA-NADC for hosting, training, and allowing me to conduct my experiments in their facility. Thanks Amy and Kelly for helping with experimental design, allowing me space to work, and training me in pig work. Thanks to Dani, Pravina, Katy, Matt, Gwen, Crystal, and Zhara for welcoming me into your lab space and providing me with everything I needed. Thanks to the animal caretakers Jason, Jason and Ty for all your efforts tending to the pigs so I could focus on the research.

I would like to thank all current and former lab members in the Perez lab. Your hard work and perseverance was always an inspiration. I greatly appreciate the time I got to spend working, talking, and coffee breaking with all of you. The success I have is merely a reflection of the intelligence and talents of those around me. Special thanks to Gloria and Erin for all the mentoring you gave when I joined that lab and helping me get started on the project presented here. Thank you all.

Thank you to all the VetMed staff throughout my time in at UMD. You guys do a fantastic job ensuring the day-to-day operations of our department happen. Whether it is

computer problems, paper jams, or animal care issues, you guys keep the engine moving. I would like to especially thank Andrea Ferrero-Perez, our building facilities manager. You do the work of three people keeping our troublesome building and BSL3 operational. I would also be remiss if I failed to mention in this permanent and public record that your flans and empanadas are the best.

Finally, I would like to thank the most important people in my life.

Mom, Dad, Cari, Alison and Greg: You guys shaped me into whom I am today. You have built me up, yet kept me ground. All my success, both past and future, is laid on the foundation you have built up around me. I know that no matter what may come, I will always have your love and support to fall back on. I love you all very much.

Kora, thank you so much for all you have done for me over the course of our time together. You have made the last few years of my education enormously more bearable. I am tremendously excited about starting the next chapter of my life with you. I love you.

Katherine, Ted, Douglas, Ben, Erin, and Sergey: I can't imagine my graduate career without you guys. You have helped me keep my sanity through this arduous process. You've properly celebrated the good times with me and drug me through the worst. You guys are the worst and though my time at the University of Maryland is drawing to a close and my future whereabouts are currently unknown, just know that you won't be rid of me that easily.

Table of Contents

Acknowledgements ..... **ii**

Table of Contents ..... **iv**

List of Tables ..... **vii**

List of figures ..... **viii**

Abbreviations ..... **x**

Chapter 1: Introduction **1**
1.1General **1**
1.2 Research objectives and outline **5**

Chapter 2: Literature Review **7**
2.1 Influenza **7**
2.1.1 Discovery of influenza and human pandemics 7
2.1.2 Classification and nomenclature 9
2.1.3 Morphology and genome organization 10
2.1.4 Viral RNA segments, gene products, and pathogenesis determinants 14
2.1.5 Influenza virus life cycle 20
2.1.6 Viral diversity 21
2.2 H9N2 influenza virus **25**
2.2.1 Overview 25
2.2.2 H9N2 in poultry 26
2.2.3 H9N2 in swine 28
2.2.4 H9N2 in humans 29
2.2.5 H9N2 and reassortment 30

Chapter 3: Characterization of sialic acid as influenza receptors in domestic poultry **34**
3.1 Abstract **34**
3.2 Introduction **35**
3.3 Materials and methods **36**
3.3.1 Animals and tissues 37
3.3.2 Viruses 38
3.3.3 Tissue preparation and sectioning 38
3.3.4 Digoxygenin sialic acid detection 38
3.3.5 Fluorescent sialic acid detection 39
3.3.6 Virus binding assay 39

3.4 Results and discussion **40**
3.4.1 Waterfowl and land-based poultry differ in SA distribution in various tissues 40
3.4.2 Age dependent variations in α2,6SA expression 47
3.4.3 Lectin binding patterns is not indicative of virus binding patterns 50

Chapter 4: Compatibility of H9N2 and pH1N1 for reassortment and aerosol transmission in ferrets **59**
4.1 Abstract **59**
4.2 Introduction **60**
4.3 Materials and methods **62**
4.3.1 Viruses and cells 62
4.3.2 Plaque assay 63
4.3.3 Infection and transmission in ferrets 63
4.3.4 Virus localization 64
4.3.5 Blocking ELISA 65
4.4 Results **66**
4.4.1 Generation of H9N2 and H9N1 influenza viruses with internal genes from pH1N1 66
4.4.2 Infection, pathology, signs of disease, and transmission in ferrets 68
4.4.3 Molecular changes associated with transmission 76
4.4 Discussion **79**

Chapter 5: Transmission and pathogenicity of H9N1 reassortants in swine **82**
5.1 Abstract **82**
5.2 Introduction **83**
5.3 Materials and methods **85**
5.3.1 Reverse genetics 85
5.3.2 Animal studies 87
5.3.3 Bronchiole lavage 87
5.3.4 Swab titration 88
5.3.5 Statistics 88
5.4 Results **88**
5.4.1 Infectivity, transmissibility, and pathogenicity 88
5.4.2 Lower respiratory infections 91
5.4.3 Serology 95
5.4 Discussion **95**

Chapter 6: Alternative reassortment events lead to the generation of respiratory transmissible H9N1 in ferrets **99**
6.1 Abstract **99**
6.2 Introduction **100**
6.3 Materials and methods **102**
6.3.1 15-plasmid transfection 102
6.3.2 Viral selection 102
6.3.3 Animal studies 103
6.3.4 Limiting dilutions 103

6.4 Results **103**
6.4.1 15-plasmid inoculation causes infection in ferrets 103
6.4.2 Rapid *in vivo* selection of aerosol transmissible H9N1 viruses 106
6.4.3 Transfection produces multiple virus subpopulations 110
6.4.4 Pathological characterization 112
6.4 Discussion **114**

Chapter 7: Conclusion and future prospects **117**
7.1 Conclusion **117**
7.2 Future prospects **120**

Appendices **124**
List of reagents **124**
Bibliography **125**

List of Tables

1. 2.1 Gene segments and translation products

2. 3.1 Relative expression of sialic acid in avian tissues

3. 3.2 Hemagglutinin binding affinity of H3N2 viruses

4. 4.1 Ferrets showed moderate weight loss following infection with reassortant H9:pH1N1 viruses

5. 5.1 Genetic content of experimental viruses

6. 6.1. Both lineages are H9N1 reassortants with mixed internal genes6.2. Limiting dilutions show a mixed population

Table of Figures

1. 2.1 Schematic structure of influenza A virus
2. 2.2 The process of reassortment produces unique progeny
3. 3.1 Sialic acid distribution in avian trachea
4. 3.2 Sialic acid distribution in avian lungs
5. 3.3 Sialic acid distribution in avian large intestine
6. 3.4 Effects of age on sialic acid distribution
7. 3.5 Virus binding to tissues correlate to sialic acid distribution in domestic ducks and geese
8. 3.6 Virus binding to tissues correlate to sialic acid distribution in domestic turkeys and pheasants
9. 3.7 Virus binding to tissues correlate to sialic acid distribution in domestic quails and guinea fowl
10. 4.1 Characteristics of H9N2:pH1N1 viruses in vitro
11. 4.2. Pathology produced by H9N2:pH1N1 viruses
12. 4.3.Virus localization in infected tissue
13. 4.4. Nasal wash titers from DI, DC, and RC ferrets
14. 4.5. Amino acid mutations during transmission of H9N2:pH1N1 viruses

15. 5.1 Nasal swab titers

16. 5.2 Lung scores

17. 5.3 BALF titers

18. 6.1. 15-Plasmid transfection system results in productive infection of ferrets

19. 6.2. Both H9N1 lineages transmit efficiently in ferrets

20. 6.3. Both H9N1 reassortants exhibit pathogenicity similar to seasonal flu

Abbreviation	Full term
1P10	A/ferret/Maryland/1P10/2010 (H9N1); mammalian H9 HA on pH1N1 backbone
1WF10	A/ferret/Maryland/1WF10/2010 (H9N1); avian H9 HA on pH1N1 backbone
2,3SA	Terminal sialic acid α2,3 linked to penultimate galactose; avian-like receptor
2,6SA	Terminal sialic acid α2,6 linked to penultimate galactose; human-like receptor
2P10	A/ferret/Maryland/2P10/2010 (H9N2); ferret H9N2 surface on pH1N1 backbone
2WF10	A/ferret/Maryland/2WF10/2010 (H9N2); avian H9N2 surface on pH1N1 backbone
A/DK	A/duck/HongKong/7/1975 (H3N2)
A/Mem	A/Memphis/31/1998 (H3N2)
A/TK	A/turkey/Ohio/313053/2004 (H3N2)
ABSL2	Animal biosafety level 2
ABSL3+	Animal biosafety level 3 enhanced
AEC	Aminoethylcarbazole
AIV	Avian influenza virus
BALF	Bronchiole airway lavage fluid
BSA	Bovine serum albumin
cRNA	Copy RNA
DAPI	4',6-Diamidino-2-phenylindole
DC	Direct contact
DI	Directly inoculated
dpc	Days post challenge
dpi	Days post inoculation
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	Fluorescein isothiocyanate
Gn. Fw.	Guinea fowl
HA	Hemagglutinin; gene segment, gene, protein

Abbreviation	Full term
HA0	immature (uncleaved) HA
HA1	Mature (cleaved) HA subunit 1
HA2	Mature (cleaved) HA subunit 2
HEK	Human embrionic kidney
HEPA	High efficiency particulate air
HPAI	Highly pathogenic avian influenza
IAV	Influenza A virus
LPAI	Low pathogenic avian influenza
M	Matrix; gene segment
M1	Matrix 1; gene, protein
M2	Matrix 2; gene, protein
MAA	*Maackia amurensis* agglutinin
MAVS	Mitochondrial antiviral signaling proteins
MDCK	Madin-Darby canine kidney
mRNA	messenger RNA
NA	Neuraminidase; gene segment, gene, protein
NADC	National Animal Disease Center
NEP/NS2	Nuclear export protein/non-structural 2; protein/gene protein
NP	Nucleoprotein; gene segment, gene, protein
NS	Non-structural; gene segment
NS1	Non-structural 1; gene, protein
P10	A/ferret/Maryland/P10/2009 (H9N2); ferret adapted H9N2:6A/Mem
PA	Polymerase acidic; gene segment, gene, protein
PB1	Polymerase basic 1; gene segment, gene, protein
PB2	Polymerase basic 2; gene segment, gene, protein
PBS	Phosphate buffered saline
pH1N1	Pandemic H1N1
RBC	Red blood cells

Abbreviation	Full term
RC	Respiratory contact
RNP	Ribonucleoprotein
SA	Sialic acid
SIV	Swine influenza
SNA	*Sambuca niger* agglutinin
TBI	Transfection based inoculation
TBS	Tris buffered saline
TCID50	Tissue culture infectious dose 50
TRIG	Triple reassortant internal genes
TRITC	Tetramethylrhodamine isothiocyanate derivative
vRNA	Viral RNA
WF10	A/guinea fowl/HongKong/WF10/1998 (H9N2)

Chapter 1: Introduction

1.1 General introduction

The *Orthomyxoviridae* family of viruses has 5 member genera: *Influenzavirus A, B* and *C*, plus *Thogotavirus*, and *Isavirus.* Influenza A viruses (IAV) represent the most well studied genera as they are major pathogens to both humans and animals, most notably swine and domestic poultry. IAV has a single stranded negative sense RNA genome composed of 8 segments. Each segment produces at least one protein and several produce multiple proteins. Depending on the specific strain, up to 12 proteins can be expressed. Each RNA segment forms the backbone of a ribonucleoprotein (RNP) complex. The viral RNA (vRNA) is coated with nucleoprotein (NP) and forms a helical hairpin structure with a heterotrimeric polymerase complex composed of polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA) bound to the end. The viral envelope is comprised of host cell plasma membrane with virus derived glycoprotein spikes incorporated. The spikes are either hemagglutinin (HA) or neuraminidase (NA) and are in an approximate 5:1 ratio respectively. A third virus protein is found in the viral envelope as well. The matrix 2 (M2) protein forms homotetrameric complexes that function as ion channels necessary during viral entry. The matrix 1 (M1) protein initiates viral budding and also interacts with the RNPs to from fully mature virion.

The HA and NA are the major antigenic determinants. There are 16 recognized HA variants and 9 NA variants. A recent influenza-like virus isolated form bat in Guatemala has an HA-like and NA-like protein proposed to be the 17th and 10th

respective subtype (Tong et al., 2012). The HA and NA subtype are used in classification and nomenclature of different influenza subtypes. For instance, an H9N2 virus has the ninth HA subtype and the second NA subtype. The HA is responsible for cell binding and plays a large role in viral entry. The protein binds to alpha linked terminal sialic acid residues on target cells. After endocytosis, the acidification of the endosome cause a conformational change in the HA causing a fusion of the viral membrane and the endosomal membrane, releasing the RNPs into cytoplasm and allowing for viral replication. While the HA bind sialic acid, the NA has sialidase activity. This is necessary for viral egress to prevent vial agglutination and rebinding of the initially infected cell.

Wild aquatic birds are the natural reservoir for influenza A viruses. Many subtypes and strains of influenza have crossed species barriers due to the close spatial proximity and shared resources that wild aqua fowl have with other animals. However, while all subtypes can be found in waterfowl, only some subtypes can cause zoonotic transmission. With 16 HA variants and 9 NA variants there are theoretically 144 distinct subtype variations possible (Fouchier et al., 2005). Of these,103 have been isolated and all of them are found in wild birds (Alexander, 2007; Munster et al., 2007).

New strains of influenza emerge constantly and there are two methods influenza utilizes to generate novel viruses. The first, genetic drift, is the gradual buildup of mutations in the genome that can modify phenotypes, alter antigenicity, or change tissue or host specificity. The second, genetic shift, is essentially sexual replication that occurs in a process called reassortment. If two distinct viruses infect the same host cell, during packaging, their genomes become mixed and novel combinations of the two different sets

of gene segments become incorporated in the reassorted progeny. Reassorted viruses can have properties similar to either parental virus or novel phenotypic characteristics.

Genetic drift and shift are important processes in zoonotic transmission and in the emergence of human pandemics. The 2009 pandemic H1N1 (pH1N1) was the product of multiple reassortments in pigs with the final reassortment allowing zoonotic transmission to humans (Neumann et al., 2009). Reassortment in fact is thought to have played an important role in all but one of the pandemics of the last 100 years. Drift is typically a slower process, but a powerful one nonetheless in shaping emerging viruses. H9 HA's receptor-binding preferences can be significantly altered with a single mutation, and mammalian transmission has been seen with as few as five (Sorrell et al., 2009; Wan and Perez, 2007). A single mutation on the H5 HA at amino acid position 160 can alter a glycosylation site and lead to a significantly more mammalian like receptor binding ability (Stevens et al., 2008).

For a novel virus to become pandemic in humans it must satisfy certain basic requirements. It must easily infect humans, transmit from human to human efficiently, and it must be antigenically dissimilar to current strains of human influenza in order to have susceptible population to spread into. The exact genetic and phenotypic characteristics that allow for these conditions to be met are not fully known, and likely change from subtype to subtype and pandemic to pandemic. The most likely method for the novel antigenicity requirement to be met is through zoonosis, and evidence suggests this is what happened with the last three naturally occurring human pandemics. Zoonotic transmission generally leads to issues with a virus fulfilling the first two requirements. This is likely why human pandemics are relatively rare. To circumvent these issues a

virus can utilize genetic shift and genetic drift. It is theorized that the 1918 pandemic virus transmitted to humans from ducks and became adapted to humans through mutations (Taubenberger et al., 2005). The other three pandemics seemingly arose via reassortment in animals and then transmitted to humans.

There have only been 3 HA subtypes known to have become endemic in humans: H1, H2, and H3. However, many subtypes occasionally jump into humans and cause infections, but not sustained transmission, most notably H5, H7 and H9. Some H5 and H7 strains have a polybasic cleavage site that leads to high morbidity and mortality in birds and are labeled as highly pathogenic avian influenza (HPAI). These HPAI viruses have caused deaths in humans and become the subject of intense research and speculation about future pandemics. H9 viruses have been isolated from humans at a much lower incidence and are associated with much less significant clinical symptoms. This has led to less research, surveillance and understanding because of the assumption that these viruses are less threatening to the human population.

A closer look at certain lines of evidence suggests a more ominous potential future for H9 influenza and humans. First, human infections with H9 viruses maybe far more common than suggested by the number of isolated human H9 viruses. While only 11 human isolates have been recovered, serological surveys of regions in the Middle East, the Indian subcontinent, Southeast Asia, and China show a far higher rate of seroconversion against the H9 HA; antibody are present in as high as 15% of people in select populations (Pawar et al., 2012; Uyeki et al., 2012; Wang et al., 2009). When the relatively mild symptoms associated with human infection with H9 influenza is considered, it is not imagine how potentially thousands of human infections are never

detected, but seroconversion is achieved in a large swath of the population. These theoretical infections would give the virus time to adapt to humans or potentially reassort with another human virus and gain transmissibility and possibly pathogenicity.

1.2 Research objectives and outline

The main goal of my research is to gain a better understanding of the threat H9N2 viruses pose to the human population. The history of these viruses shows that wild and domestic birds are the primary reservoir with frequent infections of swine and infrequent isolated human infections. This highlights the fact that zoonotic transmission will play an important role in any future threat that these viruses pose to humans. The main factor affecting zoonosis of influenza is the receptor specificity of the virus. Dogmatically, human viruses bind α2,6 linked sialic acid (2,6SA) while avian viruses bind α2,3 linked sialic acid (2,3SA). These studies, for the first time show the receptor pattern in many of the domestic species of birds that H9N2 viruses infect. Here it is shown that these birds have both types of receptors and can bind human and avian viruses in the respiratory tract creating an environment suitable for receptor switching. All four naturally occurring pandemics resulted from reassortment. If an H9N2 is to threaten the human population, reassortment will most likely be involved. The compatibility of H9N2 with human H3N2 has previously been characterized. However, the emergence of pandemic H1N1 reestablished the need to evaluate the threat an H9N2 reassortant would pose. The final two goals of this research are to establish the compatibility of H9N2 and pandemic H1N1 and to develop and utilize a method to better mimic natural reassortment in a host to describe the most likely outcome of the two viruses reassorting.

Research objectives:

1. Characterize potential of common domestic bird species to act as 'mixing bowl' for zoonotic transmission

   A) Determine sialic acid distribution in multiple relevant tissues of domestic birds.
   B) Evaluate capacity of tissues to bind human and/or avian viruses.

2. Determine the compatibility of H9N2 and pandemic H1N1

   A) Use reverse genetics system to create multiple H9N2:pH1N1 viruses
   B) Evaluate *in vitro* behavior of reassortant viruses.
   C) Characterize reassortants *in vivo* in a mammalian model to determine infectivity and transmissibility.

3. Recreate a reassortment event and evaluate the infectivity and transmissibility in ferrets of the resulting virus.

   A) Develop a system to better mimic a natural infection *in vivo* and determine the kinetics of the system.
   B) Implement the system using H9N2 and pH1N1 viruses to determine the most likely resultant virus
   C) Characterize the infectivity and transmissibility of the selected virus.

Chapter 2: Literature review

2.1 Influenza

2.1.1 Discovery of influenza and human pandemics

Influenza has probably been infecting humans for centuries as Hippocrates gave a description of a disease similar to influenza. The name itself is derived from the Italian word for influence as the disease was originally attributed to the influence of the stars during the early Renaissance and later to the influence of the cold. Influenza in humans causes respiratory disease with symptoms that may include fever, coughing, nasal congestion, body aches, fatigue and headache. Most influenza infections are not fatal, but can lead to either viral or bacterial pneumonia. The 1918 H1N1 virus and the highly pathogenic avian influenza (HPAI) H5N1 both directly cause high mortality in humans which is believed to result from over activation of the immune system called a cytokine storm (Kobasa et al., 2007).

The first description of avian influenza was Perroncito's description of "fowl plague" in 1878 (Perroncito, 1878). In 1901 it was revealed that fowl plague was caused by a filterable agent, in the case described, HPAI H7N7 (Centanni and Savonuzzi, 1901). The first recognition of swine influenza was in 1918 and was related to the human H1N1 pandemic of that same year. The viral nature of swine influenza was demonstrated in 1930 along with the first isolation of influenza A virus, A/swine/Iowa/15/1930 (H1N1) (Shope, 1931a, b). Three years later *Smith et al.* isolated the first human influenza virus, A/Wisconsin/1933 (H1N1) (Wilson Smith et al., 1933). It was not until 1955 that the connection between fowl plague virus and human influenza virus was made (Schafer,

1955). While the natural reservoir for influenza viruses are wild aquatic birds, zoonotic transmission occur in a broad variety of other animals. Zoonosis into poultry, swine and humans have already been discussed, but the virus has also been isolated from horses, dogs, ferrets, cats of all sizes from house cats to tigers, seals, whales, and camels (Anthony et al., 2012; Crawford et al., 2005; Hinshaw et al., 1986; Mushtaq et al., 2008; Patterson et al., 2009; Sponseller et al., 2010; Waddell et al., 1963). Recently, during a survey of bats in Guatemala, influenza like genes were recovered. The HA and NA were unlike any other known genes and the researchers proposed the 17th HA and 10th NA subtype (Tong et al., 2012). However, it is unclear if the nomenclature will remain as no virus has been isolated and the expressed proteins do not function like typical influenza proteins (Li et al., 2012; Zhu et al., 2012). Mice, guinea pigs, cotton rats and ferrets have been infected experimentally as models for human influenza (Blanco et al., 2013; Henneberg and Wagner, 1953; Smith and Sweet, 1988).

Historical records of influenza pandemics are hard to evaluate due to the fact that symptoms are similar to other diseases. The first influenza pandemic that most historians agree upon was in 1580 (Potter, 2001). Four more influenza pandemics are believed to have occurred from 1580-1900. The 1830 pandemic was especially widespread, but not especially deadly (Potter, 2001). The 1918 influenza pandemic marked the first pandemic that was extensively studied using modern scientific techniques. It is also the worst human pandemic infection ever recorded as an estimated 50 million people died (Taubenberger and Morens, 2006). It is believed that the virus was a direct transmission from birds into humans of an H1N1. (Taubenberger et al., 2005)1957 saw the next human influenza pandemic. Reassortment between and avian and the human H1N1

created a virus with three genes from an avian H2N2 (PB1, HA, NA) and the five remaining genes from the human H1N1. This virus caused a pandemic that started in 1957 and swept around the globe for 2 years. The next pandemic arose 11 years after the H2N2 virus. Again reassortment drove the genesis of the pandemic. This time, the H2N2 reassorted with an avian H3 virus that donated the HA and PB1 gene. The 1968 virus remains in the human population today as seasonal H3N2. Finally, the most recent influenza pandemic began in the spring of 2009. Reassortment played heavily into its creation as well. Classical swine H1N1, an avian influenza virus, and human sH3N2 reassorted to create a triple reassortant swine virus in the late 1990's (Ma W, 2008; Vincent AL, 2008). One of these triple reassortant viruses reassorted with a Eurasian avian-like swine H1N1 that resulted in the virus that spread across the human population (Neumann et al., 2009).

2.1.2 Classification and nomenclature

Orthomyxoviridae is a family of enveloped viruses with a segmented negative sense RNA genome. Recently, the family expanded to six member genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus, Isavirus,* and the new genus, *Quaranjavirus. Influenzavirus A, B,* and *C* are three genera that cause respiratory disease in humans. *Thogotovirus* has two member species, thogoto virus and Dhori virus. Jos virus and Araguari virus are two proposed members of the genus (Bussetti et al., 2012; Da Silva et al., 2005). These viruses are tick born, and thogoto virus has infected two humans naturally while Dhori virus has accidently infected 5 humans (Butenko et al., 1987; Moore et al., 1975). All cases resulted in fever and encephalitic disease. *Isavirus* has one member species, **i**nfectious **s**almon **a**nemia virus that causes significant disease in

Atlantic salmon, especially farmed fish (Clouthier et al., 2002). *Quaranjavirus* has two member species, Quaranfil virus and Johnston Atoll virus. Both are found in ticks and Quaranfil virus has been isolated from humans with mild febrile illness (Presti et al., 2009; Taylor et al., 1966). The final three genera contain influenza A, B and C virus. Influenza A is the most study of all *Orthomyxovirdae* members. It is a major disease of humans, swine, and avian species and has been isolated from a myriad of different species. In humans it causes respiratory symptoms that very from mild to severe with associated mortality depending on the strain. Influenza B is typically a human pathogen similar to influenza A in symptoms and has been isolated from seals (Osterhaus et al., 2000). Influenza C is typically of low consequence and is seen in humans and swine (Muraki and Hongo, 2010).

Influenza viruses are named by their genus, the organism it was isolated from (except humans), strain identification, year of isolation, and antigenic subtype (Palese and Shaw, 2007). The best influenza virus in the world, A/guinea fowl/Hong Kong/WF10/1999 (H9N2), came from a guinea fowl in Hong Kong in 1999 and was an H9N2 with strain identification of WF10.

2.1.3. Morphology and genome organization

Influenza virus is enveloped by a lipid bilayer derived from the host cytoplasmic membrane. The two main surface proteins are HA and NA and have been measured at approximately a 4:1 ratio (Webster et al., 1968). Matrix protein 2 (M2) is also present on the surface of the virus at a much lower concentration. Below the envelop in a layer is the matrix protein 1 (M1) which surrounds the ribonucleoprotein (RNP) complex. M1 plays

the major role in morphology, but studies altering the transmembrane portions of HA, NA, and M2 also alter morphology (Bourmakina and Garcia-Sastre, 2003; Elleman and Barclay, 2004; Iwatsuki-Horimoto et al., 2006; Jin et al., 1997)

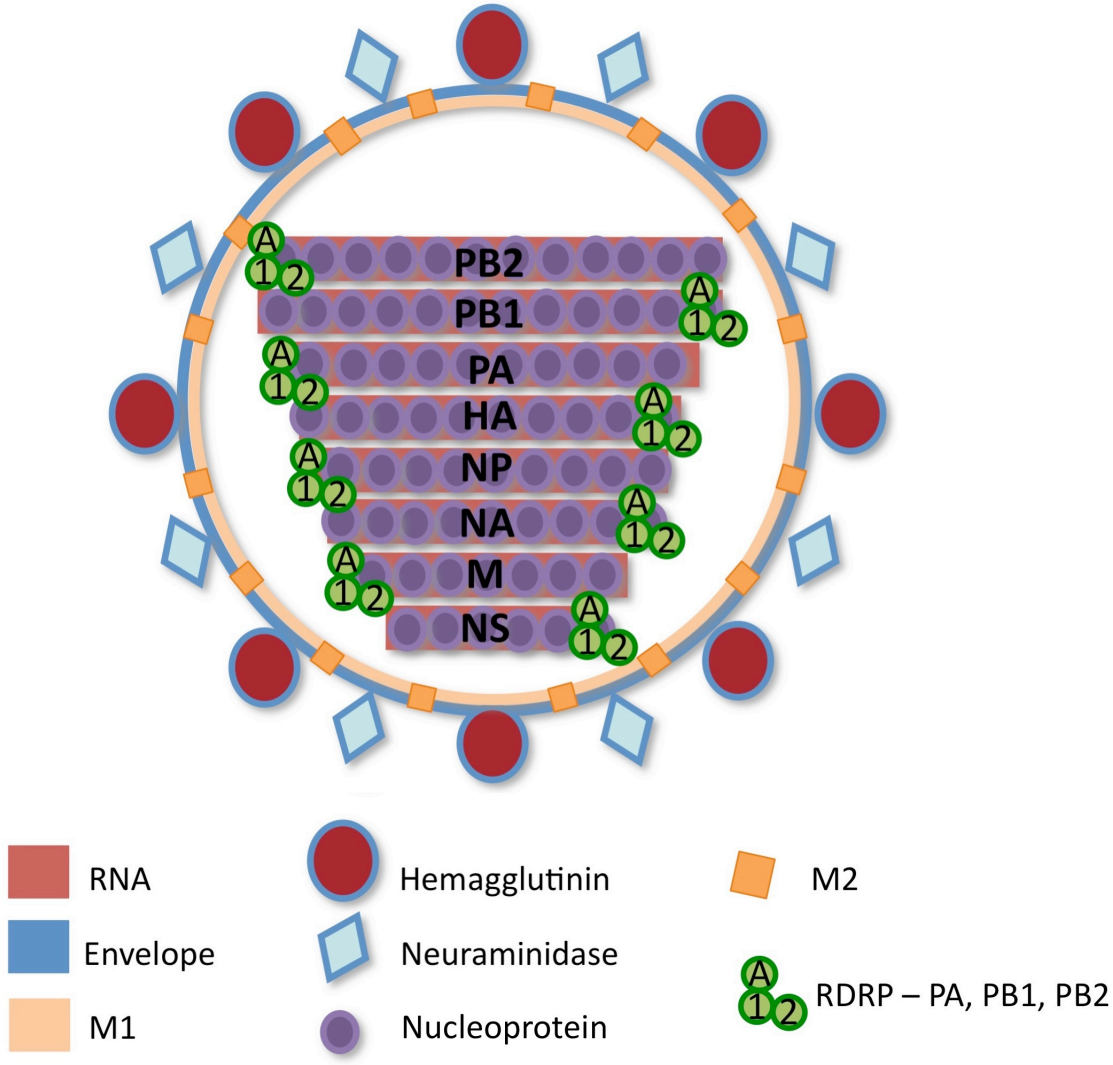


Figure 2.1. Schematic structure of influenza A virus. The cartoonistic representation shows the host derived membrane sandwiched between the surface spikes (HA and NA) and the layer of M1 with M2 inclusions. Inside shows the 8 RNA segments coated with NP and paired with the polymerase complex (PB1, PB2, PA). Not shown are low levels of NEP and host factors.

HA forms homotrimers at the surface of a mature virion. Each HA monomer is composed of HA1 that is covalently linked by a disulfide bond to the HA2 subunit (Cross et al., 2001). The HA trimers serves several biological purposes. They are responsible for host cell binding and then membrane fusion to allow the entry of the genome into the cell. HA is also highly reactive antigenically allowing for the production of neutralizing antibodies. There are approximately 500 HA spikes on each virion surface making up 80% of the radiating surface spikes (Alexander, 2007; Ruigrok et al., 1984; Webster et al., 1968).

The NA forms a homotetramer that constitutes the final 20% of the spikes seen on surface on the virion. The NA has opposite functions to the HA. While the HA is responsible for binding sialic acid receptors to gain entry into the cell, the NA cleaves these same sugar moieties to release budding virion and prevent agglutination (Gong et al., 2007). The NA also functions early in the course of an infection by clearing away the mucus coating the airway epithelium (Matrosovich et al., 2004). The opposing activities of these proteins require that each protein must compliment and balance out the other in order for the virus to function properly. If one has a significant higher enzymatic activity than the other or altered receptor specificity then replicative efficiency is quickly lost, but can be regained by compensatory mutations (Hulse et al., 2004; Lu et al., 2005; Yen et al., 2011).

The RNP is composed of viral RNA, nucleoprotein (NP), and the RNA dependent RNA polymerase made of polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acid (PA). Nuclear export protein (NEP) or nonstructural protein 2 (NS2) is

also found in purified virus (Richardson and Akkina, 1991). Electron micrographs show pleomorphic morphology with HA and NA spikes on the surface. Fresh clinical isolates can be 300nm filamentous particles while cell culture or egg derived viruses become spherical (Chu et al., 1949). Internally, the RNP complexes form a 7-around-1 formation. Each individual RNP complex is made up of a polymerase (PB2, PB1, PA) and a single RNA strand that is wrapped around multiple NP proteins.

2.1.4. Viral RNA segments and gene products

Influenza contains 8 gene segments and encodes up to 15 gene products. The segments are numbered in decreasing order of size and sometimes referred to by the predominant gene product produced. The segments are: segment 1/PB2, Segment 2/PB1, segment 3/PA, segment 4/HA, segment 5/NP, segment 6/NA, segment 7/M, and segment 8/NS (Table 2.1).

Segment #	Protein products	length (nt/aa)	Function
1	PB2	2341/759	RNA polymerase complex; cap binding
2	PB1	2341/757	RNA polymerase complex; polymerase
	PB1-F2	--/87-90	strain dependent; modulate host response
	N40	--/718	unclear
3	PA	2231/716	RNA polymerase complex; endonuclease
	PA-X	--/41,61	Down regulation of host response
	PA-N155	--/562	unclear; viral polymerase enhancement
	PA-N182	--/535	unclear
4	HA	1742-1778/562-566	Cell binding; membrane fusion; antigenicity
5	NP	565/498	RNP structure; viral assembly
6	NA	1467/454	viral egress; surface antigen
7	M1	1027/242	Assembly; virus structure
	M2	--/97	proton channel; uncoating
8	NS1	890/230	Cellular environmental modulation
	NS2/NEP	--/121	Assembly; Nuclear export of RNPs

Table 2.1. Gene segments and translation products.

Segment 1 is 2341 nucleotides long and encodes the polymerase protein PB2. PB2 is 759 amino acids long and functions in the RNA polymerase complex. PB2 is essential for viral replication and its primary purpose is to bind 5'methylated caps of host mRNA (Blaas et al., 1982). This initializes the production of viral mRNA. The protein also is a virulence determinant. This is managed by its effect on the polymerase activity as well as by interacting with mitochondrial antiviral signaling proteins (MAVS) (Graef et al., 2010; Salomon et al., 2006). Position 627 is especially important. A lysine (K) here is a marker for increased mammalian pathogenicity while a glutamic acid (E) is a marker for avian pathogenicity (Baigent and McCauley, 2003; Subbarao et al., 1993). It should be noted that some high virulent mammalian viruses contain E627, the avian marker (Chen et al., 2007). This highlights the fact that influenza virulence is a polygenic trait.

Segment 2 is 2341 nucleotides long and can encode the PB1, PB1-F2, and N40 proteins. PB1 is 757 amino acids long and is the backbone of the polymerase complex. It binds both PB2 and PA serving as the connector of the polymerase complex and also contains the enzymatic activity that actually replicates the viral genome. (Biswas and Nayak, 1994; Gonzalez et al., 1996; Perez and Donis, 2001) PB1-F2 is an 87-90 amino acid protein encoded by some segment 2 variants (Chen et al., 2001). The role of PB1-F2 is unclear, but seems to be strain, cell, and host specific. The protein is not necessary for viral replication, but seems to increase polymerase activity in some viral strains (Conenello and Palese, 2007; Mazur et al., 2008). The protein also has effects on the innate host immunity in some model organisms (Dudek et al., 2011; Le Goffic et al., 2011; Varga et al., 2011). Finally, segment 2 also encodes the N40 protein. This is a

nonessential N-truncated version of PB1 that lacks polymerase activity, interacts with the polymerase complex, and does not appear to affect virulence (Le Goffic et al., 2011; Wise et al., 2009).

Segment 3 is 2231 nucleotides long and can encode 4 proteins: The PA and PA-X proteins as well as the newly described PA-N155 and PA-N182 (N155 and N182 respectively) (Muramoto et al., 2013). PA is 716 amino acids long and is essential for replication. Its main function is to act as an endonuclease that cleaves the cap from host mRNA (Dias et al., 2009). This allows the stolen cap to prime viral synthesis. It is also a virulence determinant in HPAI H5 in ducks (Song et al., 2011). PA-X is a recently discovered nonessential protein whose role is still being elucidated. The protein is usually 61 amino acids long, but is 41 amino acids long in many strains (Jagger et al., 2012). It contains the endonuclease activity of PA, but not the PB1 binding site allowing it to act independently of the polymerase complex. It is produced by a +1 frame shift caused by a slippery sequence motif. Its role in the cell appears to be a host cell transcription inhibitor and loss of PA-X increases virulence (Jagger et al., 2012). N155 and N182 are produced by alternative start codons and result in N-truncated PA genes. They have no polymerase activity, but knock out viruses grew slower in cell culture and showed reduced pathogenicity in mice (Muramoto et al., 2013).

Segment 4 is 1742-1778 nucleotides long and encodes only the HA protein. The protein is from 562-566 amino acids long and is responsible for binding and entry into the cell. HA plays a major role in host specificity, virulence, and host adaptive immunity (Cross et al., 2001). The protein is processed as a single polypeptide HA0, but is altered in the rough endoplasmic reticulum by the removal an amino terminal leader sequence,

the addition of 6 disulfide bonds, and glycosylation (Cross et al., 2001). In order for a virus to be fully infectious, the HA0 must be cleaved by secreted host proteases into HA1 and HA2 (Lazarowitz and Choppin, 1975). For most strains of influenza, the cleave site has a single basic amino acid and is only recognized by trypsin like proteases found in only a few tissues. For HPAI viruses, the cleavage site is altered to have multiple basic residues that allow for cleave by furin, which is found in many different tissues allowing systemic infections (Rott et al., 1995). The HA trimer consists of a globular head composed completely of HA1 subunits and a stalk that is composed of HA1 and HA2. The globular head contains the receptor-binding site while the stalk region contains the transmembrain domain (Cross et al., 2001).

Segment 5 is 1565 nucleotides in length and only encodes the NP protein. NP is 498 amino acids long and primarily acts as the backbone for RNPs. NP coats viral RNA and functions as the structural protein of the RNP. NP is also involved in the transport of RNPs and packaging of RNPs into budding virion. NP interact with cellular cargo proteins of the karyopherin family (Melen et al., 2003). Additionally, it interacts with M1 during viral budding indicating a role in associating the RNP with budding virus (Baudin et al., 2001; Ye et al., 1999).

Segment 6 encodes the NA protein, which is 454 amino acids long. As previously mentioned, NA is responsible for viral egress because of sialidase activity of it globular head domain. This accomplishes to things. It clears sialic acid at the budding site so that budding virion do not agglutinate, and it clears sialic acid from late stage infected cells to prevent superinfections (Huang et al., 2008). It is also recognized by the host adaptive immune response and while most antibody pressure is placed on the HA, neutralizing NA

antibodies are produced (Subbarao and Joseph, 2007). The NA has been associated with the virulence of some strains of influenza (Pappas et al., 2008; Sorrell et al., 2010).

Segment 7 is 1027 nucleotides long and produces 2 proteins. M1 is 242 amino acids long, while M2 is 97. M1 is coats the inside of the viral envelope and lends rigidity to the virion. It is crucial for assembly and is the only protein required for the formation of virus like particles (Gomez-Puertas et al., 2000). M1 interacts with NP and vRNA as well as nuclear export protein (NEP/NS2) to bring vRNPs out of the nucleus and into the cytoplasm. It also interacts with both lipid bilayers and the cytoplasmic tails of surface proteins to facilitate viral budding (Nayak et al., 2004; Schmitt and Lamb, 2005). M2 is processed by an alternate splicing event of segment 7. It forms homotetramers on the virus surface that function as a proton channel that is vital for viral uncoating. The M segment has been associated with increased infectivity and transmissibility of pH1N1 in several model hosts (Angel M, 2013; Chou YY, 2011; Ma W, 2012).

Segment 8 is the smallest segment at 890 nucleotides and encodes two proteins: NS1 and NEP/NS2. NS1 has a long list of interacting partners (both cellular and viral) and is responsible for a variety of tasks. In general, it is responsible for modulating the cellular environment to better suit influenza's needs. Major functions include evasion of the innate immune response, down regulation of cellular mRNA export, and up regulation of viral transcription. NEP/NS2 is 121 amino acids long and produced by alternate splicing. As mentioned before, NEP associates with nascent RNPs and is responsible for nuclear export.

2.1.5. Influenza virus life cycle

The HA protein binds sialic acid residues on the surface of target cells. The specific sialic acid moiety is dependent on the specific strain of influenza, but typically, avian-like viruses have HAs that bind terminal α2,3 sialic acid residues, while human-like viruses bind α2,6 sialic acid terminating residues (Connor et al., 1994). The bound virus is taken into the cell by receptor-mediated endocytosis. As the endosome acidifies, a conformational change in the HA occurs. This exposes the fusion peptide of HA2, which allows the fusion of the viral and endosomal membranes. As the endosomal compartment acidifies, the M2 ion channel acidifies the core of the virus as well, destabilizing the M1-RNP interaction and allowing for uncoating of the viral genomic content and its release into the cytoplasm (Cross et al., 2001). The RNPs are then directed to the nucleus by host machinery because of nuclear localization signals in the NP (Wang et al., 1997).

Once in the nucleus, transcription and replication occur. The viral RNA (vRNA) serves as a template for both viral mRNA and the positive sense cRNA replication intermediate, which in turn serves as a template for more vRNA. Transcription is dependent on host cell polymerase II as capped host mRNA are used initiate the process (Palese and Shaw, 2007). PB2 binds capped mRNA, which brings the mRNA in contact with PA. PA cuts the strand between 10-13 bases from the cap. This is then recognized by PB1 as a primer and viral mRNA is replicated off the end of the capped primer. Polyadenylation is achieved via stuttering at a polyuridine stretch near the 5' end of the vRNA (Robertson et al., 1981). Thus, a capped, polyadenylated mRNA encoding the viral proteins is exported to the cytoplasm and host machinery produces viral proteins.

Unlike mRNA synthesis, cRNA is made in a primer independent fashion. The exact mechanism of how the switch from mRNA to cRNA is not clearly understood. Accumulating NP concentration has been implied, but small RNA may play a role as well (Perez et al., 2010; Portela and Digard, 2002).

As viral protein and vRNA begin to form new RNPs in the nucleus, they are exported to the cytoplasm associated with M1 and NEP. Newly made HA and NA are associated with apical surface lipid rafts. The accumulation of HA and NA in lipid rafts may cause the enlarging of the rafts and deformation of the membrane. M2 is incorporated into the budding virus by its association with M1, which interacts with the cytoplasmic tails of NA and HA. Though the process is not completely understood, vRNPs are incorporated in a selective manner, i.e. all 8 unique gene segments are incorporated into each virion (Noda et al., 2012). Each gene segment has a unique packaging signal, but packaging signals for each gene are conserved amongst influenza viruses (Fujii et al., 2005; Hutchinson et al., 2010; Liang et al., 2005; Watanabe et al., 2003). Additional to the viral proteins and RNA, a number of host proteins are packaged in the virions, but their purpose is yet to be determined (Shaw et al., 2008).

2.1.6 Viral diversity

There are many factors that affect the diversity of influenza A viruses including environmental and genetic components. There are two main environmental forces driving diversity. First, there are two main antigenic proteins, HA and NA, and each one comes in an assortment of different antigenic varieties. To date, there are 16 HA and 9 NA types (Fouchier et al., 2005). These different HA and NA genes pair up to form up to 144

unique combinations; 103 of these subtypes have been isolated in nature (Alexander, 2007). The second driving force is the species tropism of certain subtypes. Zoonotic transmission is often a dead end process for influenza. However, sometimes a virus population will become endemic in a novel species and increase the natural viral diversity. Currently, there are two subtypes endemic in humans: H1N1 and H3N2. These are also frequently found in swine and birds. Virus populations evolve differently in each host species, thus increasing overall diversity of a subtype. For instance, due to adaption in specific hosts, H1N1 has evolved into three unique lineages: avian H1N1, swine H1N1, and human H1N1.

Most organisms have two main forces that drive diversity mechanistically: replication associated mutations and sexual recombination. Influenza viruses are no different. The two processes are called antigenic drift and reassortment and function similarly.

Antigenic drift is simply the build up of genetic changes over time associated with replication. Like most RNA viruses, the polymerase of influenza does not have high sequence fidelity. It is estimated that one in 10,000 bases is misincorporated which comes out to just over one mutation every time the genome is replicated (Drake, 1993; Nobusawa and Sato, 2006). During an infection the overall genome of the viral population is consistent, but an in depth look at individual viruses shows a wide array of different genotypes at the nucleotide level. For this reason influenza populations are viewed as quasispecies. Some mutations persist and overtime populations of viruses arise with unique genomes. This becomes apparent when looking at the phylogeny of certain subtypes. Subtypes get broken down into sublineages, clades and subclades.

Antigenic shift is essentially viral sexual replication. The process is relatively simple (Figure 2.2). Two viruses infect the same cell. The genetic material of both viruses is replicated in the nucleus of the cell and the distinct gene segments are mixed up. When packaging occurs, there is no process to ensure the homogeneity of the incorporated genome, thus mixed genomes are packaged and genetically unique viruses emerge. Two genetically distinct viruses can create 256 unique reassortant progeny. Most of these progeny are replication deficient and quickly disappear (Sun Y, 2011). Some ordinarily are replication competent, but are unable to outcompete the parental viruses and eventually die out. Infrequently, a reassortant gains some advantage (higher infectivity, better transmissibility, or novel host tropism) and is able to outcompete or escape the parental viruses and establish a new viral population. This process is a major driving force in the genesis of pandemic viruses. The exact forces and how they shape reassortment are unclear, but a better understanding is required for true pandemic preparation.

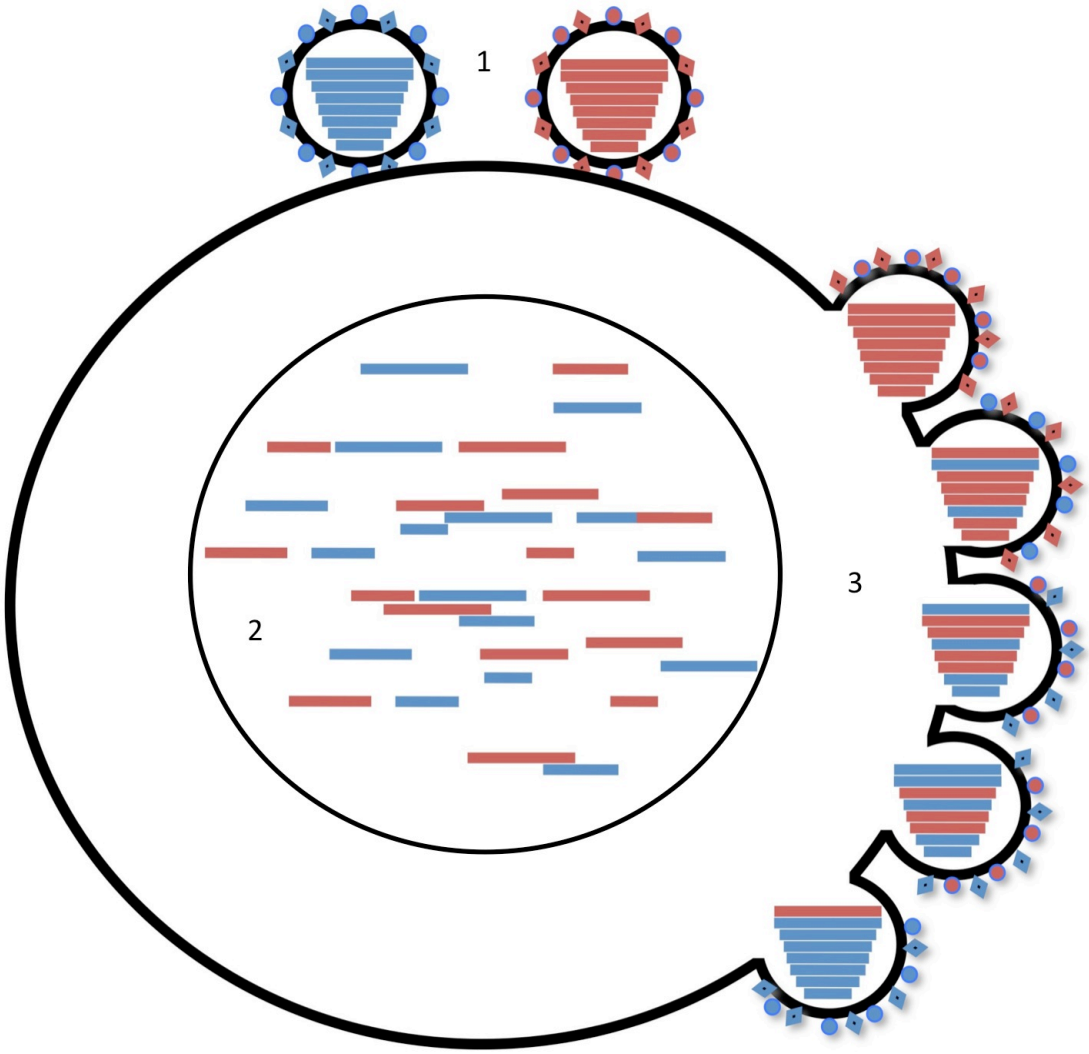


Figure 2.2. The process of reassortment produces unique progeny. 1. Two unique viruses infect the same cell. 2. Both genomes are replicated in the nucleus and mixed together. 3. Packaging randomly selects segments 1-8 resulting in potentially mixed genome progeny.

2.2 H9N2 influenza

2.2.1 Overview

H9N2s can be subdivided into multiple sublineages. *Guo et al*. proposed a four sublineage categorization that was standard for several years (Guo et al., 2000). They called for one North American sublineage and 3 Eurasian sublineages, namely, North American, G1-like, Y280-like, and Korean like. These divisions are very useful and many new isolates and novel viruses, including those discussed in this writing, are still described in these terms. However, recent phylogenetic studies have called for further breakdown of the subtype. *Fusaro et al.* examined many modern isolates of H9N2 form northern Africa, the Middle East, and central Asia. Their results called for the Eurasian viruses to be broken up into 4 sublineages (Fusaro et al., 2011). Finally, the most thorough study of H9N2 phylogenetics examined the nearly 600 complete H9N2 genomes gathered from all over the world and broke the subtype into 7 sublineages (Dong et al., 2011). The new breakdown had Eurasian sublineages based on the sublineages described by *Guo et al.*, but also included two new Eurasian sublineages. The North American H9N2s were divided into two sublineages. One that typically was found from turkeys, and one that represented H9N2 found in wild birds. Their results reflect the very complex viral ecology of H9N2 viruses. Many isolates revealed a past of reassortment between sublineages and some viruses isolated in Asia grouped with the North American lineages indicating a global heterogeneous distribution of H9N2s (Dong et al., 2011).

Domestic land based poultry and wild aquatic birds are the largest reservoirs of H9N2 across the globe. In the United States and Canada they are predominately found in wild birds such as ducks and gulls (Kawaoka et al., 1988; Sharp et al., 1997; Sharp et al., 1993). However, they have also cause periodic epidemics in turkeys (Banks et al., 2000). The first H9N2 isolated and described came from an infected turkey in Wisconsin in 1966 (Homme et al., 1970), Recently, the first H9N2 was isolated from South America from a duck in Argentina and characterized (Xu et al., 2012). It was related to North American H9N2 viruses and expanded the geographical range of the North American sublineage. This finding also highlights the fact that much of South America is a black box in terms of avian influenza. Many recent efforts have been made to shed light on the ecology and epidemiology in the region. The finding of an H9N2 in Argentina hints at an expanded genetic diversity of the subtype.

2.2.2 H9N2 in birds

The first Eurasian H9N2 was isolated from a duck in 1976 (Banks et al., 2000). For the next several years the virus was only isolated from wild ducks, however, beginning in the late 1980's land based poultry began turning up with H9N2 infections (Perez et al., 2003). By the early 1990's H9N2 was a common subtype of influenza in many Asian poultry populations (Shortridge, 1992). Today, in many regions of Asia, H9N2 is the dominant subtype of influenza in poultry. Geographically, these viruses span from Western Europe to Eastern Asia (Banks et al., 2000; Guan et al., 1999). They have been isolated from northern Africa in Tunisia, Libya, and Egypt and all across the Middle East (Aamir et al., 2007; Arafa et al., 2012; Fereidouni et al., 2010; Golender et al., 2008; Slomka et al., 2013; Tombari et al., 2011). The viruses have been found as far

south as South Africa and throughout the Indian subcontinent as well (Abolnik et al., 2007; Tosh et al., 2008).

H9N2 viruses are classified as low pathogenic avian influenza (LPAI) and typically cause mild respiratory signs and slight decrease in egg production in land based poultry (Brown et al., 2006). Most wild bird isolations have come from apparently healthy ducks. However, under certain undefined circumstances, the subtype has been known to cause high morbidity and mortality. In an H9N2 outbreak in Iranian broiler chickens beginning in 1998 mortality rates reached as high as 65% of individual flocks (Nili and Asasi, 2002). However, when filtered field samples were used as inoculums for an experimental study the mortality rates fell to 19%. This suggests that environmental conditions on poultry farms were exacerbating the disease. Additionally, it is known that certain bacterial co-infections can have a similar effect. While no secondary infection was determined in the mentioned study, the authors speculated that a bacterial co-infection was responsible for the high mortality based on similar pathological characteristics with another avian influenza outbreak in Italy with a known secondary infection (Capua et al., 2000).

Many H9N2 isolates from poultry in Asia isolated from 1997 and onwards contain mutations in the HA, and more specifically, in the receptor binding site of HA that confer human like receptor binding characteristics (Matrosovich et al., 2001). Historically, the H9N2 had a glutamine (Q) at position 226. However, more modern isolates commonly contain leucine (L) at position 226. This is very similar to H2N2 and H3N2 human isolates. This specific mutation is sufficient to change the receptor binding profile significantly. *Wan and Perez* mutated two wtH9N2 isolates, one containing Q226

and one with L226, and made single mutations to switch the amino acids at 226 (Wan and Perez, 2007). Making the L226Q mutation, human-like to avian-like, resulted in a reduction of α2,6 expressing cell binding by approximately half. Similarly, making the Q226L mutation, avian-like to human-like, resulted in about a doubling of α2,6 expressing cell binding. The rise of L226 containing H9N2 viruses in the late 1990's correlated with the first swine and then human H9N2 confirmed infections (Peiris et al., 2001; Peiris et al., 1999).

2.2.3 H9N2 in swine

In 1998, the first swine H9N2 was recovered in Hong Kong (Peiris et al., 2001). There have been multiple outbreaks of H9N2 in swine since 1998, but all confirmed cases have come from China, Hong Kong, or South Korea. The 1998 isolation came from surveillance of apparently healthy pigs coming into a slaughterhouse in Hong Kong. However, outbreaks have occurred in China that resulted in high morbidity and mortality (Xu et al., 2004). H9N2 are not endemic in swine and swine H9N2 isolates have been directly related to regional avian isolates (Rui-Hua et al., 2011)

In general, there is not a lot of published data about H9N2 in swine. Aside from a few outbreaks with high morbidity the subtype is not considered of high significance in pigs. However, swine possess both human and avian like receptors in their respiratory tract, and are susceptible to both avian and human viruses (Ito et al., 1998). Previous studies have found H9N2 co-circulating in swine with human viruses and H9N2 swine reassortant viruses have been found as well (Peiris et al., 2001). This highlights the

possibility that an H9N2 could reassort with a human virus in a pig and result in a novel virus that could be dangerous to humans.

2.2.4 H9N2 in humans

There have only been 11 isolations of H9N2 from humans all associated with mild to moderate influenza like symptoms and all cases eventually recovered (Cheng et al., 2011). The first three were isolated in 1998 was from a four-year-old boy, a one-year-old girl, and a 36-year-old woman in Shantou, China (Guo Y et al., 1999). Two more isolates were recovered in neighboring Shaoguan later that same month in a one-year-old male and a 75-year-old male (Guo Y et al., 1999). Three more cases were identified the following year. A four-year-old girl and a one-year-old girl from Hong Kong were identified in the spring and a two-year-old girl from Guangzhou (Gou et al., 2000; Peiris et al., 1999). In 2003 a five-year-old boy in Hong Kong was confirmed to have H9N2 (Butt et al., 2005). These nine cases all had mild or no underlying conditions. In 2008 and 2009 the final two human cases of H9N2 were discovered. The cases were in a three-month-old girl and a 47-year-old woman (Cheng et al., 2011). These last two are notable for two reasons. First, these are the only two human isolates with Q226 mutation. Based on previous observations of H9 viruses these two isolates probably had reduced α2,6 binding. This normally would prevent a human infection if not for the second unique fact about these two cases. Both isolations came from immune compromised individuals. The baby girl was diagnosed with leukemia four days after the initial isolation and the woman

had a blood-stem cell transplantation and was being treated for chronic graft versus host disease (Cheng et al., 2011).

These relatively few human isolations may lead some to conclude that H9N2 do not regularly infect humans. However, serological surveys of various populations across Asia suggest a different conclusion. Anti-H9 antibody prevalence has been recorded as high as 15% in live bird market retailers in China (Wang et al., 2009), although most surveys range from 1-6%. People with higher exposure risks (poultry farmers, poultry processing workers, etc.) typically show higher seroconversion, but the general population usually measures from 1-3%. These results hold up across the geographical distribution of the Eurasian lineages. A serological survey done in Vietnam found that poultry workers had a 3.5% seroconversion rate while the general population was at 3% (Uyeki et al., 2012). A study conducted in Guangzhou, China found that people regularly exposed to poultry had a 5% seroconversion rate, while the general populations was 1.3% (Wang et al., 2009). Finally, a survey of poultry workers in Pune, India revealed a 6.2% seroconversion rate (Pawar et al., 2012).

2.2.5 H9N2 and reassortment

Like most other influenza subtypes, H9N2 will reassort given the right circumstances. Multiple sublineages of H9N2 are prevalent in both wild birds and poultry across the Middle East and Asia. Phylogenetic analysis of H9N2 isolates regularly reveals homosubtypic reassortment, especially in places with higher surveillance such as Egypt and China (Arafa et al., 2012; Wang et al., 2012; Zhou et al., 2012). The identification of a six-way homosubtypic reassortment in chickens reveals the heavy

extent in which these viruses mix together (Xie et al., 2012). Homosubtypic reassortments have also been detected. An outbreak of H6N2 in chickens revealed to be a result of a reassortment between H9N2 and H6N8 (Abolnik et al., 2007). In Pakistan, several H9N2 were recovered with NS segments that more closely matches HPAI H5 or H7 NS than any H9N2 from the region suggesting multiple reassortment events between H9N2 and multiple different HPAI viruses (Iqbal et al., 2009). The most well known H9N2 reassortment however involves the 1997 HPAI H5N1 from Hong Kong. Analysis of the internal genes of the HPAI viruses that spread from chicken to humans reveals homology with the A/quail/Hong Kong/G1/1997 (H9N2) internal genes suggesting a reassortment between the two viruses (Guan et al., 1999). While inconclusive, evidence suggests that the H9N2 donated the genes to the HPAI virus.

H9N2 reassortant viruses have also been found in swine. Similar to in avian species, both heterosubtypic and homosubtypic reassortants have been isolated from swine (Cong et al., 2007; Xu et al., 2004; Yu et al., 2011) Two reports of swine H9N2 with internal genes similar to H5 viruses have come from Chinese swine surveys (Cong et al., 2007; Yu et al., 2011). It is unclear if these are two instances of swine reassortment or avian reassortant viruses that transmitted to pigs. However, a third swine reassortant virus was described that had genes from classical swine H1N1 indicating a reassortment event between avian H9N2 and a swine virus (Guo et al., 2005). H9N2 apparently readily reassort in nature, are found in a mixing bowl species, and has reassorted with influenza viruses from other species. All these facts highlight the possible danger an H9 virus might pose to humans.

While and H9N2 and human influenza reassortant virus has not yet been isolated in nature, there have been several studies conducted to determine the characteristics of such reassortants. A possible H9N2:sH3N2 reassortant with the HA and NA from an avian H9N2 and the internal genes from a human seasonal H3N2 virus readily infected ferrets and showed increased transmission over the parental H9N2 virus (Wan et al., 2008). It efficiently transmitted from infected ferrets (directly inoculated, DI) to naive ferrets that were in direct physical contact (direct contact, DC), but did not transmit to naïve ferrets that were physically separated and shared the same air (respiratory contact, RC). Later studies with the same virus showed that repeated passage in ferrets resulted in adaption of several genes including the HA that allowed for respiratory transmission of the virus (Sorrell et al., 2009).

The emergence of the 2009 pandemic H1N1 necessitated similar studies. One such study used reverse genetics to recreate every possible reassortant virus that could result from an H9N2:pH1N1 reassortment that contained the H9 HA (Sun Y, 2011). The 128-virus panel was tested for infectivity and pathogenicity in mice. The results indicated that many reassortants had increased infectivity over one or both parental viruses in mice and several had increased pathogenicity over both. This study showed that H9N2 viruses and pH1N1 viruses are compatible for reassortment and that some of these reassortment viruses could result in increased pathogenicity in a new host. Another study conducted in pigs took the surfaces genes form an avian H9N2 that did not transmit in swine and made reassortant viruses using pH1N1 as the genetic backbone (Qiao et al., 2012). They found that both the H9N2 and the H9N1 reassortant viruses infected the pigs, transmitted via direct contact, and showed increased pathogenicity compared to the wholly H9 virus.

These results indicate that an H9:pH1N1 reassortant virus could arise in swine and transmit in a herd which increase the chances of a zoonotic transmission.

Chapter 3: Characterization of sialic acid as influenza receptors in domestic poultry

Published:Kimble JB, Ramirez-Nieto G, Perez DR. Characterization of influenza virus receptors in minor poultry species. Virol J. 2010 Dec. 9(365)[1]

3.1 Abstract

It is commonly accepted that avian influenza viruses (AIVs) bind to terminal α2,3 sialic acid (SA) residues whereas human influenza viruses bind to α2,6 SA residues. By a series of amino acid changes on the HA surface protein, AIVs can switch receptor specificity and recognize α2,6 SA positive cells, including human respiratory epithelial cells. Animal species, like pigs and Japanese quail, that contain both α2,3 and α2,6 SA become ideal environments for receptor switching. Here, the SA patterns and distributions in 6 common minor domestic poultry species is described: Peking duck, Toulouse geese, Chinese ring-neck pheasant, white midget turkey, bobwhite quail, and pearl guinea fowl. Lectins specific to α2,3 and α2,6 SA (*Maakia amurensis* agglutinin and *Sambuca nigra* agglutinin, respectively) were used to detect SA by an alkaline phosphotase-based method and a fluorescent-based method. Differences in SA moieties and their ability to bind influenza viruses were visualized by fluorescent labeling of 4 different H3N2 influenza viruses known to be specific for one receptor or the other. The geese and ducks

[1] BK carried out the animal care, tissue staining, virus binding assays and drafted the manuscript. GRN carried out the animal care and participated in the study design. DRP conceived of the study, and participated in its design and coordination.

showed α2,3 SA throughout the respiratory tract and marginal α2,6 SA only in the colon. The four other avian species showed both α2,3 and α2,6 SA in the respiratory tract and the intestines. Furthermore, the turkey respiratory tract showed a positive correlation between age and α2,6 SA levels. The fact that these birds have both avian and human flu receptors, combined with their common presence in backyard farms and live bird markets worldwide, mark them as potential mixing bowl species and necessitates improved surveillance and additional research about the role of these birds in influenza host switching.

3.2 Introduction

Waterfowl act as the natural reservoir of influenza A viruses. Virus isolates from these birds show high binding preference towards glycans that terminate in sialic acids linked to galactose in an α2,3 conformation (α2,3 SA), the same receptor that dominates the duck intestinal and respiratory tracts (Alexander, 2000; Ito et al., 1998). These isolates typically show low infectivity in humans due in part to the prevalence in the respiratory tract of glycans terminating in sialic acid (α2,6) galactose (α2,6 SA) (Beare and Webster, 1991; Couceiro et al., 1993). However, stable, species specific, viral lineages have jumped from the natural reservoir to wild non-aquatic birds, domestic poultry, and many mammalian species, most notably swine and humans.

In order for an avian virus to infect a human, several changes must occur in the virus, most notably in the HA protein. This can happen in one of two ways: the build up of specific mutations (genetic/antigenic drift) or the recombination with a second virus with a suitable HA gene (genetic/antigenic shift). Both of these processes are facilitated by infection in a 'mixing bowl' species, a host that can accommodate both types of

receptors. For example, swine express both sialic acid moieties and allowed it to play a critical role in the current H1N1 pandemic (Ito et al., 1998; Scholtissek et al., Nicholson, K.G., Webster, R.G., Hay, A.J., 1998).

The emergence of highly pathogenic avian influenza (HPAI) in people who have direct contact with poultry underscore the role poultry play in the transmission of influenza into humans, yet very little is known about the distribution of sialic acid receptors in most poultry species (Peiris et al., 1999; Subbarao et al., 1998). Thus, little is known of the potential of poultry species to act as mixing bowls. Previous studies have shown that mallard and Peking ducks display predominately α2,3 SA in both the intestinal tract and the respiratory tract (Gambaryan et al., 2002; Kuchipudi et al., 2009; Wan and Perez, 2006). White leghorn chicken and, particularly, Japanese quail show more α2,6 SA expression in the respiratory tract (Perez et al., 2003; Wan and Perez, 2006).

Typically, plant lectins that specifically bind to terminal SA are used to identify the distribution of SAs in tissues via lectin histochemistry. *M. amurensis* agglutinin (MAA) binds most predominantly to any glycan terminating in α2,3 SA while *S. nigra* agglutinin binds to terminal α2,6 SA (Baum and Paulson, 1990; Shinya et al., 2006). Here two methods of lectin staining were used to describe the distribution of α2,3 SA and α2,6 SA in six poultry species: Peking duck, Toulouse goose, Chinese ring-neck pheasants, white midget turkey, bobwhite quail, and pearl guinea fowl. The first method is based on digoxigenin-linked lectins and HRP (horseradish peroxidase)-linked anti-digoxigenin antibodies that interact with a substrate to precipitate a marker visible by light microscopy. The second is based on fluorescently-labeled lectins that are visible under a fluorescent microscope.

These methods, however, do not directly measure a tissues capacity to bind influenza virus as there are many other variables that determine binding ability. Specific amino acid sequence and glycosylation in and near the receptor binding site of HA can shift binding specificity from α2,3 SA to α2,6 SA and vice versa. Additionally, these changes can shrink or expand the pool of specific glycans terminating in α2,3 SA or α2,6 SA that HA can bind (Stevens et al., 2008; Suzuki, 2005). Various modifications to the receptors can also change binding specificity (Gambaryan et al., 2006; Wu and Air, 2004). To assuage these issues, virus-binding histochemistry technique was used to directly measure the virus binding patterns as they correlated to the SA distribution.

3.3 Materials and methods

3.3.1 Animal tissues

One day-old Peking ducks, Toulouse geese, Chinese ring-neck pheasants, white midget turkeys, bob white quail, and pearl guinea fowl were received from McMurray Hatchery (Webster City, IA). Animals were maintained in ABSL2 conditions in the Department of Veterinary Medicine for 4 weeks. In the case of ducks and geese, one animal was sacrificed for tissue collection at the age of 1, 2 and 4 weeks of age. For all other birds 2 animals were sacrificed for tissue collection at 1, 2, and 4 weeks of age. Japanese quail were hatched at the Department of Veterinary Medicine and maintained in ABSL2 conditions for 4 weeks. Two animals were sacrificed for tissue collection. The Institutional Animal Care and Use Committee of the University of Maryland, College Park, approved all animal studies. Animal studies adhere strictly to the US Animal Welfare Act (AWA) laws and regulations.

3.3.2 Viruses

A/duck/Hong Kong/375/1975 (H3N2) and A/turkey/Ohio/313053/2004 (H3N2) were kindly provided by Robert Webster, St Judes Children's Research Hospital, Memphis, TN and Yehia Saif, Ohio State University, Wooster, OH, respectively. These viruses were grown in 10 day old embrionated chicken eggs and stocks prepared and maintained at -70°C until use. A/Memphis/31/1998 (H3N2) was propagated in MDCK cells, stocks prepared and maintained at -70°C until use.

3.3.3 Tissue preparation and sectioning

Trachea, lung, middle, and lower intestine were collected from each animal and rinsed in PBS for 5 minutes. Appropriate sized samples were wrapped in aluminum foil and frozen on dry ice. Samples were embedded in OCT and cut into 5 μm thick sections by Histoserv (Germantown, MD).

3.3.4 Digoxigenin sialic acid (SA) detection

Slides containing sections of tissue were rinsed for 1 h at room temperature in tap water before being fixed for 15 minutes in cold acetone followed by a 15 minute incubation in 2% H_2O_2 in methanol. Slides were rinsed 3 times for 5 minutes in tris-buffered saline (TBS) buffer and blocked over night at 4°C in 1% BSA (Sigma, Lenexa, KS) in TBS. Tissue was stained using DIG glycan differentiation kit (Roche, Mannheim, Germany). Briefly, slides were incubated for 1 hour at room temperature in digoxigenin (DIG)-labeled *M. amurensis* agglutinin (MAA, specific for α2,3SA) or DIG-labled *S. nigra* agglutinin (SNA, specific for α2,6 SA) in TBS. Following 3 rinses in TBS, slides were then incubated for 1 hour in peroxidase labeled anti-DIG fragments at room temperature. Three more washes in TBS were followed by 10 minute incubation in

aminoethylcarbazole (AEC) (DAKO, Glostrup, Denmark) and counterstained in hematoxylin for 30 minutes. Cover slips were mounted using aqueous mounting media and tissues were observed under 400× magnification.

3.3.5 Fluorescent sialic acid detection

Slides were fixed and blocked similarly as described for the DIG-based method. Tissues were stained by incubating in FITC-labeled SNA (EY Laboratories, San Mateo, CA) and TRITC-labeled MAA or FITC-labeled MAA and TRITC-labeled SNA for 1 hour at room temperature. Following 3 rinses in TBS, slides were stained for 5 minutes in DAPI (4',6-Diamidino-2-phenylindole, dihydrocholride from Thermo Scientific Rockford, IL). Cover slips were mounted over the tissue using fluorescent mounting media (KPL, Gaithersburg, MD) and imaged at 400× or 630× magnification.

3.3.6 Virus binding assay

Allantoic fluid or tissue culture supernatant was harvested and concentrated using the Centricon Plus-70 system from Millipore (Billerica, MA). Tissue was fixed and blocked as described in Digoxigenin sialic acid detection section. Approximately 600 HAU of virus was mixed 1:1 with 1% BSA in PBS and incubated on the tissue at 37°C for 2 hours. The virus was fixed after rinsing with 50/50 acetone/methanol for 15 min. at -20°C. The tissue was then incubated for 1 hour at room temperature with a monoclonal antibody specific to NP. Following three washes in phosphate buffered solution (PBS), the tissue was incubated in FITC-labeled anti-mouse antibody for one hour at room temperature in the dark. The tissue was then stained with DAPI and visualized with a fluorescent microscope at 400×.

3.4 Results and discussion

3.4.1 Waterfowl and land based poultry species differ in sialic acid distribution in various tissues

Lectin-based staining assays were used to determine the variations in sialic acid form and tissue distribution in various poultry species. Trachea, lung, and large intestine from 6 minor poultry species were used to determine the distribution of SA receptors. Ducks were included as a control as it has previously been reported that they show predominantly α2,3 SA in the trachea with increasing α2,6 on epithelial lining farther along the respiratory tract and only minimal α2,6 in the large intestine (Kuchipudi et al., 2009). All other species were chosen for their presence in live poultry markets across the world.

The results indicate that there is a distinct difference between waterfowl (duck and goose) and land-based poultry (pheasant, turkey, bobwhite quail, and guinea fowl) (Table 3.1) in terms of presence and distribution of SA receptors, particularly α2,6. There were also age-based differences observed, particularly in turkeys (Table 3.1).

Species	Age (wk)	Lung		Trachea		Large Intestine		Species	Age (wk)	Lung		Trachea		Large Intestine	
		2,3	2,6	2,3	2,6	2,3	2,6			2,3	2,6	2,3	2,6	2,3	2,6
Duck	**1**	+	+	+	-	+	-	**Turkey**	**1**	+	+	++	+	+	-
	2	++	+	+	-	++	+		**2**	+	+	++	+	+	-
	4	++	+	+	-	++	+		**4**	++	++	++	++	+	-
Goose	**1**	+	+	+	-	+	-	**Quail**	**1**	+	+	+	+	+	-
	2	+	+	+	-	++	+		**2**	+	+	+	+	+	-
	4	+	-	+	-	+	+		**4**	+	+	+	+	+	-
Pheasant	**1**	+	+	++	+	+	+	**Gn.Fw.**	**1**	+	+	+	+	+	-
	2	+	+	++	+	+	+		**2**	+	+	+	+	+	-
	4	+	+	++	+	+	+		**4**	+	+	+	+	+	-

Table 3.1. Relative expression of sialic acid in avian tissues.
No expression -, minimal expression +, moderate to high expression ++

In the trachea, the ducks showed moderate to high levels of α2,3 SA (Table 3.1 and Figure 3.1A, B, C), consistent with previous reports (Kuchipudi et al., 2009; Pillai and Lee, 2010). There was no expression of α2,6 SA, consistent with one report (Kuchipudi et al., 2009), but not the other (Pillai and Lee, 2010). The geese trachea also showed an abundance of α2,3 SA and absence of α2,6 SA at any age (Table 3.1 and Figure 3.1D, E, F). On the contrary, the four land-based species showed both forms of sialic acid at all ages tested with positive staining of mucin-producing cells lining the lumen of the trachea (Table 3.1 and Figure 3.1G-R). Farther down the respiratory tract, the lungs (Figure 3.2) tested positive for both SA forms in all birds of all ages with the only exception being in the goose. Staining was present on cells lining the lumen of the lungs. Strong positive staining for both types of SA receptors was observed in the lungs of turkeys (Figure 3.2J, K), consistent with the observation of influenza outbreaks in turkeys caused by swine influenza viruses with human-like receptor specificity. The lungs of guinea fowl showed also significant staining for both SA receptors, which is consistent with the circulation in these birds of H9N2 viruses with human-like receptor specificity. At 4 weeks of age, no α2,6 SA was detected in the goose's lung (Figure 3.2E, F). However, both α2,3 SA and α2,6 SA were seen in the lung samples from geese at weeks 1 and 2 (not shown).

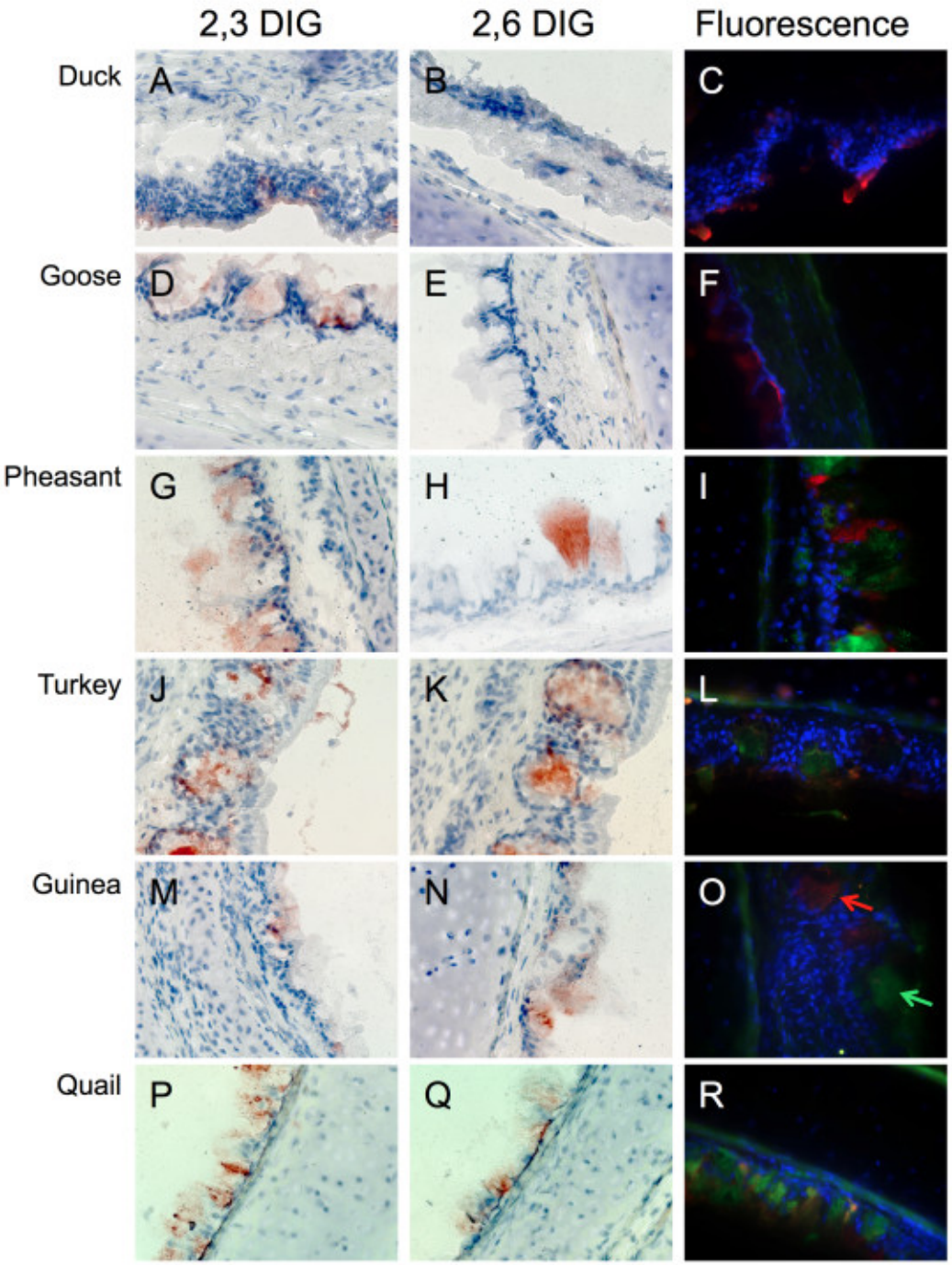


Figure 3.1. Sialic acid distribution in avian trachea. Representative sections of trachea from 4 week old duck (A, B, C), goose (D, E, F), pheasant (G, H, I), turkey (J, K, L), quail (M, N, O), and guinea fowl (P, Q, R) stained with either DIG labeled MAA (α2,3 specific, first column), DIG labeled SNA (α2,6 specific, second column) or FITC SNA (green α2,6) and TRITC MAA (red α2,3). Duck and goose trachea show only α2,3 SA while all other birds display both α2,3 and α2,6 SA.

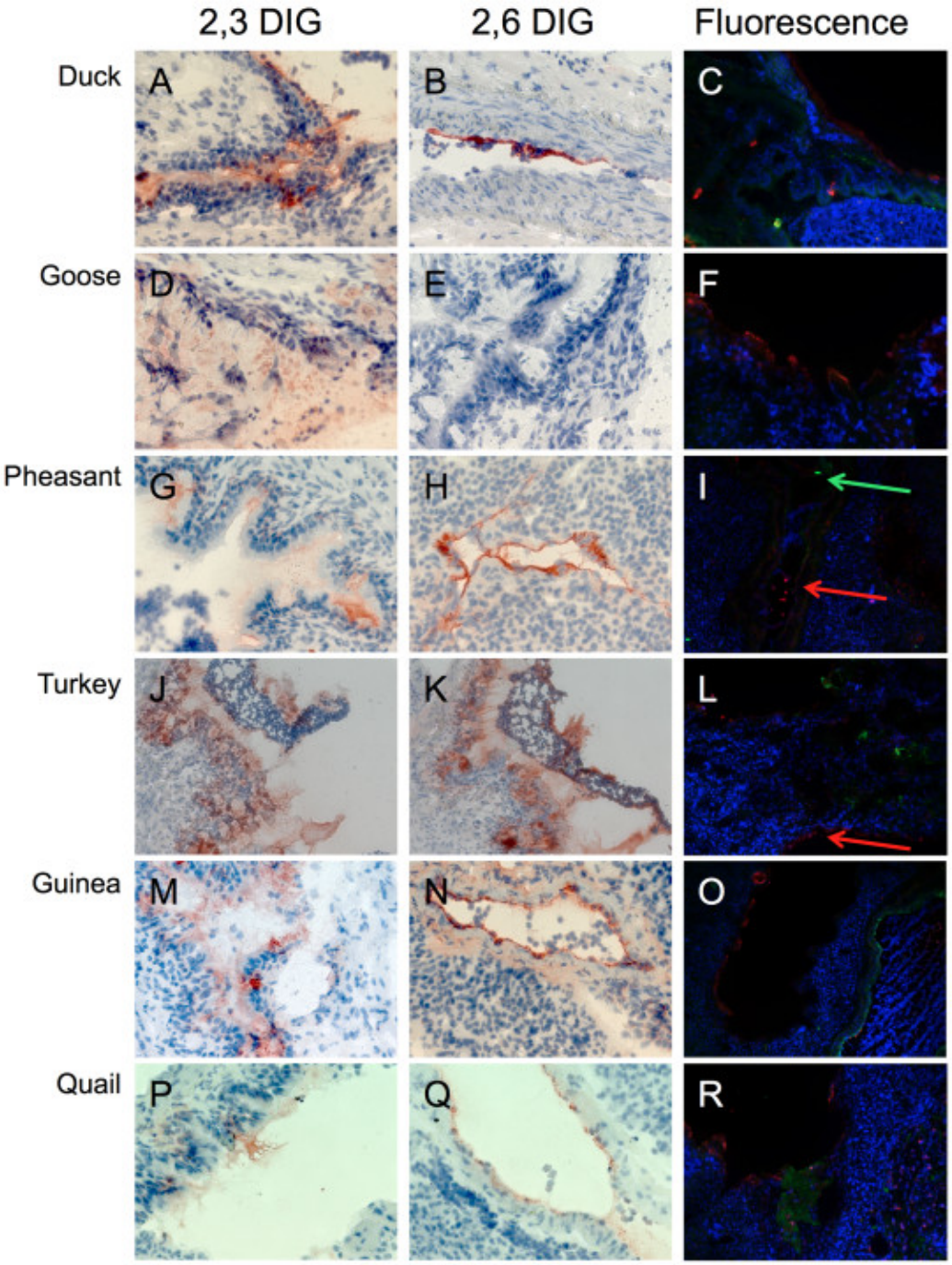


Figure 3.2. Sialic acid distribution in avian lung. Representative sections of lung from 4 week old duck (A, B, C), goose (D, E, F), pheasant (G, H, I), turkey (J, K, L), quail (M, N, O), and guinea fowl (P, Q, R) stained with either DIG labeled MAA (α2,3 specific, first column), DIG labeled SNA (α2,6 specific, second column) or FITC SNA (green α2,6) and TRITC MAA (red α2,3). Goose lung shows only α2,3 SA while all other birds display both α2,3 and α2,6 SA.

Testing of the large intestine once again showed a divide between the species. All six species tested positive for α2,3 SA in the large intestine in cells facing the lumen (Figure 3.3). However, duck, goose, and pheasant large intestine also showed minimal positive results for α2,6 SA (Figure 3.3B, E and 3.3H) while turkey, guinea fowl and quail tested negative (Figure 3.3K, N and 3.3Q; please note that significant α2,6 SA staining was observed on the basolateral side - opposite to the intestinal lumen - of epithelial cells in guinea fowl.)

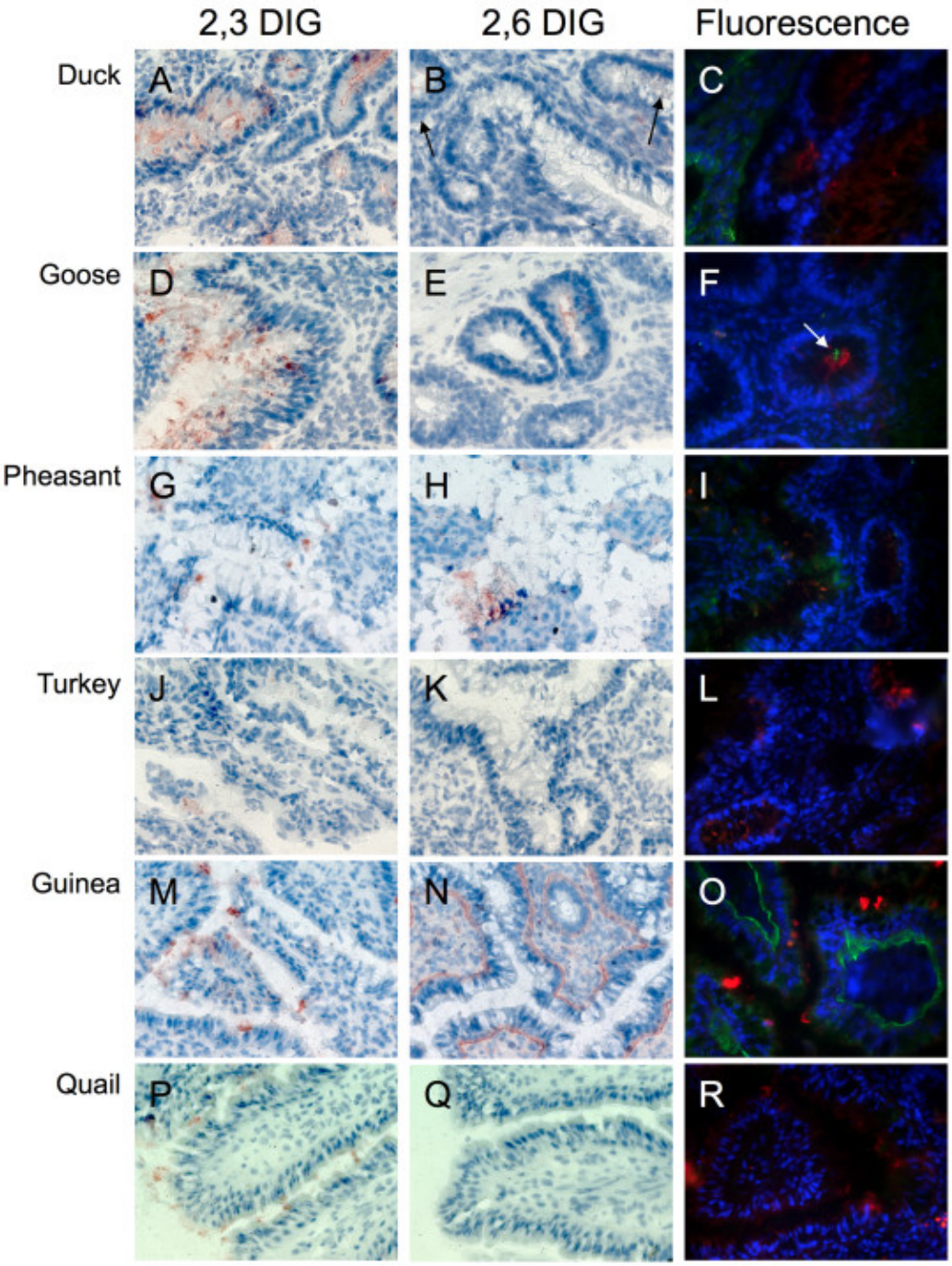


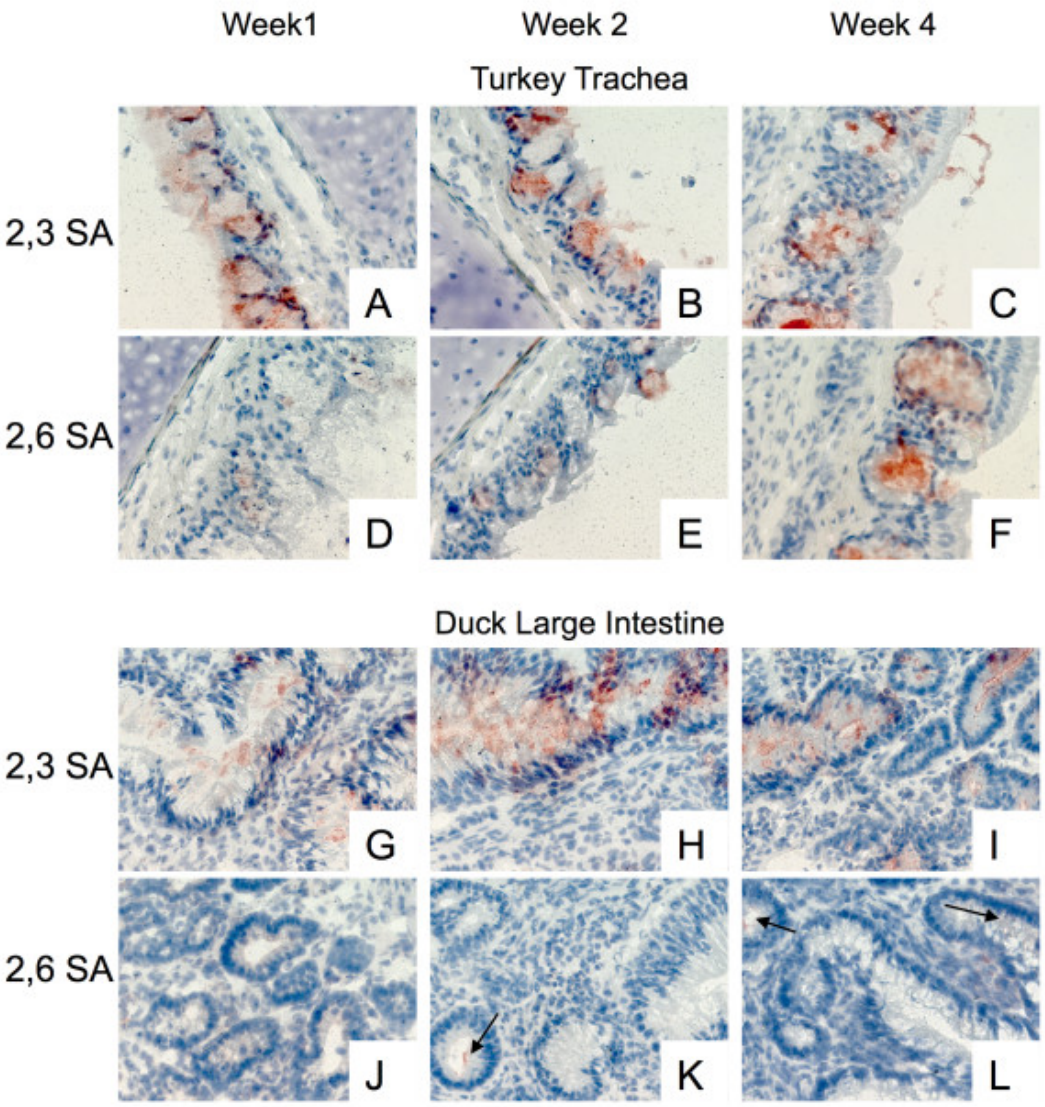
Figure 3.3. Sialic acid distribution in avian large intestine. Representative sections of large intestine 4 week old from duck (A, B, C), goose (D, E, F), pheasant (G, H, I), turkey (J, K, L), quail (M, N, O), and guinea fowl (P, Q, R) stained with either DIG labeled MAA (α2,3 specific, first column), DIG labeled SNA (α2,6 specific, second column) or FITC SNA (green α2,6) and TRITC MAA (red α2,3). Duck, goose, and pheasant large intestine show both α2,3 SA and α2,6SA while the other species show only α2,3SA. Arrows highlight positive reactions.

The birds can be divided into three groups based on the distribution of sialic acids in the tissues examined. The waterfowl, the natural host of avian influenza viruses, show predominantly α2,3 SA in their tissues. α2,6 SA is only seen in the lower respiratory tract and minimally in the large intestine. The land-based birds also express α2,3 SA in all the tissues tested, however, they also express significant levels of α2,6 SA in the upper respiratory tract. This could help explain why these birds are susceptible to AIVs resulting in the emergence of strains with altered receptor specificity, including with human-like receptor binding (Wan and Perez, 2007). This also underscores the potential role of these birds in influenza virus reassortment. Finally, the pheasants showed α2,6 SA in the trachea similar to the other land birds, but also showed α2,6 SA in the large intestine like the aquatic birds. This could make the pheasant more likely than other species to facilitate viral reassortment or to act as a "mixing bowl" species.

3.4.2 Age dependent variations in α2,6 SA expression

While performing the experiments described above a trend was noticed in three species. The ducks and geese showed an increasing expression of α2,6 SA in the large intestine as they aged. Similarly, an increase in α2,6 SA detection was seen in the trachea of turkeys as they aged. The age dependence in turkeys was later reported by Pillai and Lee (Pillai and Lee, 2010), however, they did not see any increase in α2,6 in Pekin ducks. There was no detection of α2,6 SA in the large intestine of ducks and geese at week 1 (Figure 3.4J for duck, not shown for geese). However, by week 2 there was a very low level positive reaction and at week 4 this reaction was slightly increased (Figure 3.4K and

3.4L arrows). Expression levels of α2,3SA remained relatively constant (Figure 3.4G-I) at all three time points.



<u>Figure 3.4.</u> Effects of age on sialic acid distribution. Sections from 1, 2, and 4 week old turkeys trachea (A-F) and 1, 2, and 4 week old duck large intestine (G-L) were stained with either DIG labeled MAA (α2,3 specific, A-C and G-I) or DIG labeled SNA (α2,6 specific, D-F and J-L). Little to no variation was seen in the staining of α2,3 SA in the turkey trachea or duck large intestine across the age range. However, both species show an increase in α2,6SA as the birds age. Arrows highlight positive reactions.

In the turkey trachea this change in expression was even more pronounced. At week 1 (Figure 3.4D) only minimal α2,6 SA was detected. A week later (Figure 3.4E) there was a moderate positive response. By week 4 (Figure 3.4F) there was high level of expression. Again, there was no change in expression α2,3 SA at all time points (Figure 3.4A-C). No major age-related changes were observed in the other avian species tested for either 2,3 or 2,6 SA expression. This changing receptor pattern could have effects for live attenuated vaccines against viruses with a α2,6 binding preference in young turkeys and *in ovo* inoculations.

3.4.3 Lectin binding patterns are not indicative of virus binding patterns

Glycan micro arrays have shown that not all α2,3 SA or α2,6 SA bind to influenza HA proteins equally well (Stevens et al., 2008). One glycan terminating in α2,3 SA might not bind HA while another may bind exceedingly well (Stevens et al., 2008). Unfortunately, both will show a positive reaction to the lectin-binding assays. Thus, determining the influenza virus-binding profile in tissues of different animal species is a condition *sine qua non* to better understand the role of these receptors.

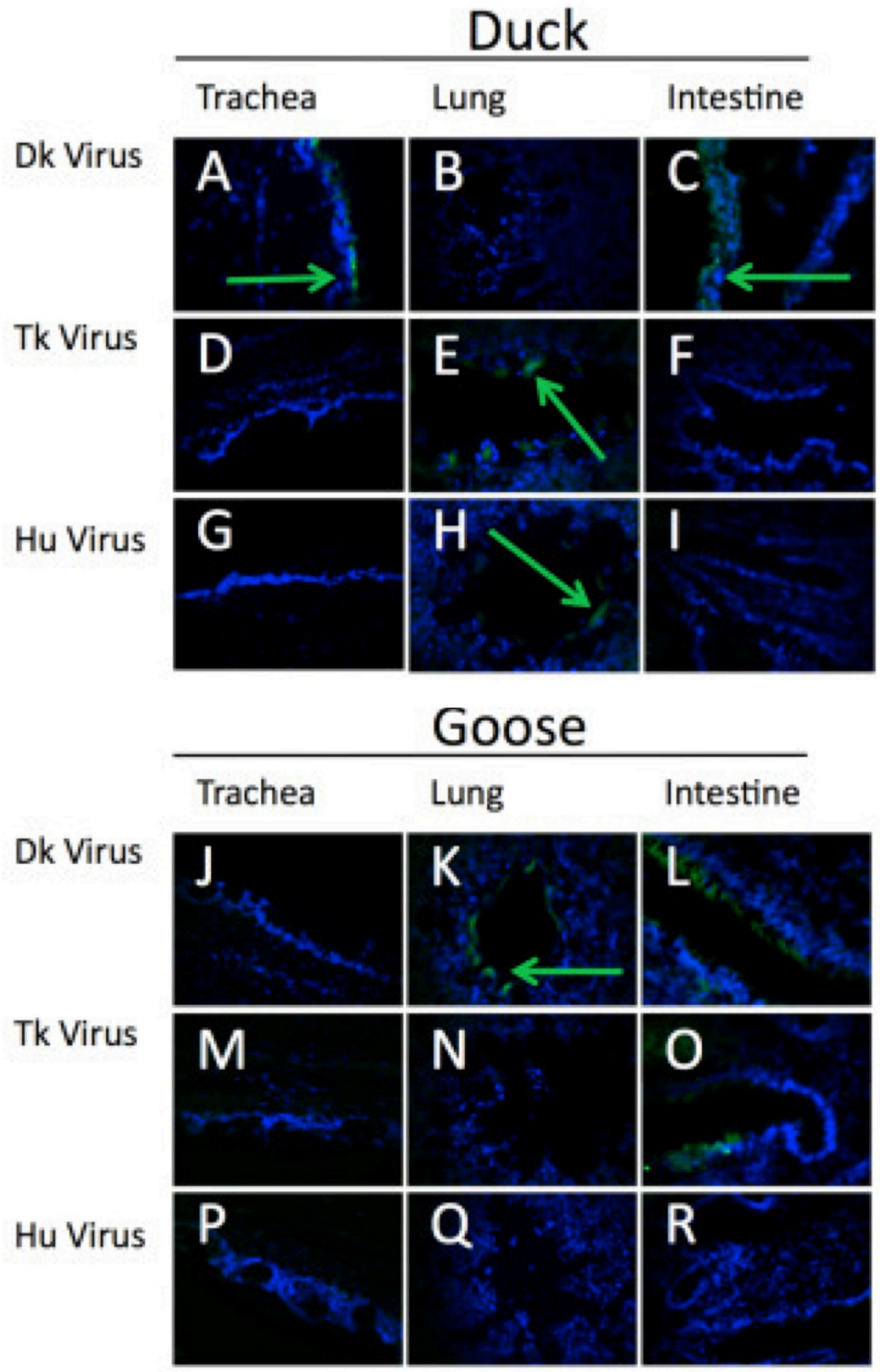
Three H3N2 influenza viruses were selected to determine the correlation between lectin binding and virus binding using 3 prototypic H3N2 viruses to ensure differences were due to receptor specificity and not differences between subtypes. To determine the binding affinity of each virus, hemagglutinin agglutination assays were performed for each virus. According to previous reports, horse red blood cells (RBCs) express solely α2,3 SA on their surface while pig RBCs express predominantly α2,6 SA (Ito et al., 1997). By comparing HA titers determined with each blood type, a binding preference

can be ascertained. A/Dk/HK/7/75 (A/Dk) is a typical AIV duck isolate that bound horse RBCs twice as readily as pig RBCs, indicating a strong α2,3SA preference (Table 3.2). A/Tk/OH/313053/04 (A/Tk) was isolated from a turkey and bound pig RBCs slightly higher than horse RBCs, indicating a slight preference for α2,6SA (Table 3.2). A/Memphis/31/98 (A/Mem) is a human origin virus that shows no α2,3SA binding (Kumari et al., 2007). Accordingly, A/Mem only showed HA titer with the pig RBCs (Table 3.2). These three viruses were used to determine the accuracy and resolution of the lectin binding results.

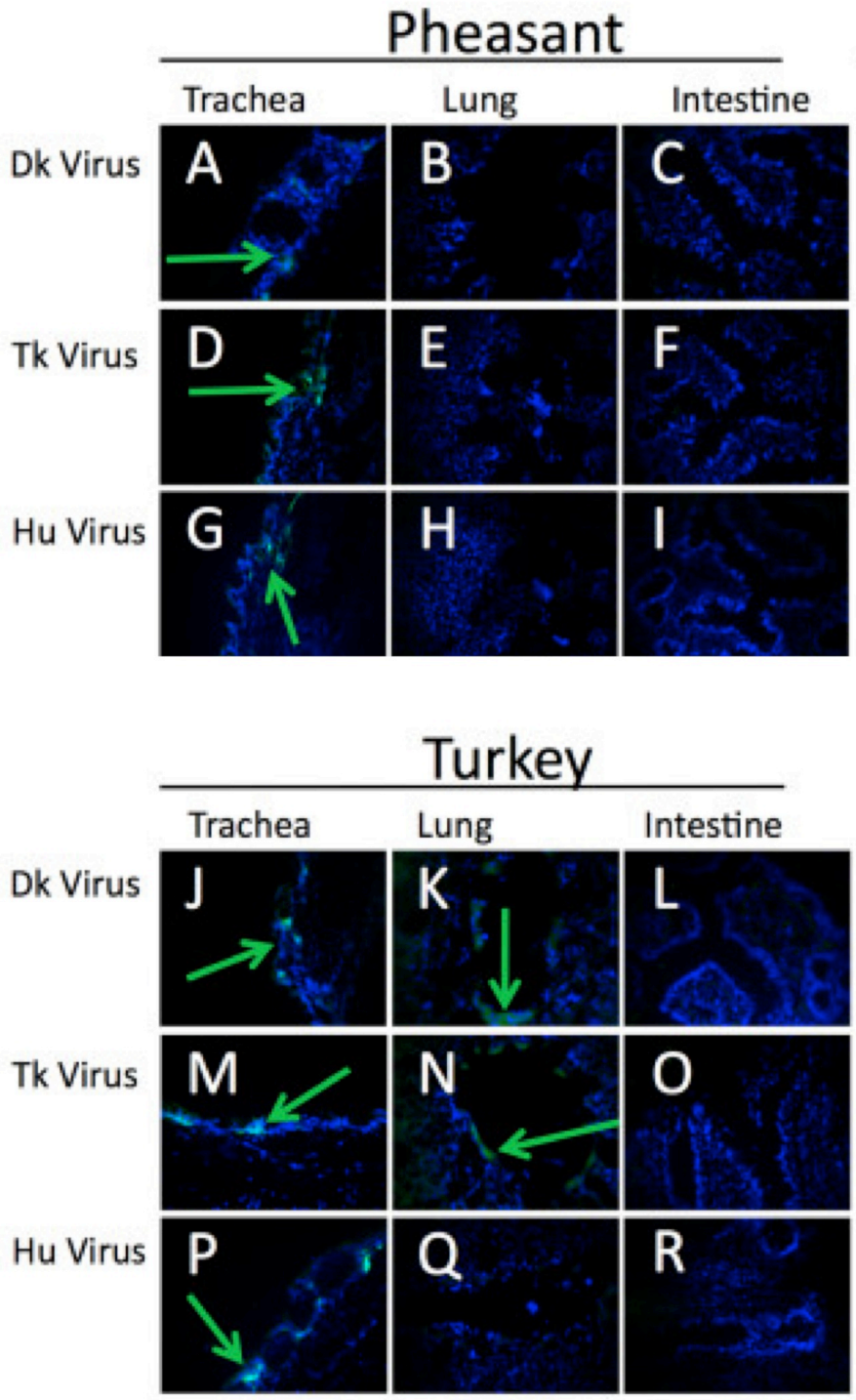
	Horse red blood cells		Pig red blood cells	
	HA titer *	**StDv**	**HA titer ***	**StDv**
A/DK	64	0	32	0
A/TK	3	+/- 1.15	7	+/- 2
A/Memphis	0	0	20	+/- 8

Table 3.2. Hemagglutinin binding affinity of H3N2 viruses.
* average of 4 assays

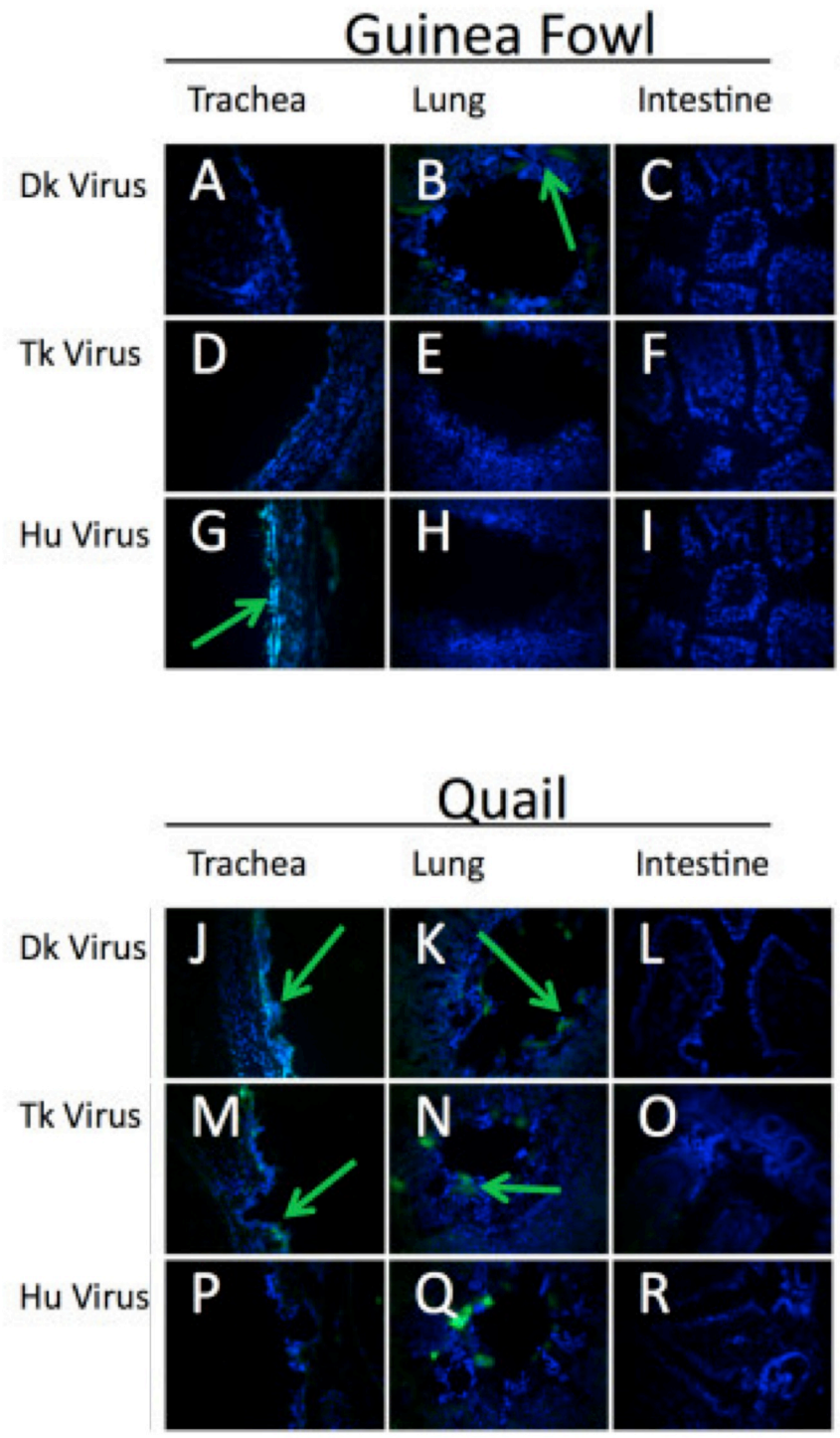
The trachea of the duck and geese showed no α2,6 SA. The virus-binding assay showed no binding to the A/Mem or the A/Tk viruses (Figure 3.5A, D, J and 3.5M). Additionally, there was minimal binding of A/Dk to the duck trachea (Figure 3.5G) and no virus binding of the A/Dk to the goose trachea despite ample expression of α2,3SA (Figure 3.5P). This is not unexpected as the typical route of infection in waterfowl is through the cloacae. In contrast, pheasant and turkey trachea exhibited the ability to bind all three viruses (Figure 3.6A, D, G, J, M and 3.6P). Based on fluorescent intensity and distribution of the fluorescent signal, in the pheasant the A/Dk virus showed the lowest levels of binding while the turkey showed equal binding between the three viruses. The quail trachea showed low binding with A/Dk and A/Tk, and no binding of the human A/Mem virus (Figure 3.7J, M, and 3.7P). The guinea fowl, on the other hand showed low levels of binding with A/Mem but no binding with A/Dk or A/Tk (Figure 3.7A, D and 3.7G).



<u>Figure 3.5.</u> Viruses binding to tissues correlates to sialic acid distribution in domestic ducks and geese. Sections from 4 week old Peking duck (A-I) and Toulouse goose (J-R) tissues were exposed to A/DK/HK/7/75 (A-C, J-L), A/TK/OH/313053/04 (D-F, M-O), or A/Memphis/31/98 (G-I, P-R). Virus presence (green) was detected by αNP monoclonal antibodies and FITC linked α-mouse antibodies. Cells nuclei were stained with DAPI (blue).



<u>Figure 3.6.</u> Viruses binding to tissues correlates to sialic acid distribution in domestic turkeys and pheasant. Sections from 4 week old white midget turkey (A-I) and Chinese ringneck pheasants (J-R) tissues were exposed to A/DK/HK/7/75 (A-C, J-L), A/TK/OH/313053/04 (D-F, M-O), or A/Memphis/31/98 (G-I, P-R). Virus presence (green) was detected by αNP monoclonal antibodies and FITC linked α-mouse antibodies. Cells nuclei were stained with DAPI (blue).



<u>Figure 3.7.</u> Viruses binding to tissues correlates to sialic acid distribution in domestic quail and guinea fowl. Sections from 4 week old bobwhite quail (A-I) and pearl guinea fowl (J-R) tissues were exposed to A/DK/HK/7/75 (A-C, J-L), A/TK/OH/313053/04 (D-F, M-O), or A/Memphis/31/98 (G-I, P-R).
Virus presence (green) was detected by αNP monoclonal antibodies and FITC linked α-mouse antibodies. Cells nuclei were stained with DAPI (blue).

To visualize the virus binding in the lungs, transversal sections of the parabronchi were imaged to minimize variations from section to section and from species to species. Whenever virus was seen in these sections, it was seen binding to the smooth atrial muscles lining the parabronchi regardless of bird species or virus. The lungs of ducks showed moderate binding of A/Tk and A/Mem but no binding to A/Dk (Figure 3.5B, E and 3.5H). The goose lung however showed binding with A/Dk (Figure 3.5K) but no binding with the other two viruses (Figure 3.5N and 3.5Q). Pheasants showed no binding of any virus in the parabronchi (Figure 3.6B, E and 3.6H). Turkey showed low to moderate binding of A/Dk and A/Tk but no binding of A/Mem (Figure 3.6K, N and 3.6Q) while the guinea fowl had A/Dk binding but neither of the other two viruses (Figure 3.7B, E and 3.7H). Finally the quail were the only species to show binding of all three viruses in the lungs (Figure 3.7K, N and 3.7Q).

Despite the fact that all birds expressed α2,3 SA in the intestines, only the ducks and the geese showed any ability to bind A/Dk in the intestines. The four land based poultry species showed no binding despite showing expression of α2,3SA. The duck, goose and pheasant intestines also showed minor α2,6 SA expression. However, only A/Tk was able to bind and only in the intestines of the geese (Figure 3.5L). These results highlight the complexities associated with understanding the host range of influenza viruses. Although many studies, including ours, have looked at the expression of SA receptors in tissues of several animal species, these receptors are not necessarily capable of binding influenza viruses (at least not under the conditions tested in this report). More studies are needed to better ascertain to which extent different animal species are likely hosts of influenza

viruses and which minimal changes in receptor binding are needed to establish productive infections in these hosts.

Chapter 4: Compatibility of H9N2 and pH1N1 for reassortment and aerosol transmission in ferrets

Published: Kimble B, Sorrell E, Shao H, Martin P, Perez D. Compatibility of H9N2 avian influenza surface genes and 2009 pandemic H1N1 internal genes for transmission in the ferret model. Proc Natl Acad Sci U S A. 2011 Jul 5.[2]

4.1 Abstract

In 2009, a novel H1N1 influenza (pH1N1) virus caused the first influenza pandemic in 40 years. The virus was identified as a triple reassortant between avian, swine, and human influenza viruses, highlighting the importance of reassortment in the generation of viruses with pandemic potential. Previously, it has been shown that a reassortant virus composed of wild-type avian H9N2 surface genes in a seasonal human H3N2 backbone could gain efficient respiratory droplet transmission in the ferret model. Here, ability of the H9N2 surface genes in the context of the internal genes of a pH1N1 virus were determined to efficiently transmit via respiratory droplets in ferrets. Reassorted viruses carrying the HA gene alone or in combination with the NA gene of a prototypical H9N2 virus in the background of a pH1N1 virus were generated. Four reassortant viruses were generated, with three of them showing efficient respiratory droplet transmission. Differences in replication efficiency were observed for these viruses; however, the results clearly indicate that H9N2 avian influenza viruses and

---

[2] Author contributions: J.B.K., E.S., and D.R.P. designed research; J.B.K., E.S., H.S., and P.L.M. performed research; H.S. contributed new reagents/analytic tools; J.B.K., E.S., H.S., P.L.M., and D.R.P. analyzed data; and J.B.K., E.S., and D.R.P. wrote the paper.

pH1N1 viruses, both of which have occasionally infected pigs, have the potential to reassort and generate novel viruses with respiratory transmission potential in mammals.

4.2 Introduction

According to the World Health Organization, H9, together with H5 and H7 subtypes, are among the leading candidates for future influenza pandemics. H9N2 has been endemic in poultry populations across Eurasia since the mid 1990s (Alexander, 2000; Lee et al., 2000; Naeem et al., 1999; Perk et al., 2006). Avian H9 viruses display a typical avian host range; however, many recent isolates contain a leucine (L)—instead of glutamine (Q)—at position 226 in the receptor binding site of the HA protein, which facilitates preferential binding to sialic acid receptors in an α2,6 conformation (SAα2,6), typical of human influenza viruses. L226-containing H9N2 viruses show efficient replication in human airway epithelial cells and in the ferret model (Matrosovich et al., 2001; Wan and Perez, 2007; Wan et al., 2008). Since the late 1990s, 12 cases of human H9N2 infection have been identified. In addition, H9N2 viruses have been isolated sporadically but consistently from pigs in Hong Kong special administrative region, China, and South Korea (Maines et al., 2008; Peiris et al., 2001; Peiris et al., 1999). Clinically, human H9N2 infections present as typical seasonal influenza infections, potentially allowing cases to go unreported and increasing the opportunities for the virus to transmit and reassort. Seroepidemiological studies indicate that human infections are more prevalent than reported (Butt KM, 2005; Guo Y et al., 1999; Jia et al., 2008; Smith et al., 2009b). Experimentally, H9N2 surface genes reassorted with seasonal H3N2 internal genes have shown efficient transmission in a ferret model only after adaptation

by serial passage and incorporation of amino acid changes on the surface and internal genes (Sorrell et al., 2009).

In Mexico in 2009, an H1N1 virus emerged from swine into the human population, quickly spread throughout the globe, and became the first pandemic virus in more than 40 y. This novel pandemic H1N1 (pH1N1) originated from a reassortment event involving human, swine, and avian influenza viruses. Recent studies indicate that this pH1N1 is present in swine populations and continues to reassort with other swine influenza viruses (Vijaykrishna et al., 2010). The original swine triple reassortant virus, as well as the ensuing reassortant swine and the pH1N1, all contain the same internal gene cassette (Ma W, 2008; Vincent AL, 2008). This triple reassortant internal gene (TRIG) cassette consists of the PB2 and PA from a North American avian virus, the PB1 from a human H3N2 seasonal virus, and the NP and NS from the classical swine H1N1. Many of the currently circulating swine viruses contain the TRIG cassette with a myriad of different surface genes. This finding has lead to the hypothesis that the TRIG cassette is a stable collection of internal genes that allows for easy surface switching (Ma W, 2008). The continued presence of pH1N1 in swine, the propensity of pH1N1 to reassort with other influenza viruses, the occasional isolation of H9N2 viruses in swine and human populations, and the "humanization" of the receptor binding preference of H9 HA protein all underscore the real threat of a novel H9N2:pH1N1 reassortant with significant threat to the human population.

A novel pandemic virus must be antigenically distinct from currently circulating influenza viruses to have a naive population through which to spread (Smith et al., 2009a). An H9N2:pH1N1 reassortant virus with a H9 surface protein on a pH1N1

backbone would be antigenically novel. The isolation of both subtypes of influenza from swine and humans makes it feasible that a natural reassortant between the two could occur. The ability of four H9N2:pH1N1 reassortant viruses were able to infect and transmit in ferrets, an animal model that resembles human influenza infection and transmission It was found that these reassortant viruses can transmit by respiratory droplet transmission in ferrets, highlighting their pandemic potential.

4.3 Materials and Methods

4.3.1 Viruses and Cells.

The reverse genetic systems for WF10 (H9N2), P10 (H9N2), and pH1N1 viruses have been previously described (Hoffmann et al., 2000.). The plasmid set for pH1N1 was kindly provided by Ron A. Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands. The viruses were generated by reverse genetics as previously reported (Hoffmann et al., 2000.). Virus stocks were produced in MDCK cells. Full-length sequencing of viral stocks was performed to verify gene combinations and later for mutation analysis of respiratory droplet contact samples. Sequences were generated using the Big Dye Terminator v3.1 Cycle Sequencing kit 1 in a 3500 Genetic Analyzer (Applied Biosystems).

Four reassortant viruses were generated: the 1WF10 virus encodes the HA from WF10 and the remaining seven genes from pH1N1; the 2WF10 virus contains the HA and NA from WF10 and the remaining six genes of pH1N1; the 1P10 virus contains the HA from (P10) and the other seven genes from pH1N1, whereas the 2P10 contains the HA and NA

genes from P10 and the remaining six genes from pH1N1. The median $TCID_{50}$ of each virus as well as titers for the growth curve experiments was determined in MDCK cells.

4.3.2 Plaque Assays.

Briefly, confluent MDCK cell monolayers in six-well plates were infected with 10-fold dilutions of virus for 1 h at 37 °C. Cells were washed twice with PBS and covered with an overlay of modified Eagle's medium containing 0.9% agar, 0.02% BSA, 1% glutamine, and 1 μg/mL trypsin. After 3 d of incubation at 37 °C, 5% CO_2, the overlays were removed and the cells were stained with Crystal violet.

4.3.3 Infection and Transmission in Ferrets.

Infection and transmission were carried out as described previously (Wan et al., 2008). Briefly, 3- to 7-mo-old ferrets were purchased from Triple F Farms. Ferrets were housed in BSL2 animal rooms for 1 week to monitor general health and establish baseline body temperature and weight. Before infection, ferrets were bled for serum collection and had an implantable transponder (Bio Medic Data Systems Inc.) placed subcutaneously for body temperature recording and identification. Transmission studies were performed in an $ABSL3^{+}$ facility in wire cages inside HEPA-filtered isolators (Wan et al., 2008). Animal studies were approved by the Animal Care and Use Committee of the University of Maryland (protocol RO-09-93). Each experiment consisted of three ferrets in duplicate for each virus. One ferret was inoculated intranasally with 10^{6} tissue culture infectious dose (TCID50) of virus in phosphate buffered solution (PBS) after light anesthesia with ketamine (20 mg/kg) and xylazine (1 mg/kg) administered intramuscularly. At 1 d post-inoculation (dpi), two naive ferrets were added to the cage. One ferret was added in direct contact with the inoculated ferret, but the second naïve ferret was added to the other half

of the cage separated by two layers of thin wire mesh, allowing no physical contact. Body weight and temperature were measured daily and nasal washes were collected for 14 dpi (except where noted). Nasal washes were collected by anesthetizing the ferrets as described above and inducing sneezing with 1 mL of PBS collected in a Petri dish. Washes were collected and brought up to 1-mL volume and tested for virus by FluDetect (Synbiotics Corp.), allocated, and stored at −80 °C until use. Seroconversion was detected at 14 dpi. Two additional ferrets were infected with each virus for pathology and virus localization at 3 and 5 dpi.

4.3.4 Virus localization

Virus was located and visualized in ferret tissue by standard immunohistochemistry methods. Briefly, slides were deparaffinized by two 3-min xylene washes followed by 2 min in ethanol at descending concentrations, from 100%, 90%, 80%, to 70% and rinsed in phosphate buffed solution (PBS Sigma). Endogenous peroxidases were blocked by 15-min incubation in 2% H_2O_2 in methanol at 4 °C. Tissues were blocked overnight at 4 °C with 1% BSA in PBS. The following day, tissues were immersed in anti-NP monoclonal antibodies that were diluted 1:500 in PBS for 1 h at room temperature. Following three 5-min rinses in PBS, tissues were incubated with anti-mouse antibody diluted 1:1,000 in PBS. Three more 5-min PBS rinses ensued and then tissues were covered with aminoethylcarbazole (DAKO) for 10 min and rinsed in distilled water. After a 30-min incubation in hematoxylin and rinse in tap water, cover slips were mounted and tissue was visualized at 200×magnification. Three slides were analyzed per ferret, per tissue.

4.3.5 Blocking ELISA

In the blocking ELISA, A/Guinea fowl/Hongkong/WF10 (H9N2) (WF10) virus was used as a coating antigen and HRP-conjugated monoclonal antibody against AIV-H9-HA-3G8 was selected the as detection antibody. In brief, 96-well plates were coated in WF10 diluted in carbonate/bicarbonate buffer (pH 9.6) for 12 h at 4 °C. After blocking, the plates with 5% (wt/vol) nonfat milk in PBS for 1 h at 37 °C, the serum samples were diluted 1:4 in dilution buffer (0.5% BSA in PBS) and added to the wells (100μL per well) and the mixture was incubated at 37 °C for 1 h. After washing once, 100μL HRP-conjugated 3G8 antibodies (0.1 ng/mL) in dilution buffer was added to well and the mixture was incubated for 1 h at 37 °C. After five washes, the development was performed using the TMB substrate system (KPL) for 10 min. The ratio of the OD630 value of the sample wells (S) to that of the negative control wells (N) was calculated, and S/N values less than 0.5 or 0.6 were considered as positive in the ELISA. Serum collected from each ferret at the end of the experiments was tested for seroconversion by hemagglutination inhibition assay and by a blocking ELISA. Every ferret that had detectable levels of shed virus proved positive by both hemagglutination inhibition and the blocking ELISA, with the exception of the RC2WF10 ferret with delayed infection. This ferret was killed only 6 d after first shedding virus and thus did not seroconvert. Additionally, the two RC1WF10 ferrets that never shed virus, never seroconverted.

4.4 Results

4.4.1 Generation of H9N2 and H9N1 Influenza Viruses with Internal Genes from pH1N1.

All four viruses were made with the pH1N1 internal genes (PB2, PB1, PA, NP, M, and NS) from A/Netherlands/602/2009 (H1N1) (Munster VJ, 2009). The surface genes came from either A/guinea fowl/Hong Kong/WF10/1999 (H9N2) or from A/ferret/Maryland/P10_UMD/2008 (H9N2) herein referred to as WF10 and P10, respectively (Perez et al., 2003; Sorrell et al., 2009). WF10 has a typical avian host range but has been shown to replicate efficiently in ferrets although transmission among ferrets occurs only with animals in direct contact (Sorrell et al., 2009; Wan et al., 2008). The P10 virus is the result of 10 serial passages in ferrets of an avian-human H9N2:H3N2 reassortant containing the WF10 surface on a seasonal H3N2 (A/Memphis/14/1998) backbone (Sorrell et al., 2009). The P10 virus has two mutations in the HA (T189A in HA1 and G192R in HA2) and one in the NA (I28V) compared with the WF10. These amino acid changes were shown to be crucial for efficient and reproducible respiratory droplet transmission in ferrets (Sorrell et al., 2009). The four viruses generated in this report had P10 HA and NA (2P10), P10 HA and pH1N1 NA (1P10), WF10 HA and NA, (2WF10) or WF10 HA and pH1N1 NA (1WF10) (Fig. 4.1*A*).

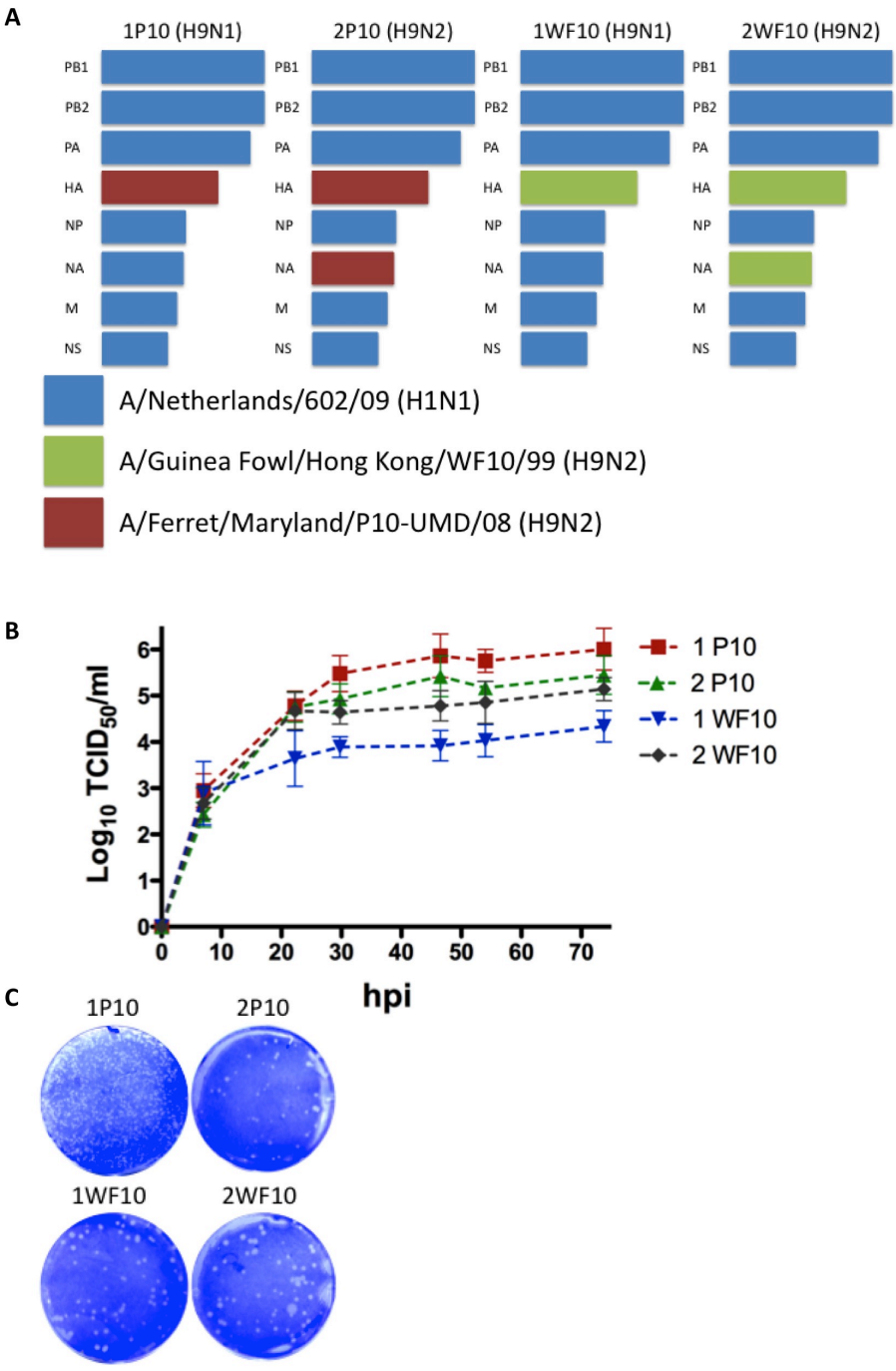


Figure 4.1. Characteristics of H9N2:pH1N1 viruses in vitro. (*A*) Genes colored black come from pdmH1N1. Red genes originate from P10 (H9N2). Green genes are from WF10 (H9N2). (*B*) Replication of four reassortant viruses in MDCK cells. Six-well plates of confluent MDCK cells were inoculated with 0.1 multiplicity of infection of either 1P10 (red), 2P10 (green), 1WF10 (blue), or 2WF10 (black). Supernatants were harvested twice a day for 3 d and titered. Mean titer and SD were calculated. (*C*) Plaque morphology in MDCK cells for 1P10, 2P10, 1WF10, and 2WF10. Cells were infected with 1×10^{-6} dilution of stock virus except for the 1P10, in which a 1×10^{-5} dilution was used because of smaller plaque size.

Viruses grew to similar titers in Madin-Darby canine-kidney (MDCK) cells, from $10^{6.5}$ to $10^{7.2}$. However, 1WF10 showed slower growth indicated by lower titers at 24 hours post infection (Fig. 4.1*B*). Wan et al. (Wan et al., 2008) had previously compared the plaque morphology of wild-type WF10 to the seasonal H3N2 and the predecessor of the P10 virus, 2WF10:6M98. Seasonal H3N2 showed large plaques but WF10 formed pinpoint plaques. The 2WF10:6M98 and P10 plaques were of an intermediate size (Sorrell et al., 2009). The 1P10 showed pinpoint plaques similar to wild-type WF10 (Fig. 4.1*C*); 2P10, 1WF10, and 2WF10 formed plaques of intermediate size that were similar to the original 2WF10:6M98 or P10 viruses (Fig. 4.1*C*).

4.4.2 Infection, Pathology, Signs of Disease, and Transmission.

The four viruses established an infection in ferrets inoculated with 10^6 tissue culture infectious dose ($TCID_{50}$) per ferret. Histopathological examination was performed on three H&E-stained sections of trachea and lung collected from directly inoculated ferrets at 3 and 5 days post inoculation (dpi) (Fig. 4.2). Virus infection in ferrets produced acute to subacute (5 dpi) tracheitis and bronchointerstitial pneumonia that was more severe at 5 dpi. The airways (trachea, bronchi, bronchioles) were minimally to mildly affected. It was in the alveolar spaces/interstitium where the lesions were most prominent. Acute to subacute bronchointerstitial pneumonia was characterized by expansion of the alveolar septae with fibrin, edema, and a mixed inflammatory infiltrate comprised of lymphocytes, plasma cells, and macrophages with fewer neutrophils. The lesions were moderate-to-severe in 1P10 and 2P10 (Fig. 4.2), with occasional foci of alveolar septal necrosis, multifocal hemorrhage, and alveolar hyaline membranes. In the

1P10 ferrets the inflammation was more severe, although there was less hemorrhage. The bronchointerstitial pneumonia was mild-to-moderate in 1WF10 and 2WF10 ferrets. Tracheal lesions consisted of minimal-to-mild acute inflammation characterized by submucosal edema with sparse inflammatory infiltrates. In 2WF10 ferrets the lesions were slightly more advanced as there was mild multifocal subacute tracheitis characterized by mild-to-moderate submucosal edema, with multifocal intraepithelial lymphoplasmacytic infliltrates with fewer neutrophils and macrophages. There was occasional individual epithelial cell necrosis, occasional cilia loss, and endothelial cell hypertrophy in the submucosal blood vessels.

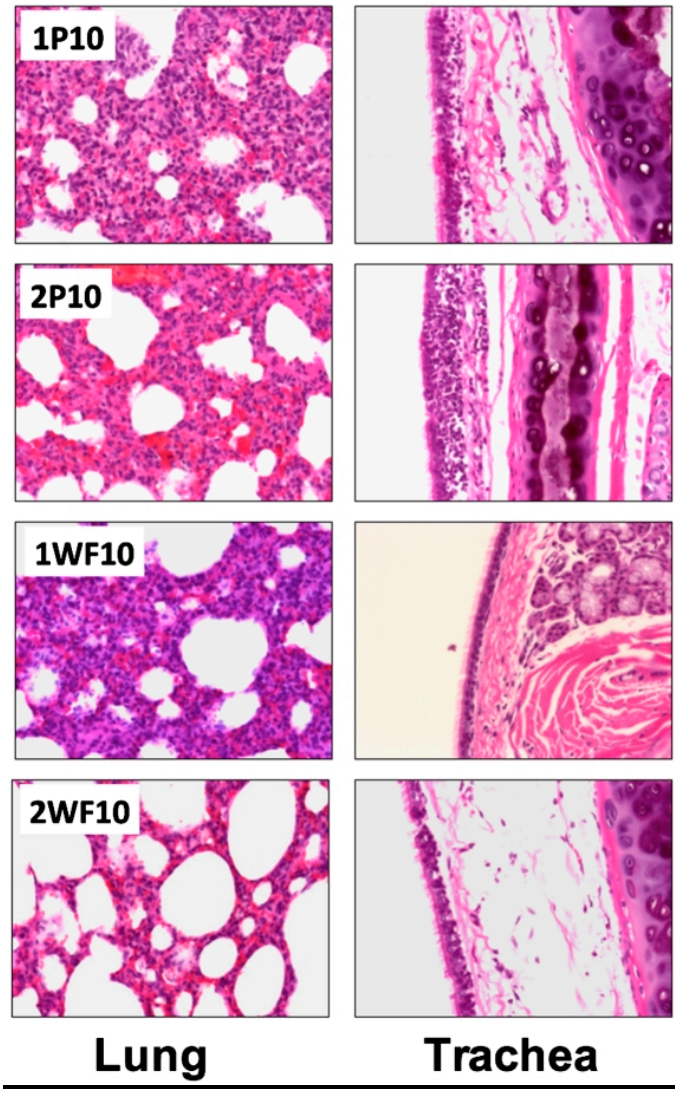


Figure 4.2. Pathology produced by H9N2:pH1N1 viruses. Ferrets were inoculated with 1×10^6 $TCID_{50}$ of either 1P10, 2P10, 1WF10, or 2WF10 and tissues were collected at 5 dpi. Samples were cut into 5-µm thick sections and stained using a standard H&E protocol by Histoserv Inc. (Magnification: 200×.)

NP viral antigen was localized by immunohistochemistry in lung and tracheal samples from 5 dpi. NP was clearly seen in the alveolar septae in regions characterized by interstitial pneumonia (Fig. 4.3). Areas of the lungs that did not show alveolar expansion, particularly in regions of the 2WF10-infected lung, did not show positive NP reaction. The airways, most notably the trachea (Fig. 4.3), showed far less NP production. Typically, virus in the trachea was mostly localized to the subepithelial mucosal layer, with infrequent localization to the epithelial layer. Virus was not located in the trachea of the WF10-infected ferrets and is consistent with the minimal pathology seen.

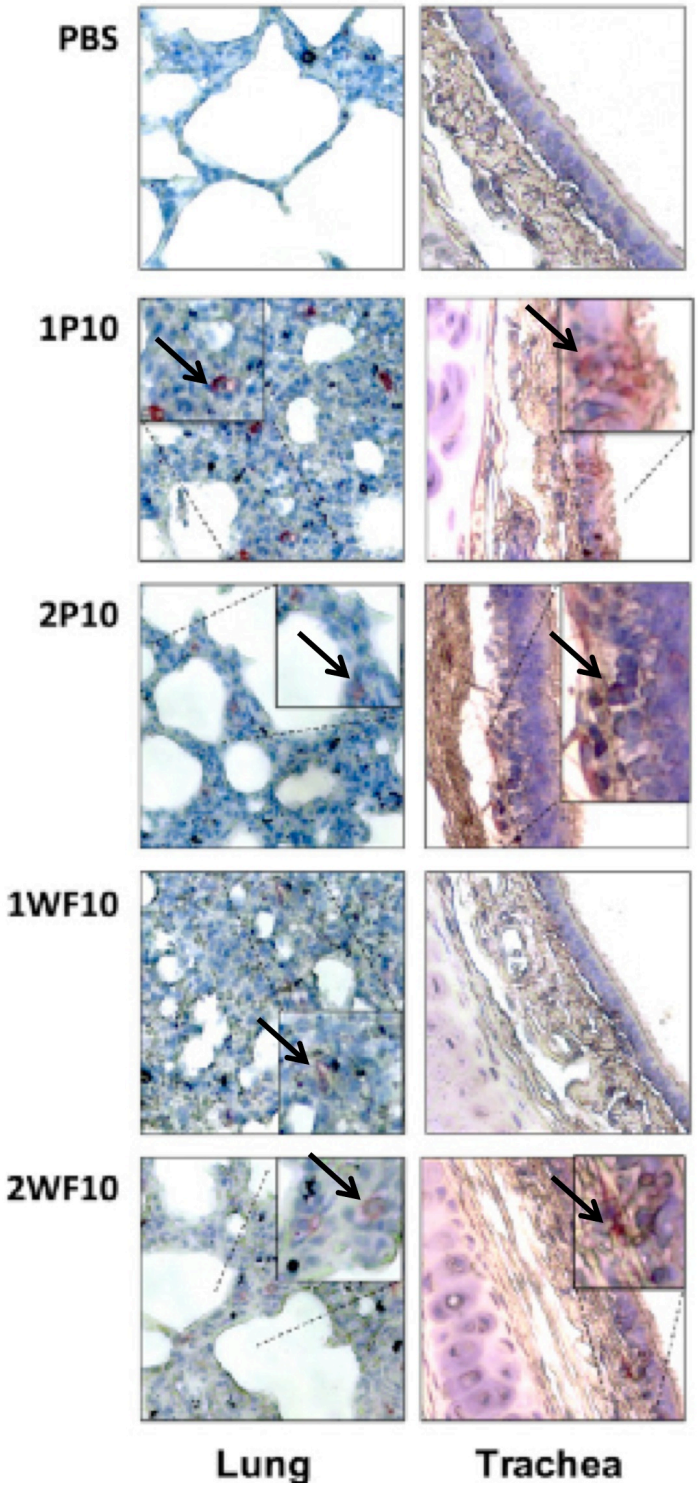


Figure 4.3.Virus localization in infected tissue. Unstained tissue from ferrets after 5 dpi with 1×106 TCID50 of either 1P10, 2P10, 1WF10, or 2WF10 underwent anti-NP based immunohistochemistry, as described in Materials and Methods. Blue/purple represents nuclear staining. Red marks area positive for NP protein. Insets show selected areas with higher magnification for visualization purposes. (Magnification: 200×)

The four viruses grew to similar titers in inoculated (DI) ferrets and were cleared in a similar time frame (Fig. 4.4, red lines). Additionally, all four viruses were able to transmit to direct contact (DC) ferrets (Fig. 4.4, blue lines), which shed virus and cleared the infection in a similar fashion. Differences were seen, however, in the respiratory contact (RC) ferrets (Fig. 4.4 green lines). Only three viruses were able to transmit in RC ferrets. Differences were apparent in the efficiency of respiratory droplet transmission. The 1P10 transmitted the fastest (4–6 dpi) and grew to the highest titers of any of the RC groups (Fig 4.4A). The 2P10 transmitted slower (6–8 dpi) and grew to slightly lower titers (Fig. 4.4*B*). The 1WF10 failed to transmit to ferrets via respiratory droplets (Fig. 4.4*C*). Finally, the 2WF10 showed a more diverse transmission profile (Fig. 4.4*D*). The first RC ferret became positive for infection 6 d before the other RC, but the virus grew to a lower titer. Ferrets lost weight and had at least 1 d of observed fever (Table 4.1), with the exception being the two RC ferrets for the 1WF10 virus that did not become infected. Additional signs of disease observed included increase in sneezing, mild depression, and mild diarrhea. Despite the small number of animals used, our studies clearly show significant compatibility between the HA of H9 influenza viruses and the pH1N1 backbone to produce pathology and transmission in the ferret model.

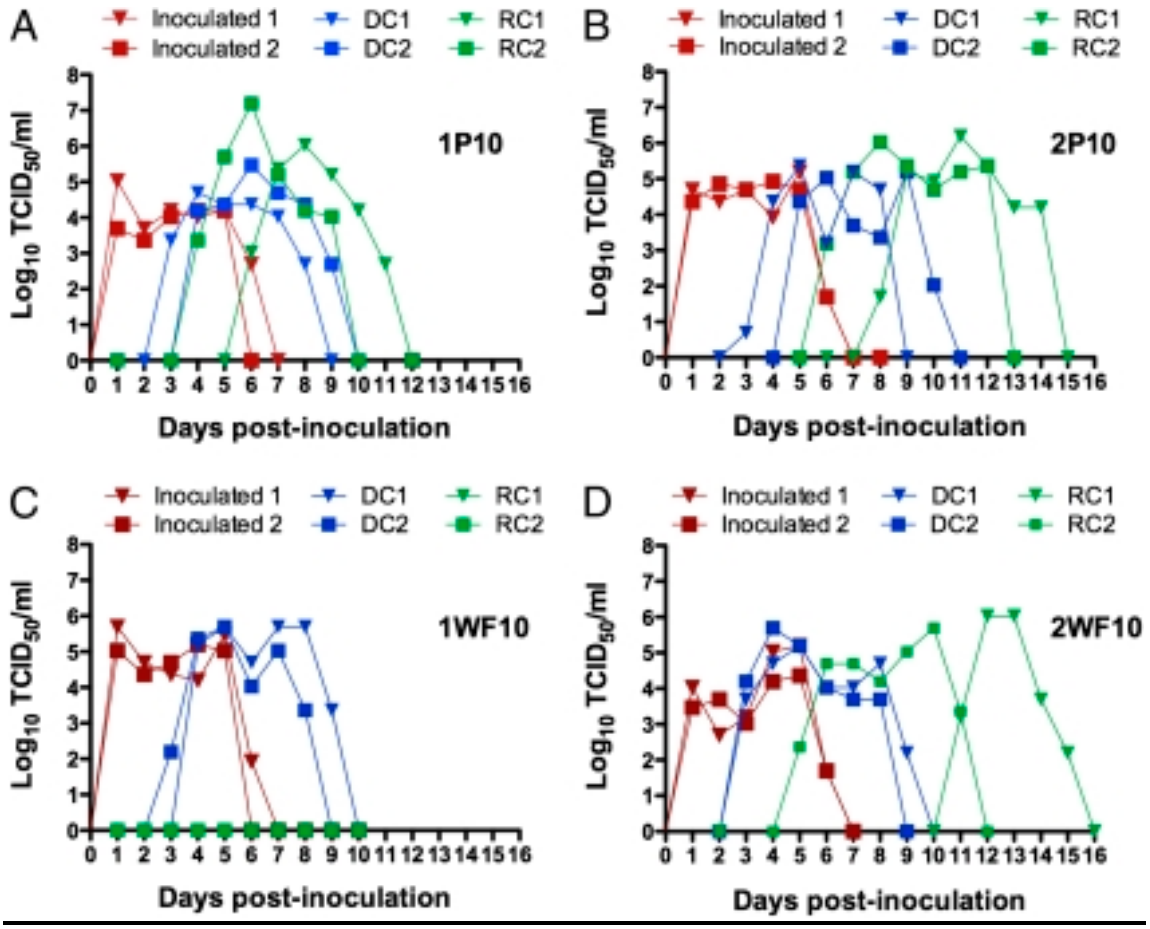


Figure 4.4. Nasal wash titers from DI, DC, and RC ferrets. Ferrets (red lines) were infected with 1×10^6 $TCID_{50}$ of 1P10 (*A*), 2P10 (*B*), 1WF10 (*C*), or 2WF10 (*D*). At 1 dpi, one inoculated ferret was moved to a clean isolator with a naive ferret in direct contact (DC, blue lines). Additionally, another naive ferret was placed in the same isolator in a manner such that no direct contact was possible, only respiratory droplet contact (RC, green lines). Nasal washes were collected daily and titrated in MDCK cells. Each virus was tested in duplicate.

Virus	Group	Body weight loss
1P10	DI	5.976 ± 0.592
	DC	5.877 ± 2.654
	RC	6.041 ± 1.652
2P10	DI	8.279 ± 0.902
	DC	2.947 ± 0.336
	RC	6.497 ± 2.523
1WF10	DI	3.708 ± 0.479
	DC	6.34 ± 6.788
	RC	
2WF10	DI	4.298 ± 2.388
	DC	5.406 ± 0.856
	RC	4.647 ± 2.796

Virus	Serum (HI titer)	Blocking ELISA
1P10	2,560, 2,560	+, +
	640, 640	+, +
	2,560, 1,280	+, +
2P10	2,560, 2,560	+, +
	1,280, 640	+, +
	320, 320	+, +
1WF10	1,280, 1,280	+, +
	2,560, 1,280	+, +
	<10	−, −
2WF10	1,280, 1,280	+, +
	640, 320	+, +
	<10, 1,280	−, +

Table 4.1. Ferrets showed moderate weight loss following infection with reassortant H9:pH1N1 viruses. Direct inoculated (DI), direct contact (DC), and respiratory contact (RC) ferrets peak percent body weight loss during infection. The "–" in the Body weight loss column indicates no weight loss observed. HI, hemagglutination inhibition. In the Blocking ELISA column, "+" indicates positive and "–" indicates negative for anti H9 HA antibodies

4.4.3 Molecular Changes Associated with Transmission.

Nasal washes from RC ferrets on the day of peak shedding were passed once in MDCK cells and then sequenced to determine if genetic changes had arisen that could account for differences seen in titer and transmission between and within the infected groups. The virus recovered from 1P10 was found to have accrued no mutations in the ferrets. The 2P10 showed two nonsynonymous mutations that were identical in the two RC ferrets. The alanine residue at position 30 of the NA protein was mutated to a threonine (A30T) and the threonine residue at position 58 of PB2 was altered to an isoleucine (T58I). The 2WF10 also showed two nonsynonymous mutations. Serine at position 261 of the PB1 was changed to an asparagine (S261N) and was seen in both RC ferrets. Finally, valine at position 104 of the HA protein was switched to alanine (V104A). The V104A mutation was only seen in the 2WF10 RC ferret with significantly reduced speed of transmission (Fig. 4.4*D*, green line with triangle).

To confirm that these changes had occurred during transmission in ferrets and not in tissue culture cells, sequencing at the site of the mutations was performed on viral RNA samples obtained directly from nasal washes from every positive sample (Fig. 4.5). The NA A30T mutation in the RC2P10 group was found in both ferrets on the first day virus was shed. There was a reversion to wild-type (A30) on the final 2 d of shedding for one RC ferret, but the other consistently shed virus with the mutation (T30) on all days. The mutation was never detected in the DI or DC ferrets. The PB2 T58I mutation of the RC2P10 group arose post-transmission for both RC ferrets. Likewise, the PB1 S261N mutation in the RC2WF10 ferrets appeared days after transmission. The HA V104A

mutation seen in only one RC2WF10 ferret occurred in the DC2WF10 ferret associated with it on the last day of shedding (9 dpi). The RC2WF10 ferret began shedding virus with the A104 11 dpi, but on 13 dpi shed a mixed population and fully reverted to wild-type (V104) for the final 2 d of shedding.

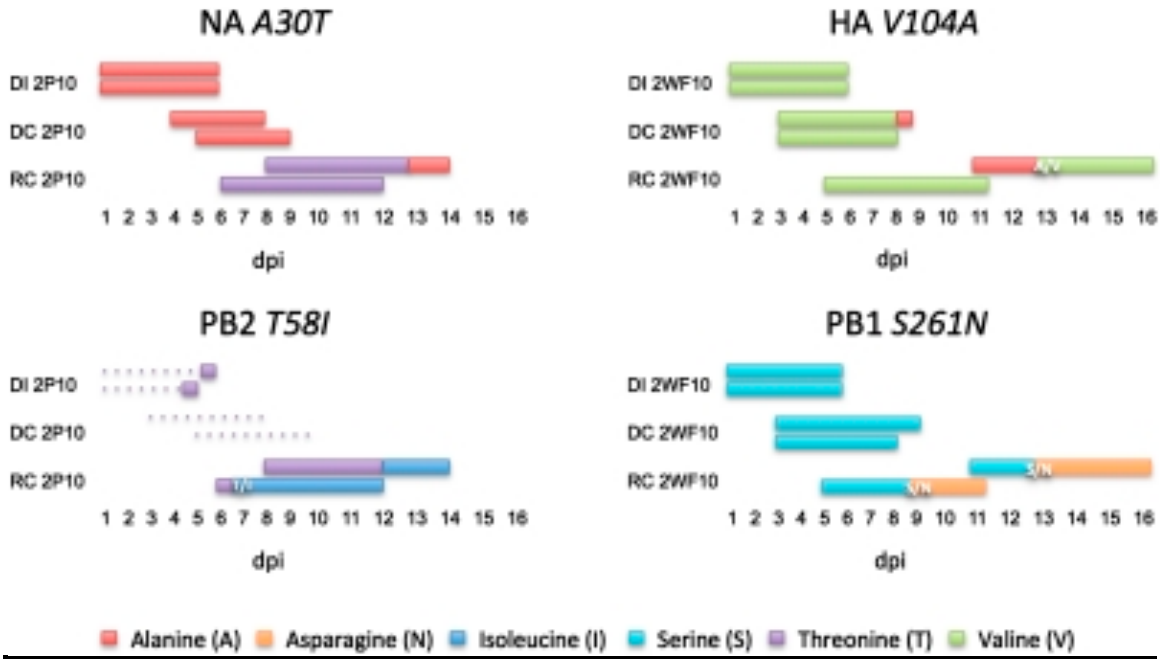


Figure 4.5. Amino acid mutations during transmission of H9N2:pH1N1 viruses. Sequences from virus-positive nasal washes were generated for every ferret at the site in which a mutation was observed during peak shedding compared with the wild-type virus. Solid lines indicate days in which sequences were performed. Dotted lines indicated days in which viruses were detected but no sequences were generated (PB2 T58I DI and DC 2P10). Amino acids are indicated in color. A/V, T/I, and S/N indicate mixed virus population on the date shown.

4.5 Discussion

In this study, the compatibility between the HA of an H9 subtype influenza virus and the rest of genes from a prototypical pH1N1 was evaluated. The results indicate that the pH1N1 and a wild-type avian H9N2 are compatible for reassortment and capable of creating viruses with the ability to infect and transmit via respiratory contact in ferrets. Previous results indicate that an H9N2:H3N2 (2WF10:6M98) reassortant could infect and transmit to DC ferrets. However, transmission to RC ferrets only occurs after serial passage and adaption in ferrets (P10) (Sorrell et al., 2009). Interestingly, it was shown that the wild-type WF10 surface genes in the background of the pH1N1 virus can infect and transmit to RC ferrets with no need of adaption, which is consistent with our previous observations on the efficient transmission of pH1N1 viruses in the ferret model. Nevertheless, adapting the H9N2 surface genes to ferrets results in viruses with more efficient transmission profiles, similar upper respiratory tract pathology, and increased lung pathology. These results are significant because they show that the adaptive changes identified on the surface of the P10 virus do provide a transmission advantage in more than one virus background.

It is also notable to compare the 1WF10 and the 2WF10 viruses. These viruses differ only in the origin of their NA segment. The 1WF10 (pH1N1 NA) shows reduced titer (Fig. 4.1) compared with the 2WF10 (H9N2 NA). Additionally, the mismatched surface proteins of the 1WF10 caused a phenotypic change in respiratory transmission (Fig. 4.4). This finding indicates that the 2WF10 virus with surface genes of shared origin

is more fit than the 1WF10 with surface genes of mixed origin. However, and perhaps more importantly, because the 1P10 (H9N1) virus showed the fastest transmission profile of all four viruses, it also suggests that just two amino acid changes (P10 mutations T189A in HA1 and G192R in HA2 compared with WF10), provide the necessary balance between HA and NA activities to improve viral fitness and transmission. This phenomenon has been seen in pandemic viruses before. The 1957 H2N2 pandemic arose from a reassortment in which both surface genes were replaced with an avian set of surface genes; however, the H3 HA of avian origin was perfectly compatible with the N2 NA from the human strain in the 1968 H3N2 pandemic (Scholtissek et al., 1978; Wagner et al., 2002).

These results also support the idea that the TRIG cassette confers an infection/transmission advantage to a virus regardless of surface genes. The H9N2 with seasonal H3N2 internal genes could not transmit to respiratory contacts. However, the same surface genes with the TRIG cassette transmitted via respiratory contact. These results are consistent with the fact that, after 80 years as the dominant subtype in swine population in North America, the classic swine H1N1 was replaced in a few years by an assortment of TRIG viruses.

Pathologically, these viruses are similar to the seasonal strains of human influenza. The infected lungs show mild-to-moderate bronchointerstitial pneumonia similar to what is seen with seasonal H3N2 (Memoli MJ, 2009), seasonal H1N1, and pH1N1 (Munster VJ, 2009; Rowe T, 2010) infections in ferrets. Tracheal pathology was again similar to pathology seen in typical human virus infections (Memoli MJ, 2009; Munster VJ, 2009; Rowe T, 2010). Recently, Sun et al. determined the growth

characteristics and pathology associated with 127 H9 containing H9N2:pH1N1 reassortants in mice (Sun Y, 2011). Although ~25% of these reassortants showed similar pathology to either parental virus, about 10% showed pathogenicity higher than either virus. These results and ours highlight the potential for a H9:pH1N1 reassortant virus with increased pathogenicity and respiratory transmissibility.

Although amino acid changes were identified in RC contact ferrets for some of these viruses, it is unlikely that these changes were determinants for transmission because they were not found in nasal washes from DI or DC ferrets. It is not known if these four artificially created reassortants would be created during a co-infection in the field. It is unknown if these reassortants would be the most fit or if some other combination would be more suitable. It needs to be determined if these or some other reassortant virus would out-compete either parental virus in a naive host and what effects preexisting immunity would have. These experiments were performed in ferrets, a model of human infection, but other intermediate species, such as swine or poultry, might act as better hosts for reassortment. Our results highlight the need to develop better H9N2 surveillance in both swine and humans as these viruses continue to show features consistent with pandemic potential.

Chapter 5: Transmission and pathogenicity of H9N1 reassortants in swine.

Pending publication: J. Brian Kimble, Daniela RaJao, Kelly Lager, Amy Vincent, Daniel R. Perez[3]

5.1 Abstract

Swine possess both avian- and human-like influenza receptors in their respiratory tracts. This leaves them susceptible to both human and avian influenza. This makes the pig an ideal environment for reassortment between avian and human influenza viruses and for the generation of potential human pandemic viruses. H9N2 influenza virus has human-like receptor binding, has infected humans in the past, more frequently infects swine, and can undergo reassortment to create mammalian transmissible viruses. These facts together create a framework for the possible generation of a human transmissible H9 virus with pandemic potential. Previously it was reported that H9N2:pH1N1 reassortant viruses that could efficiently transmit in ferrets. As swine are a reported mixing bowl that have served as the environment for the generation of a previous pandemic, it is vital to determine the behavior of these H9N2:pH1N1 reassortants in pigs. The mammalian adapted H9N2 surfaces genes on a pH1N1 genetic backbone were used to create an H9N1 and an H9N2 reassortants. It was found that both viruses transmit to direct contact pigs and show increased pathogenicity compared to a fully H9N2 virus. These findings show the potential danger of the co-circulation of H9N2 and pH1N1 in swine.

---

[3] Author contributions: JBK, AV, KL and DRP designed research. JBK, AV, KL, and DR performed research; JBK, AV, and DRP analyzed data; and JBK and DRP wrote the paper.

5.1 Introduction

H9N2 avian influenza was first isolated in 1966 from a turkey in Wisconsin (Homme et al., 1970). Since then, the subtype has been isolated across North America, Eurasia, and northern Africa. Dogmatically, the Eurasian lineage has been broken down into three sublineages: G1-like, Y280-like, and Korean-like (Guo et al., 2000). However, recent phylogenetic studies suggest further sub lineage divides (Dong et al., 2011; Fusaro et al., 2011). All isolated H9N2 viruses have been categorized as low pathogenic viruses; however, outbreaks have been associated with high morbidity and mortality in poultry in the past (Brown et al., 2006).

The first human isolates were recovered in 1999 in Hong Kong from two patients reporting mild respiratory symptoms (Peiris et al., 1999). Studies showed that these isolates were genetically and antigenically of the G1-like sublineage. All of the subsequent sporadic human isolations have been from the G1-like or Y280-like sublineages (Butt KM, 2005; Cheng et al., 2011). These relatively few human infections appear to be the result of direct zoonosis from birds and no human to human transmission has been recorded (Uyeki et al., 2002). Swine have also played host to H9N2 influenza. In 1998 the first swine H9N2 was isolated in Hong Kong and proved to be of the Y280-like sublineage. Swine infections are more common than human infection, are associated with all multiple sublineages, and can be associated with high morbidity and mortality (Cong et al., 2007; Rui-Hua et al., 2011; Xu et al., 2008).

In the four most thoroughly studied human influenza pandemics, 1918, 1957, 1968, and 2009, reassortment, zoonosis, or both played a key role in the generation of the

virus. Homosubtypic and heterosubtypic reassortments have involving H9N2 have been isolated from both birds and swine (Abolnik et al., 2007; Cong et al., 2007; Wang et al., 2012; Yu et al., 2008; Yu et al., 2011). Sequencing demonstrates that a H9N2 G1 like virus has the same internal genes as the 1997 HPAI H5N1, suggesting a history or reassortment (Guan et al., 1999). Additionally, many H9N2 isolates contain mutations in the HA gene that suggest human-like receptor binding patterns. (Choi et al., 2004; Li et al., 2005; Li et al., 2003) Pandemic H1N1 (pH1N1) has been also isolated from swine populations across the globe and the pH1N1 viruses have shown a propensity for reassortment in swine (Pereda A, 2010; Vijaykrishna et al., 2010). In the laboratory, H9N2 viruses also demonstrate a readiness for reassortment, especially with regards to human seasonal H3N2 (sH3N2) and pandemic H1N1 (pH1N1) (Kimble et al., 2011; Sorrell et al., 2009; Sun Y, 2011; Wan et al., 2008). The full panel of H9N2:pH1N1 reassortants were tested in mice and indicated that several reassortants showed increased infectivity and pathogenicity (Sun Y, 2011). An H9N2 with sH3N2 internal genes infected and directly transmitted in ferrets, however, aerosol transmission was only seen after adaption to the ferret host (Sorrell et al., 2009; Wan et al., 2008). A reassortant with fully avian H9N2 surface genes on the pH1N1 backbone was able to transmit via aerosol contact in ferrets while a similar H9N1 virus was not. However, switching the surface genes to the ferret adapted version resulted in more efficient transmission for both subtypes (Kimble et al., 2011). A similar study in swine using only the avian H9N2 surface genes and pH1N1 internal genes showed that both the H9N2 and the H9N1 reassortants transmitted in swine (Qiao et al., 2012).

Together, these observations create a potential scenario in which H9N2 and pH1N1 reassort in swine, spread to humans, and begin a future human pandemic. Furthermore, the results indicate that an avian like H9N2 reassortant and H9N1 reassortant both transmit in swine, but only an H9N2 with avian like surface will transmit via aerosol in ferrets. When the surface genes are adapted to ferret, the reassortants demonstrate increased infectivity, pathogenicity and transmission efficiency. Additionally, the results indicate that an H9N1 would be favored over an H9N2 in the ferret model. What is not known is how adaption to a mammalian host would affect the behavior of the reassortants in swine. The ferret adapted surface genes were used to generate similar H9N2 and H9N1 reassortants to test infectivity, pathogenicity and transmissibility in pigs.

5.3 Material and Methods

5.3.1 Reverse genetics

Four viruses were grown by reverse genetics using previously described plasmids containing the genes for A/Netherlands/602/2009 (H1N1), A/Guinea fowl/Hong Kong/WF10/1999 (H9N2), and the ferret adapted surfaces genes from the WF10 (H9N2) virus (Sorrel et al. 2009) designated P10. The four viruses were: A/Netherlands 602/2009 (pH1N1), the P10 HA and NA on the WF10 backbone (H9N2), the P10 HA with the 7 other genes from pH1N1 (1P10), and the P10 HA and NA with the 6 remaining genes from pH1N1 (2P10) (Table 5.1).

Name	HA subtype	NA subtype	Internal genes origin
Control	-	-	-
H9N2	H9	N2	Avian H9N2
pH1N1	H1	N1	pandemic H1N1
2P10	H9	N2	pandemic H1N1
1P10	H9	N1	pandemic H1N1

Table 5.1. Genetic content of experimental viruses.

5.3.2 Animal studies

Swine studies were performed in appropriate, high-containment facilities of the National Animal Disease Center (NADC), Ames, IA, by following protocols approved by the NADC and the University of Maryland animal care and use committees. The study was done on 3-week-old crossbred pigs that were obtained from a high-health herd free of swine influenza virus (SIV) and porcine reproductive and respiratory syndrome virus. The pigs were divided into four groups of 21 and one group of 15. One virus per group was used to inoculate 15 of the 21 pigs that were then housed separately. The group of 15 was used as a negative control group. Two days post inoculation (dpi) the 6 remaining naïve pigs from each virus group were reintroduced to the 15 inoculated pigs as direct contacts. All pigs were swabbed on 2, 4, 6, 8, 10, and 12dpi. On 3 and 5dpi 5 pigs from the four virus groups and control group were swabbed, bled, and sacrificed. The lungs were scored for macroscopic lesions, bronchiole lavage was done, and lung and tracheal tissue was collected for microscopic lesion analysis.

5.3.3 Bronchiole lavage

Bronchiole lavage was performed on freshly collected lungs immediately post necropsy. The entire trachea, heart and lung section was removed and 50ml of DMEM media was washed down the trachea and into the lung. Media was then recollected and stored on ice until processing. Processing involved taking two 2ml aliquots, centrifuging the remaining bronchiole airway lavage fluid (BALF) for 20 minutes at 2000g, and

collecting two more 2ml aliquots of cell free BALF. Whole BALF was used for bacterial isolation and virus titration, while cell free BALF was used for cytokine analysis.

5.3.4 Swab titration

All swabs were stored in 2ml of collection media and tested for virus isolation by inoculating with 200ul of filtered collection media and 200ul of OptiMEM with TPCK added on a well of a 24 well plate confluent with MDCK cells. After 48 hours the cells were fixed and stained using anti NP mouse antibodies, rabbit anti mouse HRP conjugated antibodies, and AEC substrate. Any swab that produced a well positive for NP was then titrated by $TCID_{50}$ in MDCK cells.

5.3.5 Statistics

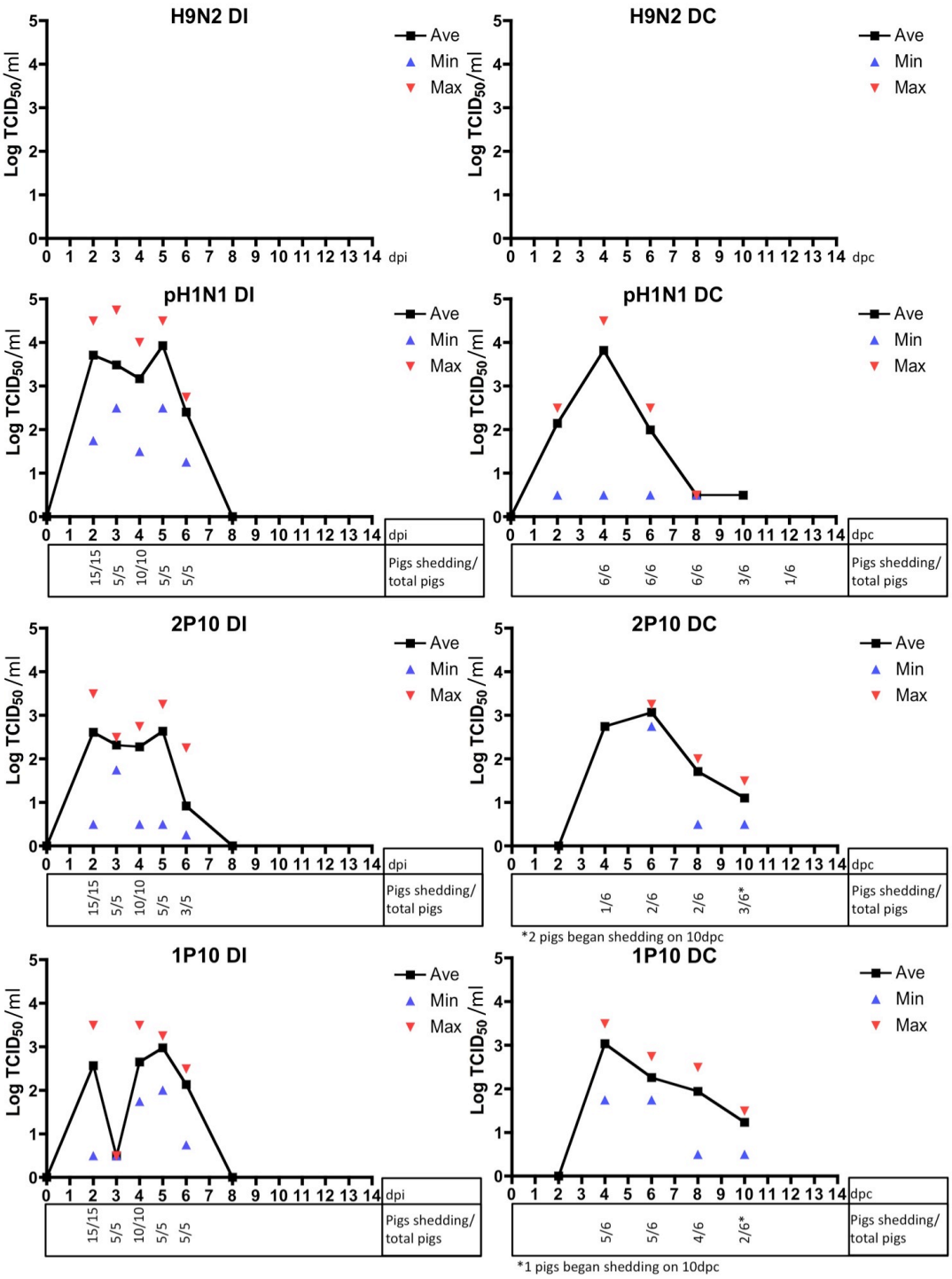
All statistical analyses were performed using GraphPad Prism software version 4.00 (GraphPad Software Inc., San Diego, CA). Comparisons between means were done using a one-way ANOVA followed by Bonferoni's multiple comparison test. $P<.05$ was considered significant.

5.4 Results

5.4.1 Infectivity, transmissibility, and pathogenicity

All swine were swabbed prior to, but on the day of inoculation (0dpi). Swab samples were tested for the presence of influenza by virus isolation. No pigs were shedding virus at 0dpi. The pigs showed no clinical signs of disease over the course of the study. No control pig was positive for virus in the nasal swabs throughout the course of the experiments as well.

All 15 swine inoculated with the H9N2 virus showed no viral shedding at any of the sampling time points. No H9N2 direct contact (DC) pig had a positive nasal swab through out the experiment (Fig. 1). All nasal swabs from the pH1N1 group were positive from 2-6dpi. However, by 8dpi none of the remaining directly inoculated (DI) pigs were still shedding virus. All 6 DC pigs were positive for virus by 2 days post contact (dpc) and continued shedding through 6dpc before beginning to clear the virus on 8dpc (3 /6 positive) and 10dpc (1/6 positive). The H9N2 reassortant (2P10) DI pigs shed virus from 2dpi through 6dpi. One H9N2 DC pig began shedding at 4dpc and another began on 6dpc. There was an apparent second round of transmission from the infected DC pigs as two more previously negative DC pigs began shedding at 10dpc, well after the DI pigs had cleared the virus. In total, four the six contact pigs became infected. Finally, the reassortant H9N1 (1P10) DI pigs all shed virus from 2dpi through 6 dpi before all stopped shedding virus at 8dpi. The 2P10 virus proved to be more transmissible as 5 of 6 DC pigs began shedding at 4dpc with clearance by 8-10dpc. There was also a secondary transmission event in this group as the one previously negative DC pig began shedding virus at 10dpc making six of six contact pigs that became infected.



<u>Figure 5.1.</u> Reassortant H9 transmit in swine with slightly reduced efficiency compared with the pandemic H1N1. Nasal swabs were collected, frozen, thawed, filtered and then titrated by $TCID_{50}$ on MDCK cells. All positive samples for each group, at each time point were averaged (black squares). The high (red triangle) and low (blue triangle) titer of each group is also marked. The number of pigs positive for viral shedding in each group vs. the total number of pigs is listed under each time point. Note the DC graphs are measured in days post contact (dpc) not dpi.

5.4.2 Lower respiratory infections

Pathogenically, the H9N2 group showed very few visible lung lesions at 3dpi and was not any different than the control group at 5dpi. The pH1N1 showed the most visible lesions of any group. The 5 lungs averaged 16.5% of visible lung surface affected by lesions at 3dpi and averaged 20.5% of visible area at 5dpi. The pH1N1 group was the only inoculated group that showed an increase in lesions from 3dpi to 5dpi. The two reassorted viruses showed an intermediate level of gross pathology. The 2P10 group averaged 5.6% and 3.3% on 3dpi and 5dpi respectively. The 1P10 had lesions covering 6.7% and 4.3% of visible lung surface at 3dpi and 5dpi (Fig. 2).

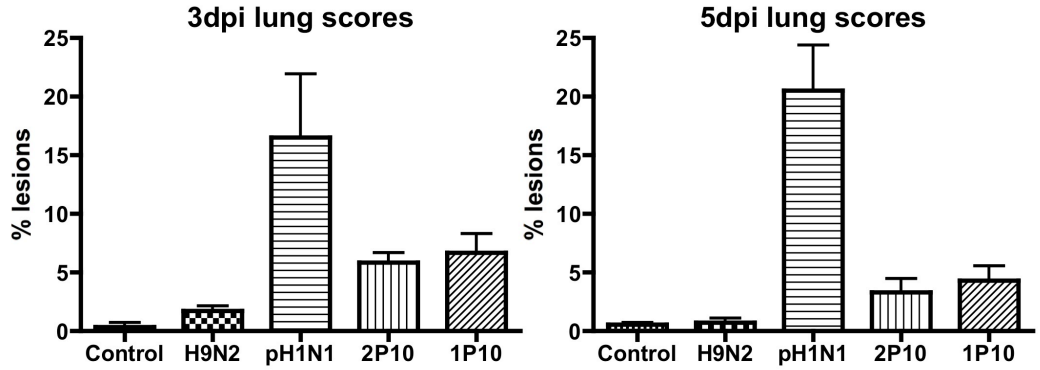


Figure 5.2. H9 reassortants result in intermediate lung pathology. Five pigs from each direct inoculation group were euthanized on 3dpi and 5dpi. Each lung lobe was individually scored for visible lesions at the time of the necropsy and then a weighted total percentage of the lung surface affected was calculated. The bars represent the average of the five lungs from each group.

Bronchoalveolar lavages were performed on swine lungs at the time of necropsy on 3dpi and 5dpi. Samples of the bronchoalveolar lavage fluid (BALF) were then titrated by $TCID_{50}$. The control group and the H9N2 group had no titratable virus in any sample on either day. This correlates with the nearly complete lack of pathology in these groups at these time points. The pH1N1 group had the highest average titer of all 5 groups on both 3dpi and 5dpi. The 1P10 group had the lowest average titer for both days, however by 5dpi the titer was nearly equivalent to the 2P10 5dpi titer. The 2p10 group showed nearly consistent titers from 3dpi to 5dpi (Fig. 3).

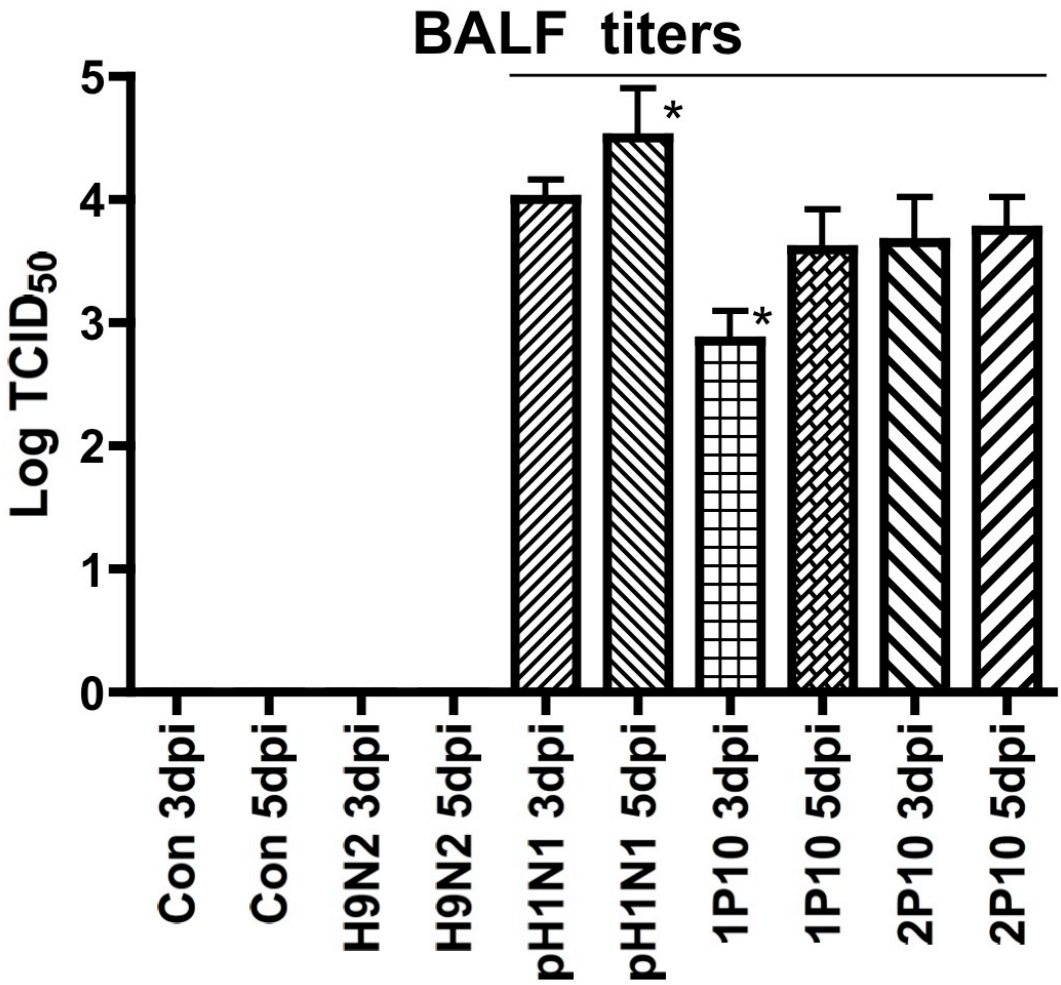


Figure 5.3. Reassortant H9 viruses replicate in the lungs of swine. Bronchiole airway lavage was performed on five pigs per group, per time point at the time of necropsy. The bronchiole airway lavage fluid (BALF) was collected and stored on ice before processing. The BALF were then titrated for virus by $TCID_{50}$ in MDCK cells. The bars represent the average titer of the five samples from each group. Results under the line are not significant at the $p<.05$ level unless noted. Asterisks indicate significance at $p<.01$.

5.4.3 Serology

All pigs were bled at -2dpi and sera samples were collected and tested by NP ELISA for prior exposure. No pig was seropositive prior to the start of the experiment. To check for exposure and seroconversion, all surviving pigs were bled at 14dpi and 20dpi. Sera samples were again collected and tested by NP ELISA for evidence of exposure. No control pig showed evidence of seroconversion. From the H9N2 group, only three of the five directly inoculated (DI) pigs that were bled on 14dpi and 20dpi and none of the DC pigs seroconverted. This is unsurprising given the lack of titratable virus from samples from this group. The DI pH1N1 group showed seroconversion in three of four pigs at 14dpi and four of four at 20dpi. All six DC pigs were seroconverted by 14 dpi. The DI 2P10 group had two of four pigs at 14dpi and four of four at 20dpi. The two DC pigs that became infected early on seroconverted by 20dpi and were the only DC pigs to do so. Finally, the 1P10 DI pigs all seroconverted by 20dpi. Five of six 1P10 DC pigs shed virus by 6dpi and the sixth pig began shedding at 12 dpi. Four of the five Dc pigs that shed were seroconverted at 14dpi and the sixth pig was negative. Interestingly, one of the 14dpi-seroconverted pigs was seronegative at 20dpi leaving only three of the six DC pigs seropositive.

5.5 Discussion

H9N2 viruses are endemic in poultry populations across Asia. Many of the recent H9N2 isolates from poultry in this region possess hallmarks of human receptor specificity. A leucine at amino acid position 226 in the HA gene is commonly found in avian H9N2 viruses. Typically, avian H9N2 have a glutamine at his position. Studies have shown that the Q226L mutation is sufficient for avian to human-like receptor

switching (Wan and Perez, 2007). Since the late 1990's H9N2 have in fact occasionally infected mammalian hosts, both swine and human. Pandemic H1N1 (pH1N1) is also found in both species. While the possibility of a human H9N2:pH1N1 reassortant has been address, less is clear about a similar swine reassortant.

Swine carry both avian (α-2,3 sialic acid) and human (α-2,6 sialic acid) like influenza receptors in their respiratory tract (Ito et al., 1998). This allows for infection with both avian and human viruses and creates an environment where two different viruses may reassort and lead to novel genotypes. Heterosubtypic reassortment involving either H9N2 or pH1N1 have occurred in swine in the past, but as of yet, these events pose little threat to humans. However, recent studies have shown that an H9N2:pH1N1 reassortant can infect and transmit in ferrets, the best model of transmission of influenza in humans (Kimble et al., 2011). Kimble et al. showed that a reassortant with wtH9N2 surface on a pH1N1 backbone would transmit in ferrets, but a wtH9N1 on the same backbone would not. Qiao et al. conducted a similar study in pigs and found that while both transmitted in swine, the H9N1 reassortant was more efficient (Qiao et al., 2012). This suggests that a reassortment in swine with pH1N1 and avian H9N2 would favor a reassortant virus that would not transmit in humans. Kimble et al. went on to show that similar reassortant viruses with ferret adapted version of the H9N2 instead of avian H9N2 genes both transmitted in ferrets, but the ferret adapted H9N1 showed higher efficiency. This leaves the possibility of a swine H9N2:pH1N1 reassortant adapting to the mammalian respiratory tract and transmitting to humans. What was not clear is how the mammalian adaption would affect the behavior of the virus in swine. Here it is shown that mammalian adapted H9N2 reassortants are able to infect and transmit in swine.

Similar to the avian wtH9N2:pH1N1 reassortants, an H9N1 is more efficient. This also matches the studies done with similar mammalian adapted reassortants in ferrets.

Compared to the pH1N1 virus, these reassortants show reduced pathogenicity. While no immediately observable signs of disease were apparent in any infectious group during the course of the experiment, differences were readily apparent upon inspection of the lungs at the time of necropsy. Swine infected with pH1N1 showed relatively high coverage of lesions at both 3 and 5dpi, with the highest lesion scores on 5dpi. The two reassortant viruses showed less severe affects in the lung than the pH1N1 did. This is consistent with what was seen in ferrets with similar viruses. The 2P10 group had slightly higher lesion scores than the 1P10 group on both days, but the difference was not significant. In contrast to what was seen in pH1N1 group the pathological effects were less severe on 5dpi than on 3dpi for both groups.

This study highlights the role pigs may play as mixing vessel species. It also underscores the potential threat that H9N2 viruses could pose to humans as a future pandemic subtype. Swine play hosts to both H9N2 and pH1N1 and both viruses have a history of reassortment in swine. A future meeting of these two viruses in porcine respiratory cells could result in a problematic virus. Pigs could also serve as suitable environment for the adaption to the mammalian host of any potential reassortment. These results support the role of swine in both genetic drift and genetic shift towards a potential future pandemic. The findings here emphasize the need for better surveillance of influenza in swine. Increased swine surveillance will lead to a better understanding of zoonosis and transmission and will lead the world to better pandemic preparedness.

Additionally, they also stress the need for future research on how H9N2 influenza behaves in pigs and how the swine host can effect reassortment.

Chapter 6: Alternative reassortment events lead to the generation of respiratory transmissible H9N1 in ferrets.

Pending Publication: J. Brian Kimble, Matthew G. Angel, Troy C. Sutton, Hongquan Wan, Courtney Finch, and Daniel R. Perez[4]

6.1 Abstract

Influenza A H9N2 viruses have been previously isolated from human hosts in the past. Serological surveys suggest that human infections with H9N2 are not a rare event, but infections are masked by minimal clinical symptoms. It has been shown previously that H9N2 viruses are capable of reassortment and can generate novel viruses with increased transmissibility and pathogenicity, especially in regards to the 2009 pandemic H1N1 (pH1N1). The modeling power of a novel transfection based inoculation system is demonstrated to elucidate the most likely resultant virus of a reassortment event between an H9N2 and pH1N1 in ferrets. Plasmids containing the mammalian adapted surface H9N2 genes, wt internal H9N2 genes, and 7 pH1N1 genes (segment 4 excluded) were transfected into HEK 293T cells to generate the full panel of possible H9 reassortants. These cells were then used to inoculate ferrets. Population dynamics were studied using serial passage in ferrets and cell culture. Consistent with previous data, an aerosol transmissible H9N1 was twice selected by our method, indicating a selective pressure in ferrets for the novel combination of surface genes. While the surface genes of both

---

[4] Author contributions: JBK, TCS and DRP designed research. JBK, MGA and CF performed research; JBK, MGA, HW and DRP analyzed data; and JBK and DRP wrote the paper.

viruses were the same, the internal gene selections were not identical. These results show that a transfection based inoculation system is a fast, efficient method to model reassortment events and highlight the potential risk of a reassorted H9N2 and pH1N1 virus can pose.

6.2 Introduction

Influenza A H9N2 began appearing in domestic poultry in the late 1980's. Throughout most of the 1990's these viruses remained in domestic and wild bird populations and displayed typical avian receptor and species specificity (Alexander, 2000; Lee et al., 2000; Naeem et al., 1999; Perk et al., 2006). However, in the later part of the decade the virus began showing more human like receptor specificity and in 1998 the first swine H9N2 was isolated. A year later, the first human H9N2 virus was isolated in Hong Kong (Peiris et al., 1999). Since then there have been several more human isolations of H9N2. However, the minimal clinical symptoms associated with the subtype combined with seroprevalence surveys lead some to believe the incidence in humans is far higher than the few isolations would suggest (Pawar et al., 2012; Wang et al., 2009).

Genetic shift and drift affect all subtypes of influenza and H9N2s are no exception. H9N2 reassort in nature and these reassortments can impact the human population (Xie et al., 2012). Sequencing shows that the highly pathogenic H5N1 and the G1 like H9N2 have common internal genes. This indicates an pasts reassortment and evidences suggests that the H9N2 donated the internal genes to the HPAI H5N1 that infected humans (Guan et al., 1999). Genetic drift has cause distinct lineages of H9N2 to

emerge in Eurasia. Between three and five Eurasian H9N2 lineages have been proposed (Dong et al., 2011; Fusaro et al., 2011; Guo et al., 2000).

Recent data has shown that H9N2 viruses are capable of reassortment and genetic drift to generate novel reassortant viruses that are more transmissible and more pathogenic in mammalian models than parental viruses (Sorrell et al., 2009; Wan et al., 2008). These experiments show the threat that H9N2 could pose to the human population. The emergence of the 2009 pandemic H1N1 (pH1N1) further heightened these dangers as H9N2 has shown to be compatible for reassortment with the newly emerged pandemic viruses (Kimble et al., 2011; Sun Y, 2011).

Previous studies investigating the threat an H9N2:pH1N1 virus would pose to the human population have used a blunt force approach in mice, testing each possible combination one at a time (Sun Y, 2011). This is not a cost efficient option for any system other than the mouse model. Other studies have arbitrarily selected a reassortant genome allowing only a partial view of what is possible (Kimble et al., 2011). Finally, some studies have selected for viruses in cell culture, which lacks the selective pressure of an *in vivo* system (Schrauwen, 2011). In this study a novel 15-plasmid transfected cell based inoculation system was used to quickly and efficiently mimic a reassortment event between an H9N2 and pH1N1 in ferrets. This system is able to test all possible reassortant viruses in ferrets, the ideal animal model of transmission and pathogenicity in humans. By serially passing the resultant viral population we winnowed down to a single virus representing the most likely outcome from such a reassortment event.

6.3 Materials and Methods

6.3.1 15-plasmid infection

HEK 293T cells were transfected with equal molar amounts 15 plasmids containing the surface gene segments from A/ferret/MD/P10/2009 (H9N2) (Wan et al., 2008), the internal genes from A/guinea fowl/HK/WF10, and 7 gene segments from A/NL/602/2009 (H1N1) excluding segment 4. Transfection was done using TransIT lipofectimine (Mirus) in 6-well cell culture plates. Transfected cells were lifted 24 hours post transfection and collected in the transfection supernatant. Seronegative ferrets were inoculated with 1ml of transfected cells/supernatant, which corresponded to one well of cells. Ferrets were monitored for signs of disease and sampled daily by nasal washes with 1ml of PBS. Nasal washes were tested for viral antigen by FluDetect (Synbiotics Corps. San Diego, CA) allocated and stored at -80C.

6.3.2 Viral selection

Selection of the fittest virus was determined by serial passage of the viral quasispecies in ferrets in the following manner. The transfected cells were used to inoculated passage 1 (p1) ferrets. The day of peak shedding was determined by $TCID_{50}$ titration and 700ul of that sampled was used to directly inoculate p2 ferrets. Based on the shedding curve of p1 ferrets, samples from 4 day post inoculation (dpi) were used to inoculate the next passage of ferrets out to p5. Ferrets for p6 were added to the cage of p5 in a manner to only allow for respiratory contact (RC) at p5 1dpi.

6.3.3 Animal studies

All studies were approved by the Animal Care and Use Committee of the University of Maryland (protocol R-09-93). 3-7 month old female ferrets were purchased from Triple F farms (Sayer, PA). Ferrets were quarantined for 7 days in BSL-2 animal facility. All animals were implanted with temperature transponder (Bio Medics, Seaford, DE) to identify individual ferrets and record body temperature. Sera were used to determine the naivety of each ferret prior to experimentation.

6.3.4 Limiting dilutions

Transfection supernatant was passed in MDCK cells once for 48 hours. Cell culture supernatant was diluted 10-fold from 10^{-1} to 10^{-8} across a 96 well plate containing MDCK cells. After 72 hours, an HA assay was performed and the well that was inoculated with the most dilute sample and still resulted in an infection was selected and assumed to have a single infectious virus. The process was repeated 12 times down the 96 well plate thus selecting 12 different single viruses. The supernatant from the 12 selected wells were then partially sequenced to identify the eight gene segments present.

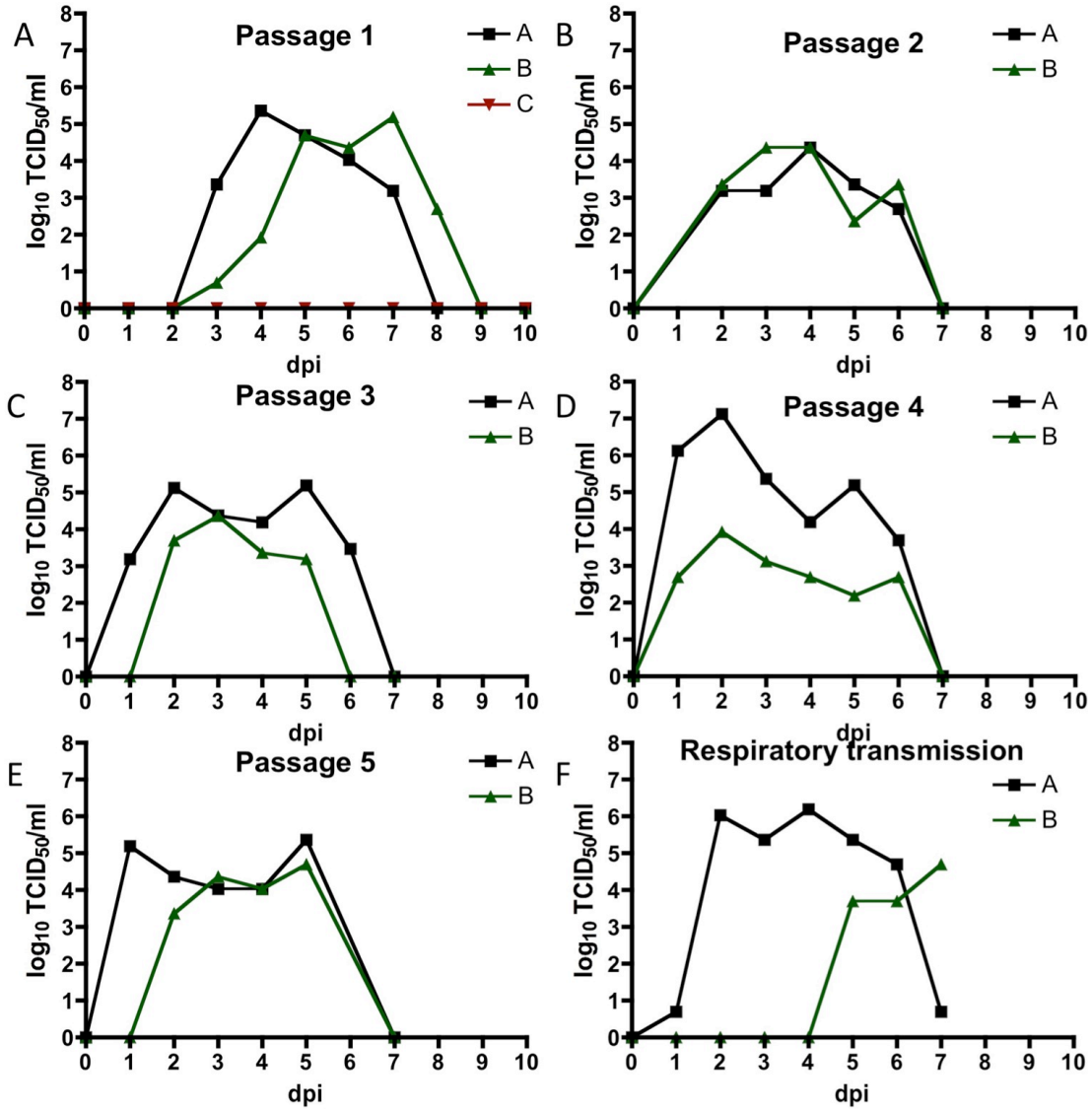
6.4 Results

6.4.1 15-plasmid inoculation causes infection in ferrets

HEK 293 T cells were transfected with 15 plasmids containing the 8 H9N2 genes and 7 pH1N1 genes. Segment 4 (HA) of pH1N1 was omitted in order to ensure an H9 virus was selected for. At 24 hours post transfection the cells were harvested and used to inoculate the nasal cavity of three isolated ferrets, designated Lineage A, B and C.

Additionally, transfected cells were passed in MDCK cells to ensure the transfection worked properly. The cell passed transfection was positive by FluDetect at 1dpi and grew to a titer of 6.3x10^6 TCID50 by 3dpi (data not shown).

Daily nasal washes revealed that Lineage A and Lineage B ferrets became infected and began shedding virus three days post inoculation (dpi). The ferrets shed virus for 5 or 6 days and then were able to clear the infection. Lineage C failed to produce virus. (Figure 1A). Sera collected at 10dpi were positive for Lineage A and B, but negative for Lineage C. Signs of disease were mild. Infected ferrets showed mild fever and mild weight loss and appeared mildly lethargic and depressed.



<u>Figure 6.1.</u> 15-Plasmid transfection system results in productive infection of ferrets. Three ferrets were inoculated with cells transfected with 15 plasmids containing genes for H9N2 and pH1N1 influenza viruses. Ferrets were nasal washed with PBS daily and monitored for signs of disease. Samples were titrated for virus by $TCID_{50}$ in MDCK cells. (A) Passage one in which the third ferret did not shed virus. (B-E) Passage 2-5 in which ferrets were directly inoculated with nasal washes from the previous passage. (F) Respiratory passage 6 (RCP6) were respiratory contact ferrets for passage 5.

6.4.2 Rapid *in vivo* selection of aerosol transmissible H9N1 viruses

A transfection with 15 plasmids is theoretically capable of producing 128 genetically distinct viruses. Based on the potential viral diversity it was assumed the ferrets were shedding a mixed population of viruses. To select for a single virus, the viral populations was serially passed via direct inoculation of nasal washes from 5dpi into a naïve ferret. This process was repeated for 5 passages. Theses ferrets displayed typical shedding profiles with peak titers between 10^4 and 10^6 $TCID_{50}$ and viral clearance after 6 or 7 days (Figure 1B-E).

A naïve respiratory contact ferret was added to the isolator of each passage 5 (p5) ferret in a manner that prevented any physical contact. These ferrets (RCP6) were monitored by nasal washes. Both Lineage A and Lineage B proved to be respiratory transmissible (Figure 1F.) However, due to time constraints the experiment was terminated prior to the clearance of the virus by the RCP6 ferrets. To more completely assess the transmissibility of these viruses, three additional ferrets for each lineage were directly inoculated with nasal washes from P4. At 1dpi a naïve ferret was added in as a respiratory contact (RC). The A lineage grew faster and to higher titers than did the B lineage virus in the DI ferrets. However, both viruses were cleared in a similar time frame, by 7 or 8 dpi. The two lineages transmitted efficiently as all three RC ferrets in each group shed virus. The A lineage virus appeared to have transmitted slightly faster and grew to slightly higher titers than the B lineage did in the RC ferrets (Fig. 2).

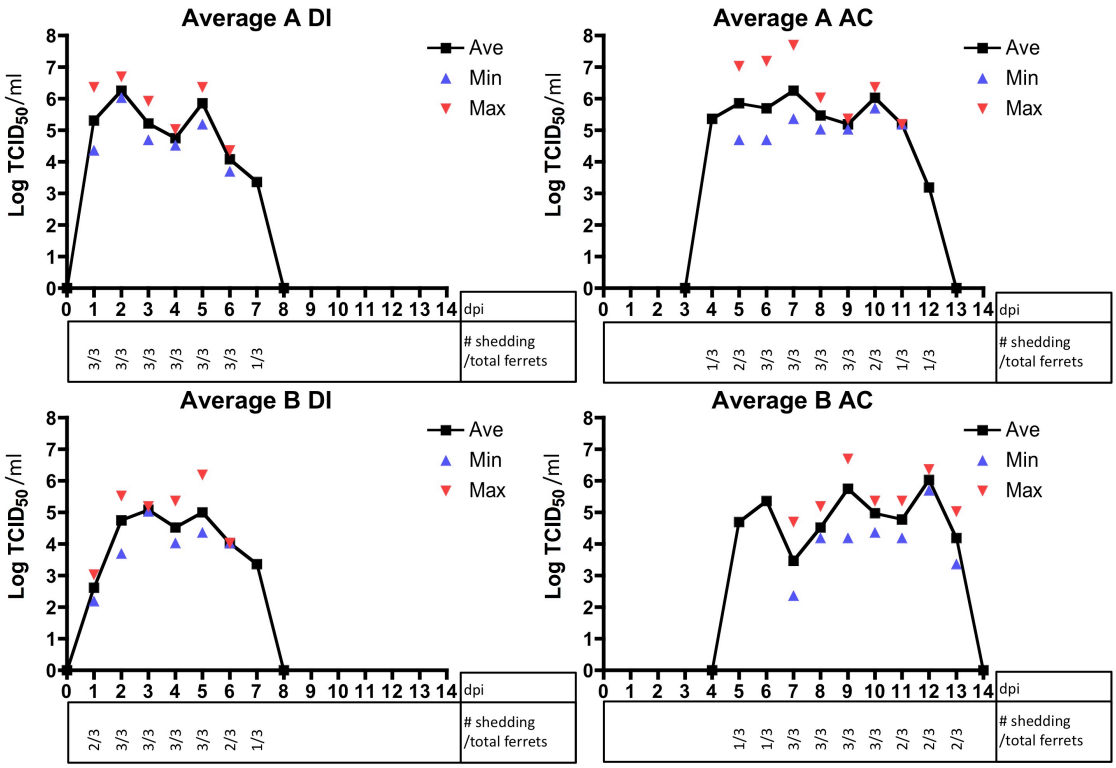


Figure 6.2. Both lineages transmit efficiently in ferrets. Three directly inoculated (DI) ferrets per lineage were inoculated with 10^5 $TCID_{50}$ of p4 virus sample. At one day post inoculation a single respiratory contact (RC) ferret was added to each DI ferret. All ferrets were nasal washed daily and the samples were titrated by $TCID_{50}$ in MDCK cells. All positive samples per lineage per time point were averaged (square) and the minimum titer (blue triangle) and maximum (red triangle) titer are noted. The number of ferrets that were positive is noted in the table below each graph.

Full-length sequencing was done on the RCP6 samples that had the highest titer. Both Lineage A and Lineage B were H9N1 viruses with mixed internal genes. The PB2, NP, and NA were of pH1N1 origin for both viruses, while the PA, HA, and NS were of H9N2 origin for both. The two lineages differed in the PB1 and M segments. Both segments were from the pH1N1 for Lineage A. The PB1 and M from Lineage B originated form the H9N2 (Table 1). A number of mutations were found in the full length sequencing of the RCP6 samples. Lineage A had three mutations: D253N in PB2, K26E in PA, and S254N in HA. Lineage B also had a total of three mutations in PB1 (D120N, D439E) and in M2 (E95K). Next, the speed at which the selection process occurred was tested. Standard Sanger sequencing was performed on passage 1 samples from 4dpi (A lineage) or 5dpi (B lineage). Results indicate that the population in passage 1 exactly matched the population that was transmitted via aerosol to the RCP6 ferrets, indicating that the viruses were selected for within five days of inoculation (Table 1).

A lineage			
	Passage 1	Passage 6	
Segment	origin	origin	Mutations
PB2	pH1N1	pH1N1	D253N
PB1	pH1N1	pH1N1	
PA	**H9**	**H9**	K26E
HA	**H9**	**H9**	S254N
NP	pH1N1	pH1N1	
NA	pH1N1	pH1N1	
M	pH1N1	pH1N1	
NS	**H9**	**H9**	

B lineage			
	Passage 1	Passage 6	
Segment	Identity	Identity	Mutations
PB2	pH1N1	pH1N1	
PB1	**H9**	**H9**	D120N, D439E
PA	**H9**	**H9**	
HA	**H9**	**H9**	
NP	pH1N1	pH1N1	
NA	pH1N1	pH1N1	
M	**H9**	**H9**	E95K in M2
NS	**H9**	**H9**	

Table 6.1. Both lineages are H9N1 reassortants with mixed internal genes. Full length sequencing of passage 6 viruses revealed 3 mutations in each lineage. Partial sequencing confirmed passage 1 virus and passage 6 virus were identical.

6.4.3 Transfection produced multiple virus subpopulations

Limiting dilutions were performed 12 times on the cell culture passed transfection to isolate individual viruses from the mixed population and ensure each of the 15 original plasmids were capable of producing viable virus. Partial sequencing was done for each gene segment in each dilution to identify the origin of each segment. Results indicated that both the H9 origin segments as well as the pH1N1 origin segments were found in at least one of the dilutions with the exceptions of the pH1N1 HA (not included in the original transfection) and the pH1N1 PB1 (found in Lineage A ferrets) (Table 2). This confirms that Lineage A and Lineage B viruses were selected out from a mixed population and that each plasmid was capable of being included in a viable virus. These results also indicate that cell culture based selection can drastically skew the results when compared to *in vivo* based selection processes.

Replicate	PB2	PB1	PA	NP	NA	M	NS
1	**H9**	**H9**	pH1N1	pH1N1	pH1N1	**H9**	**H9**
2	**H9**	**H9**	**H9**	pH1N1	pH1N1	**H9**	**H9**
3	**H9**	**H9**	pH1N1	**H9**	**H9**	pH1N1	pH1N1
4	**H9**	**H9**	**H9**	pH1N1	pH1N1	**H9**	**H9**
5	**H9**	**H9**	pH1N1	pH1N1	pH1N1	**H9**	**H9**
6	**H9**	**H9**	**H9**	pH1N1	pH1N1	**H9**	**H9**
7	**H9**	**H9**	pH1N1	pH1N1	pH1N1	**H9**	pH1N1
8	pH1N1	**H9**	pH1N1	pH1N1	pH1N1	pH1N1	**H9**
9	pH1N1	**H9**	pH1N1	pH1N1	pH1N1	**H9**	pH1N1
10	**H9**	**H9**	**H9**	pH1N1	pH1N1	**H9**	**H9**
11	pH1N1	**H9**	pH1N1	pH1N1	pH1N1	**H9**	**H9**
12	Mix	**H9**	pH1N1	pH1N1	pH1N1	Mix	Mix

Table 6.2. Limiting dilutions show a mixed population resulting from transfection. Limiting dilutions were performed as described in the materials and methods section 6.3.4. Sanger sequencing revealed the genetic constellation of each of the 12 replicates. Replicate 12 appears to be a mixed population of viruses.

6.4.4 Pathological characterization

Pathologically, these viruses are similar to seasonal flu. Alveolitis and interstitial pneumonia, characterized by evident inflammatory infiltration of neutraphils, lymphocytes and macrophages, were observed in the lungs from both groups on 3dpi and 5dpi (Figure 3). Congestion was more evident in the lungs on 3dpi than on 5dpi in both groups, while infiltration in the lung from the B lineage group (Figure 3E) was more severe than in the lung from the A lineage group (Figure 3A) on 3dpi. No evident lesion was observed in the tracheas. Additionally, the ferrets showed mild signs of disease. Typical signs were mild fever, moderate body weight loss and mild lethargy. There was no apparent difference between the two viruses.

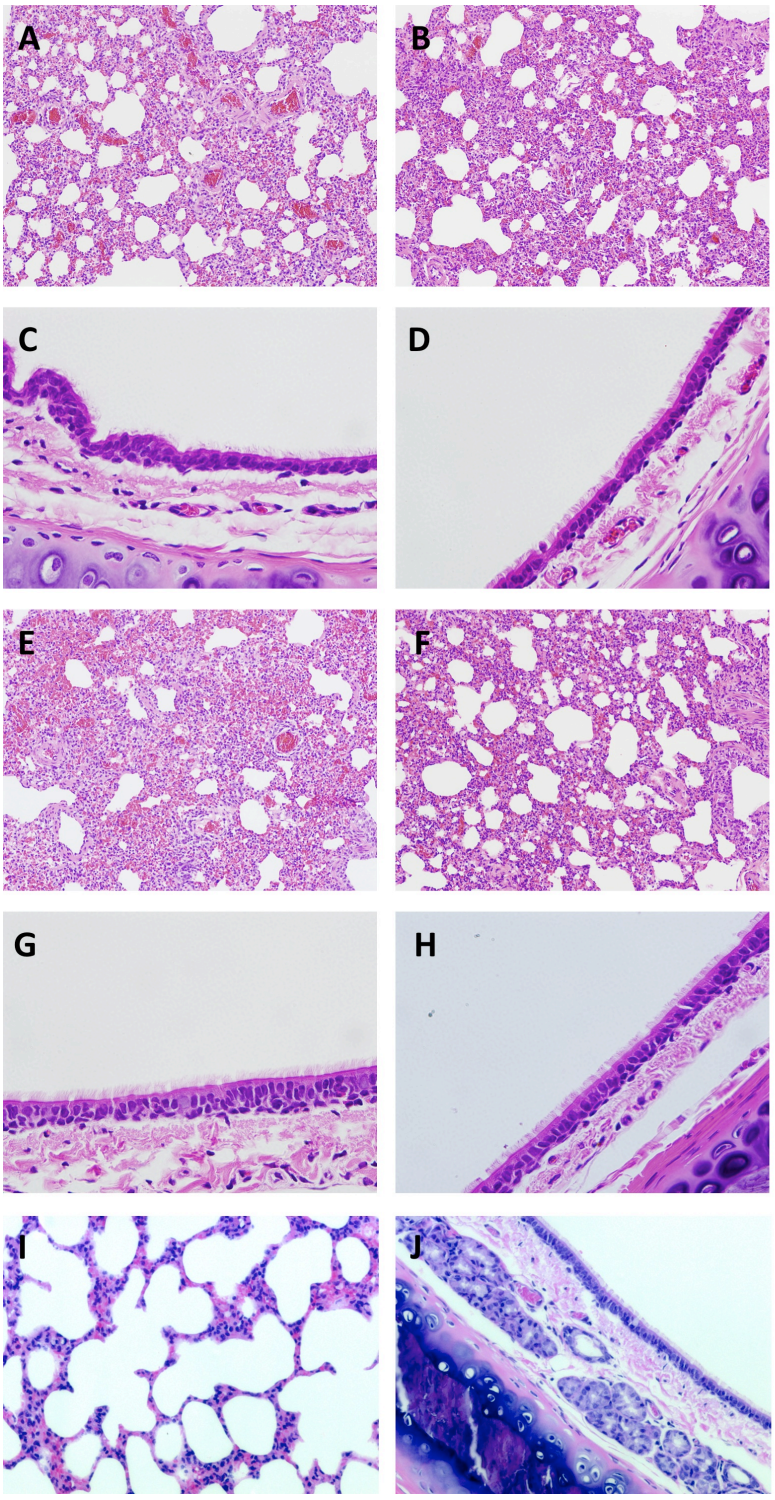


Figure 6.3. Both H9N1 reassortants exhibit pathogenicity similar to seasonal flu. Ferrets were inoculated with 10^6 $TCID_{50}$ of either A or B lineage virus. Tissues were collected at 3 or 5dpi in formalin, cut into 5um sections and stained with H&E. (A) and (C), the lung and trachea collected from an A lineage ferret at 3dpi; (B) and (D), the lung and trachea collected from an A lineage ferret at 5dpi; (E) and (G), the lung and trachea collected from a B lineage ferret at 3dpi; (F) and (H), the lung and trachea collected from a B lineage ferret at 5dpi. Lung (I) and trachea (J) from naïve ferrets serve as controls.

6.5 Discussion

Here, a novel method for modeling reassortment and selecting for reassorted viruses by using transfection based inoculation (TBI) was described. This method was used to select two alternative reassorted viruses from a reassortment between H9N2 and pH1N1. Previous methods of reassortment modeling employed reverse genetics to individually test each potential resultant virus (Sun Y, 2011), selected for a single resultant virus in tissue culture (Schrauwen et al., 2011) or co-infection in a host (Angel M, 2013). This new method has advantages over each of the previous methods. The reverse genetics method is a massive amount of work, eliminates the competition between resultant viruses, and is infeasible to test in more natural model hosts like swine or ferrets.

The tissue culture based method of reassortment modeling is far easier to do and incorporates the competitive selection, however it is not a natural selection. Our results indicate that the selective pressure applied to the population in tissue culture produces a very different virus from that which is produced in the ferret. Only the initial round of replication is done in cells. Subsequent replication is done in the ferrets and subjected to typical *in vivo* levels of selective pressure. Additionally, the selection takes multiple passes in cells to fully resolve into a single genetic constellation. TBI was able to select a single genetic reassortant virus in the nasal washes of the ferrets at 4dpi.

The most natural method of modeling reassortment is the co-infection study. However, co-infecting an animal does not ensure that reassortment will take place and a negative result does not indicate that a reassortment cannot take place. In our system, the

reassortment is forced to happen. Similar to the tissue culture based selection, co-infections typically take multiple passes to fully resolve when there is a reassortment due to the fact that, initially, the parental viruses are present at much higher titers from the inoculation. Our system resolves much more rapidly because each distinct progeny is competing against other viruses that are present at similar levels after one round of replication.

Previous results showed that an H9N1 reassortant virus grew to higher titers and transmitted faster in a limited study in ferrets compared to an H9N2 reassortant (Kimble et al., 2011). The results here corroborate those findings as two independent trials both resulted in an H9N1 being selected for. The different lineages created in this study did differ in the internal gene segments that were selected. The PB2, PA, NP, and NS genes were identical, however, the two viruses differed in the PB1 and M segments.

Our results also indicate that the A lineage virus transmits faster and grows to higher titers than the B lineage virus. This could be due to several factors. There mutational differences in several of the genes: PB2, PB1, PA, HA and M2. One or more of these mutations may influence the transmissibility and infectivity of these viruses, but there is no data to support this idea. The far more likely cause of the difference is the gene selection. As mentioned earlier, the A lineage and B lineage differ in the PB1 and the M gene. The PB1 and M of the A lineage is from pH1N1 and of human origin. The B lineage has the WF10 versions of those genes that are of avian origin.

The WF10 and pH1N1 PB1 differ drastically. Sequencing of the WF10 PB1 gene suggests that it has a full length PB1-F2 while the pH1N1 PB1 has a truncated expression

due to a stop codon at the eleventh amino acid position. That being said, PB1-F2 is a virulence marker. There is no data to suggest a role in transmission and even less on its effects in ferrets. The different M genes, however, do seem to have an effect on the transmissibility and infectivity of the viruses. It has been shown in guinea pigs (Chou YY, 2011), swine (Ma W, 2012), and ferrets (Angel M, 2013) that the M gene from the pH1N1 positively correlates with higher titers and increased transmission.

Chapter 7: Conclusions and future prospects

7.1 Conclusions

The overall goal of this body of work was to cast a better light on the processes of zoonotic transmission, especially in regards to reassortment. The viral subtype used for the majority of these studies, H9, is listed as a potential future human pandemic, which lends relevance to the work. Furthermore, we developed a novel method of modeling reassortment which make future studies easier and more in depth.

Firstly, I characterized the sialic acid distribution in several common poultry species that had not previously been described. I found that all the land-based poultry that were investigated carried both avian-like and human-like influenza receptors and were able to bind both avian and human viruses. These findings make sense of some natural observations of the past. In the late 1990's, H9N2 viruses began appearing with human-like receptor binding ability. The phenotype expanded over the years and is now a common feature of H9N2 across Africa and Asia. The logical conclusion was that the virus gained some advantage by having this binding pattern, because the birds have human-like receptors. These findings confirm that speculation. Similarly, there have been several reports of birds, particularly turkeys, being infected with the 2009 pH1N1 human influenza virus (Berhane et al., 2010; Reid et al., 2012). This is also expected in light of our findings, as a fully human-like receptor-binding virus would have plenty of human-like receptor expressing cells to infect.

Next, I investigated compatibility of H9N2 and pH1N1 for reassortment in regards to both ferrets, the animal model for humans, and swine. I found a virus with the

surface genes from an avian H9N2 virus with the internal genes of a pH1N1 was able to transmit via aerosol in ferrets. To my knowledge, this is the first avian surfaced gene to transmit via respiratory contact in ferrets. Furthermore, by using the mammalian adapted genes, we made an H9N1 virus that was highly efficient at aerosol transmission, and showed increased infectivity. I then used these viruses in pigs and found similar results. Together these results show that the two viruses are compatible for reassortment and the resulting virus would have increased risk of zoonotic transmission and pandemic risk in humans.

Finally, I developed a novel technique for modeling reassortment *in vivo.* The technique has a number of factors that make it a valuable tool over existing methods. First, it is far more efficient and flexible than existing *in vivo* methods. Co-infections are tricky to get results as novel viruses only exist as subpopulations and then must outcompete the much greater represented parental viruses during serial passaging. It is also limited to only whole viruses. Our system was able to select a single reassortant variant from a mixed population in a single animal and can utilize only specific genes from different viruses. Second, it utilizes *in vivo* pressures to more naturally simulate selective pressure on the viral population. Finally, it was able to select a single variant after a few a 4 days. Iused this technique to model two reassortment events in ferrets between H9N2 and pH1N1. Interestingly, both reassortments resulted in an H9N1 virus. This is congruent with the previous ferret study. Also, the virus with the M segment from pH1N1 showed increased transmission and replication which is consistent with previous studies.

Several conclusions can be drawn from the results presented in chapters 3 to 6. They are:

1) **Many common terrestrial poultry species contain receptors for both avian and human viruses (chapter 3)**

    A) All land-based poultry species tested showed both avian- and human-like receptors.

    B) All tested birds except for geese, were able to bind both human and avian viruses.

    C) Turkeys and ducks showed an age dependent change in expression of human-like receptors

    D) Could explain avian viruses with human like characteristics (H9N2, some HPAI H5N1) and infections of turkeys with human pH1N1.

2) **Pandemic H1N1 and H9N2 can reassort to form viruses with increased risk of zoonotic transmission and human pandemic potential (chapter 4, 5).**

    A) H9N2 and pH1N1 are compatible for reassortment in both the pig and ferret host.

    B) Two reassortant viruses that readily transmit via aerosol contact also readily transmit in swine.

    C) Adaption to ferrets increases transmissibility and replication in ferrets.

D) Highlights the risk of an H9N2:pH1N1 reassortant from either humans or swine followed by zoonosis.

E) H9N1 seems to be the favored surface combination.

3) Transfection based inoculation can be used as a superior model of reassortment compared to existing techniques (chapter 6).

A) Fast (4 days), efficient (single animal), natural (*in vivo* selective pressure) method of modeling reassortment.

B) Allows for the flexibility to pick and choose genetic input of the reassortment to be modeled.

C) An H9N1 was twice selected as the resultant virus indicating an advantage over H9N2 reassortants, confirming previous results.

D) The H9N1 with the pH1N1 M segment had slightly higher infectivity and increased transmission ability compared to the H9N2 M segmented virus in concordance with other studies.

7.2 Future prospects

Based on the findings in this work the following recommendations are made concerning future directions of the work:

1) Characterization of sialic acid influenza receptors in common poultry species

A. I found minor variations in sialic acid distribution and virus binding ability between species. The six species we tested represent a minor proportion of

species found in bird markets and farms in influenza endemic areas. More species should be characterized that represent common poultry species from different areas.

B. I found that human viruses were able to bind poultry tissues, particularly in the respiratory tract. Studies looking at actual infectivity of a wider panel of human viruses should be conducted.

C. Studies have found that pH1N1 will readily infect turkeys. Based on our results pH1N1 maybe able to infect other poultry species. This should be investigated.

2) Transmissibility of H9N2:pH1N1 reassortants (Chapters 4, 5)

A. I found H9N2:pH1N1 and H9N1:pH1N1 viruses infected and transmitted in ferrets and pigs. We also showed that poultry species are possibly susceptible to human viruses. These reassorted viruses should be tested in poultry, particularly quail (the host species of the virus used in our study) and turkey (natural infections of pH1N1 and H9N2).

B. I used a Eurasian strain of H9N2 similar to the G1 lineage viruses. North American lineages of H9N2 differ from these strains, but commonly infect turkeys, which are also susceptible to pH1N1. Other Eurasian lineages infect both humans, swine and birds. We also found that a wholly avian H9N2 surface pair on a pH1N1 backbone transmitted in ferrets. We should test different H9N2 lineages reassorted with pH1N1 and other human viruses in poultry, swine and ferrets.

3) Modeling reassortment *in vivo (*Chapter 6)

A. I tested our model in ferrets, a model for human influenza infection, however, reassortment events are common in other species, such as swine and poultry. Tests should be conducted to ensure the plasmid-based inoculation system functions properly in other natural host species such as swine and poultry, but also in other common model species such as mice and guinea pigs.

B. I used 15 plasmids in our study. 16 plasmids would represent two full viruses. 24 plasmids would be equivalent to 3 full viruses. It is unclear what the limit is to the number of plasmids used in our system and still be confident in an equal distribution of all possible combinations. Tests should be undertaken to determine the plasmid number limits of the transfection-based inoculation.

C. It is unclear how big a role the specific host environment has on the outcome of reassortment. Assuming 3A results indicate that the plasmid based system works across different species, tests should be conducted to determine the role the specific host species plays in the outcome of a reassortment event. Would an identical 15-plasmid transfection result in an H9N1? Would the internal genes be similar? What about a reassortment in poultry?

D. I twice isolated an H9N1 from our mixed populations in two attempts; however, there were differences in the internal gene cassettes selected. Studies should be conducted to determine the consistency of the method. Ferrets maybe prohibitively expensive to do large number studies, but if 3A confirms that the

system works in mice, then meaningful statistical analysis can be done to separate random selection of gene segments from selective selection of gene segments.

E. Theoretically, existing immunity could alter the course of a reassortment. If one were to transfect 16 plasmids (two full viruses) and inoculation the mixture into three groups of animals (one naïve, one with immunity to one virus, and one with immunity to the second virus) and then compare the outcome viruses, then one could begin to ascertain the effect prior immunity has on reassortment. This could also shed light on the relative immunogenicity of each gene segment.

Appendices

List of reagents

Reagent	Company	Cat. #
DIG Glycan Differentiation Kit	Roche	11210238001
BSA	Sigma	A2058
AEC	Sigma	A5754
Hematoxylin	Histoserv	--
FITC labeled MAA	EY laboratories	F-7801-2
FITC labeled NSA	EY laboratories	F-6803-1
DAPI	Thermo Scientific	62247
Mounting media	KPL	71-00-16
Centricon filter concentration	Milipore	UFC710008
BigDye terminator v3.1	Applied Biosystems	4337455
FluDetect	Synbiotics	96-6800
TMB substrate system	KPL	50-76-00
TransIT	Mirus	MIR 2305
Implatable transponders	Bio Medics	IPTT-300

Bibliography

Aamir, U.B., Wernery, U., Ilyushina, N., Webster, R.G., 2007. Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology 361, 45-55.

Abolnik, C., Bisschop, S., Gerdes, T., Olivier, A., Horner, R., 2007. Outbreaks of avian influenza H6N2 viruses in chickens arose by a reassortment of H6N8 and H9N2 ostrich viruses. Virus Genes 34, 37-45.

Alexander, D.J., 2000. A review of avian influenza in different bird species. Vet Microbiol 74, 3-13.

Alexander, D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine 25, 5637-5644.

Angel M, K.J., Pena L, Wan H, Perez DR., 2013. In vivo selection of H1N2 influenza virus reassortants in the ferret model. J Virol.

Anthony, S.J., St Leger, J.A., Pugliares, K., Ip, H.S., Chan, J.M., Carpenter, Z.W., Navarrete-Macias, I., Sanchez-Leon, M., Saliki, J.T., Pedersen, J., Karesh, W., Daszak, P., Rabadan, R., Rowles, T., Lipkin, W.I., 2012. Emergence of fatal avian influenza in New England harbor seals. MBio 3, e00166-00112.

Arafa, A.S., Hagag, N., Erfan, A., Mady, W., El-Husseiny, M., Adel, A., Nasef, S., 2012. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. Virus Genes 45, 283-294.

Baigent, S.J., McCauley, J.W., 2003. Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays 25, 657-671.

Banks, J., Speidel, E.C., Harris, P.A., Alexander, D.J., 2000. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Pathol 29, 353-359.

Baudin, F., Petit, I., Weissenhorn, W., Ruigrok, R.W., 2001. In vitro dissection of the membrane and RNP binding activities of influenza virus M1 protein. Virology 281, 102-108.

Baum, L.G., Paulson, J.C., 1990. Sialyloligosaccharides of the respiratory epithelium in the selection of human influeza virus receptor specificity. Acta Histochemistry Suppl. 40, 4.

Beare, A.S., Webster, R.G., 1991. Replication of avian influenza viruses in humans. Arch Virol 119, 37-42.

Berhane, Y., Ojkic, D., Neufeld, J., Leith, M., Hisanaga, T., Kehler, H., Ferencz, A., Wojcinski, H., Cottam-Birt, C., Suderman, M., Handel, K., Alexandersen, S., Pasick, J., 2010. Molecular characterization of pandemic H1N1 influenza viruses isolated from turkeys and pathogenicity of a human pH1N1 isolate in turkeys. Avian Dis 54, 1275-1285.

Biswas, S.K., Nayak, D.P., 1994. Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. J Virol 68, 1819-1826.

Blaas, D., Patzelt, E., Kuechler, E., 1982. Identification of the cap binding protein of influenza virus. Nucleic Acids Res 10, 4803-4812.

Blanco, J.C., Pletneva, L.M., Wan, H., Araya, Y., Angel, M., Oue, R.O., Sutton, T.C., Perez, D.R., 2013. Receptor characterization and susceptibility of cotton rats to avian and 2009 pandemic influenza virus strains. J Virol 87, 2036-2045.

Bourmakina, S.V., Garcia-Sastre, A., 2003. Reverse genetics studies on the filamentous morphology of influenza A virus. J Gen Virol 84, 517-527.

Brown, I.H., Banks, J., Manvell, R.J., Essen, S.C., Shell, W., Slomka, M., Londt, B., Alexander, D.J., 2006. Recent epidemiology and ecology of influenza A viruses in avian species in Europe and the Middle East. Dev Biol (Basel) 124, 45-50.

Bussetti, A.V., Palacios, G., Travassos da Rosa, A., Savji, N., Jain, K., Guzman, H., Hutchison, S., Popov, V.L., Tesh, R.B., Lipkin, W.I., 2012. Genomic and antigenic characterization of Jos virus. J Gen Virol 93, 293-298.

Butenko, A.M., Leshchinskaia, E.V., Semashko, I.V., Donets, M.A., Mart'ianova, L.I., 1987. [Dhori virus--a causative agent of human disease. 5 cases of laboratory infection]. Vopr Virusol 32, 724-729.

Butt KM, S.G., Chen H, Zhang LJ, Leung YH, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JS, Guan Y., 2005. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43, 5760-5767.

Butt, K.M., Smith, G.J., Chen, H., Zhang, L.J., Leung, Y.H., Xu, K.M., Lim, W., Webster, R.G., Yuen, K.Y., Peiris, J.S., Guan, Y., 2005. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43, 5760-5767.

Capua, I., Mutinelli, F., Marangon, S., Alexander, D.J., 2000. H7N1 avian influenza in Italy (1999 to 2000) in intensively reared chickens and turkeys. Avian Pathol 29, 537-543.

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

Chen, H., Bright, R.A., Subbarao, K., Smith, C., Cox, N.J., Katz, J.M., Matsuoka, Y., 2007. Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 influenza viruses in mice. Virus Res 128, 159-163.

Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J.R., Yewdell, J.W., 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7, 1306-1312.

Cheng, V.C., Chan, J.F., Wen, X., Wu, W.L., Que, T.L., Chen, H., Chan, K.H., Yuen, K.Y., 2011. Infection of immunocompromised patients by avian H9N2 influenza A virus. J Infect 62, 394-399.

Choi, Y.K., Ozaki, H., Webby, R.J., Webster, R.G., Peiris, J.S., Poon, L., Butt, C., Leung, Y.H., Guan, Y., 2004. Continuing evolution of H9N2 influenza viruses in Southeastern China. J Virol 78, 8609-8614.

Chou YY, A.R., Pica N, Lowen AC, Richt JA, García-Sastre A, Palese P, Hai R., 2011. The M segment of the 2009 new pandemic H1N1 influenza virus is critical for its high transmission efficiency in the guinea pig model. J Virol 85.

Chu, C.M., Dawson, I.M., Elford, W.J., 1949. Filamentous forms associated with newly isolated influenza virus. Lancet 1, 602.

Clouthier, S.C., Rector, T., Brown, N.E., Anderson, E.D., 2002. Genomic organization of infectious salmon anaemia virus. J Gen Virol 83, 421-428.

Conenello, G.M., Palese, P., 2007. Influenza A virus PB1-F2: a small protein with a big punch. Cell Host Microbe 2, 207-209.

Cong, Y.L., Pu, J., Liu, Q.F., Wang, S., Zhang, G.Z., Zhang, X.L., Fan, W.X., Brown, E.G., Liu, J.H., 2007. Antigenic and genetic characterization of H9N2 swine influenza viruses in China. J Gen Virol 88, 2035-2041.

Connor, R.J., Kawaoka, Y., Webster, R.G., Paulson, J.C., 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205, 17-23.

Couceiro, J.N., Paulson, J.C., Baum, L.G., 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 29, 155-165.

Crawford, P.C., Dubovi, E.J., Castleman, W.L., Stephenson, I., Gibbs, E.P., Chen, L., Smith, C., Hill, R.C., Ferro, P., Pompey, J., Bright, R.A., Medina, M.J., Johnson, C.M., Olsen, C.W., Cox, N.J., Klimov, A.I., Katz, J.M., Donis, R.O., 2005. Transmission of equine influenza virus to dogs. Science 310, 482-485.

Cross, K.J., Burleigh, L.M., Steinhauer, D.A., 2001. Mechanisms of cell entry by influenza virus. Expert Rev Mol Med 3, 1-18.

Da Silva, E.V., Da Rosa, A.P., Nunes, M.R., Diniz, J.A., Tesh, R.B., Cruz, A.C., Vieira, C.M., Vasconcelos, P.F., 2005. Araguari virus, a new member of the family Orthomyxoviridae: serologic, ultrastructural, and molecular characterization. Am J Trop Med Hyg 73, 1050-1058.

Dias, A., Bouvier, D., Crepin, T., McCarthy, A.A., Hart, D.J., Baudin, F., Cusack, S., Ruigrok, R.W., 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458, 914-918.

Dong, G., Luo, J., Zhang, H., Wang, C., Duan, M., Deliberto, T.J., Nolte, D.L., Ji, G., He, H., 2011. Phylogenetic diversity and genotypical complexity of H9N2 influenza A viruses revealed by genomic sequence analysis. PLoS One 6, e17212.

Drake, J.W., 1993. Rates of spontaneous mutation among RNA viruses. Proc Natl Acad Sci U S A 90, 4171-4175.

Dudek, S.E., Wixler, L., Nordhoff, C., Nordmann, A., Anhlan, D., Wixler, V., Ludwig, S., 2011. The influenza virus PB1-F2 protein has interferon antagonistic activity. Biol Chem 392, 1135-1144.

Elleman, C.J., Barclay, W.S., 2004. The M1 matrix protein controls the filamentous phenotype of influenza A virus. Virology 321, 144-153.

Fereidouni, S.R., Werner, O., Starick, E., Beer, M., Harder, T.C., Aghakhan, M., Modirrousta, H., Amini, H., Moghaddam, M.K., Bozorghmehrifard, M.H., Akhavizadegan, M.A., Gaidet, N., Newman, S.H., Hammoumi, S., Cattoli, G., Globig, A., Hoffmann, B., Sehati, M.E., Masoodi, S., Dodman, T., Hagemeijer, W., Mousakhani, S., Mettenleiter, T.C., 2010. Avian influenza virus monitoring in wintering waterbirds in Iran, 2003-2007. Virol J 7, 43.

Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D., 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79, 2814-2822.

Fujii, K., Fujii, Y., Noda, T., Muramoto, Y., Watanabe, T., Takada, A., Goto, H., Horimoto, T., Kawaoka, Y., 2005. Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. J Virol 79, 3766-3774.

Fusaro, A., Monne, I., Salviato, A., Valastro, V., Schivo, A., Amarin, N.M., Gonzalez, C., Ismail, M.M., Al-Ankari, A.R., Al-Blowi, M.H., Khan, O.A., Maken Ali, A.S., Hedayati, A., Garcia Garcia, J., Ziay, G.M., Shoushtari, A., Al Qahtani, K.N., Capua, I., Holmes, E.C., Cattoli, G., 2011. Phylogeography and

evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol 85, 8413-8421.

Gambaryan, A., Tuzikov, A., Pazynina, G., Bovin, N., Balish, A., Kilmov, A., 2006. Evolution of the receptor binding phenotype of influenza A (H5) viruses. Virology 344, 7.

Gambaryan, A., Webster, R., Matrosovich, M., 2002. Differences between influenza virus receptors on target cells of duck and chicken. Arch Virol 147, 1197-1208.

Golender, N., Panshin, A., Banet-Noach, C., Nagar, S., Pokamunski, S., Pirak, M., Tendler, Y., Davidson, I., Garcia, M., Perk, S., 2008. Genetic characterization of avian influenza viruses isolated in Israel during 2000-2006. Virus Genes 37, 289-297.

Gomez-Puertas, P., Albo, C., Perez-Pastrana, E., Vivo, A., Portela, A., 2000. Influenza virus matrix protein is the major driving force in virus budding. J Virol 74, 11538-11547.

Gong, J., Xu, W., Zhang, J., 2007. Structure and functions of influenza virus neuraminidase. Curr Med Chem 14, 113-122.

Gonzalez, S., Zurcher, T., Ortin, J., 1996. Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic Acids Res 24, 4456-4463.

Gou, Y., Xie, J., Wang, M., 2000. [A strain of influenza A H9N2 virus repeatedly isolated from human population in China]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 14, 209-212.

Graef, K.M., Vreede, F.T., Lau, Y.F., McCall, A.W., Carr, S.M., Subbarao, K., Fodor, E., 2010. The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. J Virol 84, 8433-8445.

Guan, Y., Shortridge, K.F., Krauss, S., Webster, R.G., 1999. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci U S A 96, 9363-9367.

Guo Y, Li J, X, C., 1999. Discovery of men infected by avian influenza A (H9N2) virus. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 13, 105-108.

Guo, Y.J., Krauss, S., Senne, D.A., Mo, I.P., Lo, K.S., Xiong, X.P., Norwood, M., Shortridge, K.F., Webster, R.G., Guan, Y., 2000. Characterization of the Pathogenicity of Members of the Newly Established H9N2 Influenza Virus Lineages in Asia. Virology 267, 10.

Guo, Y.J., Wen, L.Y., Zhang, Y., Wang, M., Guo, J.F., Li, Z., Shu, Y.L., 2005. [Origin of internal genes of two strains of swine influenza A (H1N1) virus]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 19, 358-361.

Henneberg, G., Wagner, L., 1953. [Determination of the value of influenza virus vaccine in guinea pig]. Zentralbl Bakteriol Orig 159, 482-485.

Hinshaw, V.S., Bean, W.J., Geraci, J., Fiorelli, P., Early, G., Webster, R.G., 1986. Characterization of two influenza A viruses from a pilot whale. J Virol 58, 655-656.

Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U S A. 97, 6108-6113.

Homme, P.J., Easterday, B.C., Anderson, D.P., 1970. Avian influenza virus infections. II. Experimental epizootiology of influenza A-turkey-Wisconsin-1966 virus in turkeys. Avian Dis 14, 240-247.

Huang, I.C., Li, W., Sui, J., Marasco, W., Choe, H., Farzan, M., 2008. Influenza A virus neuraminidase limits viral superinfection. J Virol 82, 4834-4843.

Hulse, D.J., Webster, R.G., Russell, R.J., Perez, D.R., 2004. Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. J Virol 78, 9954-9964.

Hutchinson, E.C., von Kirchbach, J.C., Gog, J.R., Digard, P., 2010. Genome packaging in influenza A virus. J Gen Virol 91, 313-328.

Iqbal, M., Yaqub, T., Reddy, K., McCauley, J.W., 2009. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PLoS One 4, e5788.

Ito, T., Couceiro, J.N., Kelm, S., Baum, L.G., Krauss, S., Castrucci, M.R., Donatelli, I., Kida, H., Paulson, J.C., Webster, R.G., Kawaoka, Y., 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72, 7367-7373.

Ito, T., Suzuki, Y., Mitnaul, L., Vines, A., Kida, H., Kawaoka, Y., 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology 227, 493-499.

Iwatsuki-Horimoto, K., Horimoto, T., Noda, T., Kiso, M., Maeda, J., Watanabe, S., Muramoto, Y., Fujii, K., Kawaoka, Y., 2006. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. J Virol 80, 5233-5240.

Jagger, B.W., Wise, H.M., Kash, J.C., Walters, K.A., Wills, N.M., Xiao, Y.L., Dunfee, R.L., Schwartzman, L.M., Ozinsky, A., Bell, G.L., Dalton, R.M., Lo, A.,

Efstathiou, S., Atkins, J.F., Firth, A.E., Taubenberger, J.K., Digard, P., 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337, 199-204.

Jia, N., Wang, S.X., Liu, Y.X., Zhang, P.H., Zuo, S.Q., Lin, Z., Dang, R.L., Ma, Y.H., Zhang, C., Zhang, L., Lu, S., Cao, W.C., 2008. Increased sensitivity for detecting avian influenza-specific antibodies by a modified hemagglutination inhibition assay using horse erythrocytes. J Virol Methods 153, 43-48.

Jin, H., Leser, G.P., Zhang, J., Lamb, R.A., 1997. Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. EMBO J 16, 1236-1247.

Kawaoka, Y., Chambers, T.M., Sladen, W.L., Webster, R.G., 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 163, 247-250.

Kimble, J., Sorell, E., Shao, H., Martin, P., Perez, D., 2011. Compatibility of H9N2 avian influenza surface genes and 2009 pandemic H1N1 internal genes for transmission in the ferret model. Proc Natl Acad Sci U S A 108.

Kobasa, D., Jones, S.M., Shinya, K., Kash, J.C., Copps, J., Ebihara, H., Hatta, Y., Kim, J.H., Halfmann, P., Hatta, M., Feldmann, F., Alimonti, J.B., Fernando, L., Li, Y., Katze, M.G., Feldmann, H., Kawaoka, Y., 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 445, 319-323.

Kuchipudi, S.V., Nelli, R., White, G.A., Bain, M., Chang, K.C., Dunham, S., 2009. Differences in influenza virus receptors in chickens and ducks: implications for interspecies transmission. J Mol Genet Med 3, 9.

Kumari, K., Gulati, S., Smith, D.F., Gulati, U., Cummings, R.D., Air, G.M., 2007. Receptor binding specificity of recent human H3N2 influenza viruses. Virol J 4, 42.

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

Le Goffic, R., Arshad, M.I., Rauch, M., L'Helgoualc'h, A., Delmas, B., Piquet-Pellorce, C., Samson, M., 2011. Infection with influenza virus induces IL-33 in murine lungs. Am J Respir Cell Mol Biol 45, 1125-1132.

Lee, C.W., Song, C.S., Lee, Y.J., Mo, I.P., Garcia, M., Suarez, D.L., Kim, S.J., 2000. Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the pathogenic potential of isolate MS96. Avian Dis 44, 527-535.

Li, C., Yu, K., Tian, G., Yu, D., Liu, L., Jing, B., Ping, J., Chen, H., 2005. Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 340, 70-83.

Li, K.S., Xu, K.M., Peiris, J.S., Poon, L.L., Yu, K.Z., Yuen, K.Y., Shortridge, K.F., Webster, R.G., Guan, Y., 2003. Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? J Virol 77, 6988-6994.

Li, Q., Sun, X., Li, Z., Liu, Y., Vavricka, C.J., Qi, J., Gao, G.F., 2012. Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. Proc Natl Acad Sci U S A 109, 18897-18902.

Liang, Y., Hong, Y., Parslow, T.G., 2005. cis-Acting packaging signals in the influenza virus PB1, PB2, and PA genomic RNA segments. J Virol 79, 10348-10355.

Lu, B., Zhou, H., Ye, D., Kemble, G., Jin, H., 2005. Improvement of influenza A/Fujian/411/02 (H3N2) virus growth in embryonated chicken eggs by balancing the hemagglutinin and neuraminidase activities, using reverse genetics. J Virol 79, 6763-6771.

Ma W, K.R., Richt JA, 2008. The pig as a mixing vessel for influenza viruses: Human and veterinary implications. J Mol Genet Med 3.

Ma W, L.Q., Bawa B, Qiao C, Qi W, Shen H, Chen Y, Ma J, Li X, Webby RJ, García-Sastre A, Richt JA., 2012. The neuraminidase and matrix genes of the 2009 pandemic influenza H1N1 virus cooperate functionally to facilitate efficient replication and transmissibility in pigs. J Gen Virol 93, 1261-1268.

Maines, T.R., Szretter, K.J., Perrone, L., Belser, J.A., Bright, R.A., Zeng, H., Tumpey, T.M., Katz, J.M., 2008. Pathogenesis of emerging avian influenza viruses in mammals and the host innate immune response. Immunol Rev 225, 68-84.

Matrosovich, M.N., Krauss, S., Webster, R.G., 2001. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281, 156-162.

Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D., 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol 78, 12665-12667.

Mazur, I., Anhlan, D., Mitzner, D., Wixler, L., Schubert, U., Ludwig, S., 2008. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell Microbiol 10, 1140-1152.

Melen, K., Fagerlund, R., Franke, J., Kohler, M., Kinnunen, L., Julkunen, I., 2003. Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein. J Biol Chem 278, 28193-28200.

Memoli MJ, J.B., Dugan VG, Qi L, Jackson JP, Taubenberger JK, 2009. Recent human influenza A/H3N2 virus evolution driven by novel selection factors in addition to atigenic drift. J Infect Dis 200, 1232-1241.

Moore, D.L., Causey, O.R., Carey, D.E., Reddy, S., Cooke, A.R., Akinkugbe, F.M., David-West, T.S., Kemp, G.E., 1975. Arthropod-borne viral infections of man in Nigeria, 1964-1970. Ann Trop Med Parasitol 69, 49-64.

Munster, V.J., Baas, C., Lexmond, P., Waldenstrom, J., Wallensten, A., Fransson, T., Rimmelzwaan, G.F., Beyer, W.E., Schutten, M., Olsen, B., Osterhaus, A.D., Fouchier, R.A., 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 3, e61.

Munster VJ, d.W.E., van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, van de Vijver D, Boucher CA, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA, 2009. Pathogenesis and transmission of swine-origin 2009 A (H1N1) influenza virus in ferrets. Science 325, 481-483.

Muraki, Y., Hongo, S., 2010. The molecular virology and reverse genetics of influenza C virus. Jpn J Infect Dis 63, 157-165.

Muramoto, Y., Noda, T., Kawakami, E., Akkina, R., Kawaoka, Y., 2013. Identification of Novel Influenza A Virus Proteins Translated from PA mRNA. J Virol 87, 2455-2462.

Mushtaq, M.H., Juan, H., Jiang, P., Li, Y., Li, T., Du, Y., Mukhtar, M.M., 2008. Complete genome analysis of a highly pathogenic H5N1 influenza A virus isolated from a tiger in China. Arch Virol 153, 1569-1574.

Naeem, K., Ullah, A., Manvell, R.J., Alexander, D.J., 1999. Avian influenza A subtype H9N2 in poultry in Pakistan. Vet Rec 145, 560.

Nayak, D.P., Hui, E.K., Barman, S., 2004. Assembly and budding of influenza virus. Virus Res 106, 147-165.

Neumann, G., Noda, T., Kawaoka, Y., 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459, 931-939.

Nili, H., Asasi, K., 2002. Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol 31, 247-252.

Nobusawa, E., Sato, K., 2006. Comparison of the mutation rates of human influenza A and B viruses. J Virol 80, 3675-3678.

Noda, T., Sugita, Y., Aoyama, K., Hirase, A., Kawakami, E., Miyazawa, A., Sagara, H., Kawaoka, Y., 2012. Three-dimensional analysis of ribonucleoprotein complexes in influenza A virus. Nat Commun 3, 639.

Osterhaus, A.D., Rimmelzwaan, G.F., Martina, B.E., Bestebroer, T.M., Fouchier, R.A., 2000. Influenza B virus in seals. Science 288, 1051-1053.

Palese, P., Shaw, M., 2007. Orthomyxoviridae: the viruses and their replication, 1689 ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Pappas, C., Aguilar, P.V., Basler, C.F., Solorzano, A., Zeng, H., Perrone, L.A., Palese, P., Garcia-Sastre, A., Katz, J.M., Tumpey, T.M., 2008. Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. Proc Natl Acad Sci U S A 105, 3064-3069.

Patterson, A.R., Cooper, V.L., Yoon, K.J., Janke, B.H., Gauger, P.C., 2009. Naturally occurring influenza infection in a ferret (Mustela putorius furo) colony. J Vet Diagn Invest 21, 527-530.

Pawar, S.D., Tandale, B.V., Raut, C.G., Parkhi, S.S., Barde, T.D., Gurav, Y.K., Kode, S.S., Mishra, A.C., 2012. Avian Influenza H9N2 Seroprevalence among Poultry Workers in Pune, India, 2010. PLoS One 7, e36374.

Peiris, J.S., Guan, Y., Markwell, D., Ghose, P., Webster, R.G., Shortridge, K.F., 2001. Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J Virol 75, 9679-9686.

Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Ip, P.L., Lai, R.W., Orr, W.K., Shortridge, K.F., 1999. Human infection with influenza H9N2. Lancet 354, 916-917.

Pereda A, C.J., Quiroga MA, Baumeister E, Insarralde L, Ibar M, Sanguinetti R, Cannilla ML, Franzese D, Escobar Cabrera OE, Craig MI, Rimondi A, Machuca M, Debenedetti RT, Zenobi C, Barral L, Balzano R, Capalbo S, Risso A, Perfumo CJ., 2010. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis 16.

Perez, D.R., Donis, R.O., 2001. Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. J Virol 75, 8127-8136.

Perez, D.R., Lim, W., Seiler, J.P., Yi, G., Peiris, M., Shortridge, K.F., Webster, R.G., 2003. Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. J Virol 77, 3148-3156.

Perez, J.T., Varble, A., Sachidanandam, R., Zlatev, I., Manoharan, M., Garcia-Sastre, A., tenOever, B.R., 2010. Influenza A virus-generated small RNAs regulate the switch from transcription to replication. Proc Natl Acad Sci U S A 107, 11525-11530.

Perk, S., Panshin, A., Shihmanter, E., Gissin, I., Pokamunski, S., Pirak, M., Lipkind, M., 2006. Ecology and molecular epidemiology of H9N2 avian influenza viruses isolated in Israel during 2000-2004 epizootic. Dev Biol (Basel), 201-209.

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

Pillai, S.P., Lee, C.W., 2010. Species and age related differences in the type and distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. Virol J 7, 5.

Portela, A., Digard, P., 2002. The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J Gen Virol 83, 723-734.

Potter, C.W., 2001. A history of influenza. J Appl Microbiol 91, 572-579.

Presti, R.M., Zhao, G., Beatty, W.L., Mihindukulasuriya, K.A., da Rosa, A.P., Popov, V.L., Tesh, R.B., Virgin, H.W., Wang, D., 2009. Quaranfil, Johnston Atoll, and Lake Chad viruses are novel members of the family Orthomyxoviridae. J Virol 83, 11599-11606.

Qiao, C., Liu, Q., Bawa, B., Shen, H., Qi, W., Chen, Y., Mok, C.K., Garcia-Sastre, A., Richt, J.A., Ma, W., 2012. Pathogenicity and transmissibility of reassortant H9 influenza viruses with genes from pandemic H1N1 virus. J Gen Virol 93, 2337-2345.

Reid, S.M., Cox, W.J., Ceeraz, V., Sutton, D., Essen, S.C., Howard, W.A., Slomka, M.J., Irvine, R.M., Brown, I.H., 2012. First reported detection of influenza A (H1N1)pdm09 in turkeys in the United Kingdom. Avian Dis 56, 1062-1067.

Richardson, J.C., Akkina, R.K., 1991. NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. Arch Virol 116, 69-80.

Robertson, J.S., Schubert, M., Lazzarini, R.A., 1981. Polyadenylation sites for influenza virus mRNA. J Virol 38, 157-163.

Rott, R., Klenk, H.D., Nagai, Y., Tashiro, M., 1995. Influenza viruses, cell enzymes, and pathogenicity. Am J Respir Crit Care Med 152, S16-19.

Rowe T, L.A., Crevar CJ, Carter DM, Xu L, Ran L, Fang Y, Cameron CM, Cameron MJ, Banner D, Ng DC, Ran R, Weirback HK, Wiley CA, Kelvin DJ, Ross TM., 2010. Modeling host responses in ferrets during A/California/07/2009 influenza infection. Virology 401, 257-265.

Rui-Hua, Z., Hong-Yu, C., Ming-Ju, X., Kai, L., Hua-Lan, C., Cun-Lian, W., Dong, W., Cun-Xin, L., Tong, X., 2011. Molecular characterization and pathogenicity of swine influenza H9N2 subtype virus A/swine/HeBei/012/2008/ (H9N2). Acta Virol 55, 219-226.

Ruigrok, R.W., Andree, P.J., Hooft van Huysduynen, R.A., Mellema, J.E., 1984. Characterization of three highly purified influenza virus strains by electron microscopy. J Gen Virol 65 (Pt 4), 799-802.

Salomon, R., Franks, J., Govorkova, E.A., Ilyushina, N.A., Yen, H.L., Hulse-Post, D.J., Humberd, J., Trichet, M., Rehg, J.E., Webby, R.J., Webster, R.G., Hoffmann, E., 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med 203, 689-697.

Schafer, W., 1955. Vergleichende sero-immunologische Untersuchungen über die Viren der Influenza und der klassischen Geflügelpest. Zeitschrift für Naturforschung 10b, 81-91.

Schmitt, A.P., Lamb, R.A., 2005. Influenza virus assembly and budding at the viral budozone. Adv Virus Res 64, 383-416.

Scholtissek, C., Hinshaw, V.S., Olsen, C.W., 1998. Influenza in Pigs and their role as the intermediate host., in: Nicholson, K.G., Webster, R.G., Hay, A.J. (Eds.), Textbook of Influenza. Blackwell Science Ltd., Oxford, pp. 137-145.

Scholtissek, C., Rohde, W., Von Hoyningen, V., Rott, R., 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87, 13-20.

Schrauwen, E., Herfst, S, Salin Chutinimitkul, S, Bestebroer, TM, Rimmelzwaan, GF, Osterhaus, A, Kuiken, T, and Fouchier, RAM, 2011. Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment. Emerg Infect Dis 17.

Schrauwen, E.J., Herfst, S., Chutinimitkul, S., Bestebroer, T.M., Rimmelzwaan, G.F., Osterhaus, A.D., Kuiken, T., Fouchier, R.A., 2011. Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment. Emerg Infect Dis 17, 200-208.

Sharp, G.B., Kawaoka, Y., Jones, D.J., Bean, W.J., Pryor, S.P., Hinshaw, V., Webster, R.G., 1997. Coinfection of wild ducks by influenza A viruses: distribution patterns and biological significance. J Virol 71, 6128-6135.

Sharp, G.B., Kawaoka, Y., Wright, S.M., Turner, B., Hinshaw, V., Webster, R.G., 1993. Wild ducks are the reservoir for only a limited number of influenza A subtypes. Epidemiol Infect 110, 161-176.

Shaw, M.L., Stone, K.L., Colangelo, C.M., Gulcicek, E.E., Palese, P., 2008. Cellular proteins in influenza virus particles. PLoS Pathog 4, e1000085.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., Kawaoka, Y., 2006. Avian flu: influenza virus receptors in the human airway. Nature 440, 435-436.

Shope, R.E., 1931a. The Etiology of Swine Influenza. Science 73, 214-215.

Shope, R.E., 1931b. Swine Influenza : Iii. Filtration Experiments and Etiology. J Exp Med 54, 373-385.

Shortridge, K.F., 1992. Pandemic influenza: a zoonosis? Semin Respir Infect 7, 11-25.

Slomka, M.J., Hanna, A., Mahmood, S., Govil, J., Krill, D., Manvell, R.J., Shell, W., Arnold, M.E., Banks, J., Brown, I.H., 2013. Phylogenetic and molecular characteristics of Eurasian H9 avian influenza viruses and their detection by two different H9-specific RealTime reverse transcriptase polymerase chain reaction tests. Vet Microbiol 162, 530-542.

Smith, G., Bahl, J., Vijaykrishna, D., Zhang, J., Poon, L.L., Chen, H., Webster, R.G., Peiris, J.S., Guan, Y., 2009a. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A 106, 11709-11712.

Smith, G., Vijaykrishna, D., Bahl, J., Lycett, S., Worobey, M., Pybus, O., Ma, S., Cheung, C., Raghwani, J., Bhatt, S., Peiris, J.S., Guan, Y., Rambaut, A., 2009b. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459, 1122-1125.

Smith, H., Sweet, C., 1988. Lessons for human influenza from pathogenicity studies with ferrets. Rev Infect Dis 10, 56-75.

Song, J., Feng, H., Xu, J., Zhao, D., Shi, J., Li, Y., Deng, G., Jiang, Y., Li, X., Zhu, P., Guan, Y., Bu, Z., Kawaoka, Y., Chen, H., 2011. The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. J Virol 85, 2180-2188.

Sorrell, E.M., Song, H., Pena, L., Perez, D.R., 2010. A 27-amino-acid deletion in the neuraminidase stalk supports replication of an avian H2N2 influenza A virus in the respiratory tract of chickens. J Virol 84, 11831-11840.

Sorrell, E.M., Wan, H., Araya, Y., Song, H., Perez, D.R., 2009. Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc Natl Acad Sci U S A.

Sponseller, B.A., Strait, E., Jergens, A., Trujillo, J., Harmon, K., Koster, L., Jenkins-Moore, M., Killian, M., Swenson, S., Bender, H., Waller, K., Miles, K., Pearce, T., Yoon, K.J., Nara, P., 2010. Influenza A pandemic (H1N1) 2009 virus infection in domestic cat. Emerg Infect Dis 16, 534-537.

Stevens, J., Blixt, O., Chen, L.M., Donis, R.O., Paulson, J.C., Wilson, I.A., 2008. Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity. J Mol Bio 381, 13.

Subbarao, E.K., London, W., Murphy, B.R., 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67, 1761-1764.

Subbarao, K., Joseph, T., 2007. Scientific barriers to developing vaccines against avian influenza viruses. Nat Rev Immunol 7, 267-278.

Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., Cox, N., 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279, 393-396.

Sun Y, Q.K., Wang J, Pu J, Tang Q, Hu Y, Bi Y, Zhao X, Yang H, Shu Y, Liu J, 2011. High genetic compatibliity and increased pathologenicity of reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza viruses. Proc Natl Acad Sci U S A.

Suzuki, Y., 2005. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull 28, 399-408.

Taubenberger, J.K., Morens, D.M., 2006. 1918 Influenza: the mother of all pandemics. Emerg Infect Dis 12, 15-22.

Taubenberger, J.K., Reid, A.H., Lourens, R.M., Wang, R., Jin, G., Fanning, T.G., 2005. Characterization of the 1918 influenza virus polymerase genes. Nature 437, 889-893.

Taylor, R.M., Hurlbut, H.S., Work, T.H., Kingston, J.R., Hoogstraal, H., 1966. Arboviruses isolated from ARGAS TICKS IN Egypt: Quaranfil, Chenuda, and Nyamanini. Am J Trop Med Hyg 15, 76-86.

Tombari, W., Nsiri, J., Larbi, I., Guerin, J.L., Ghram, A., 2011. Genetic evolution of low pathogenecity H9N2 avian influenza viruses in Tunisia: acquisition of new mutations. Virol J 8, 467.

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D.A., Chen, L.M., Recuenco, S., Ellison, J.A., Davis, C.T., York, I.A., Turmelle, A.S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M.R., Tang, K., Rowe, L.A., Sammons, S., Xu, X., Frace, M., Lindblade, K.A., Cox, N.J., Anderson, L.J., Rupprecht, C.E., Donis, R.O., 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A 109, 4269-4274.

Tosh, C., Nagarajan, S., Behera, P., Rajukumar, K., Purohit, K., Kamal, R.P., Murugkar, H.V., Gounalan, S., Pattnaik, B., Vanamayya, P.R., Pradhan, H.K., Dubey, S.C., 2008. Genetic analysis of H9N2 avian influenza viruses isolated from India. Arch Virol 153, 1433-1439.

Uyeki, T.M., Chong, Y.H., Katz, J.M., Lim, W., Ho, Y.Y., Wang, S.S., Tsang, T.H., Au, W.W., Chan, S.C., Rowe, T., Hu-Primmer, J., Bell, J.C., Thompson, W.W.,

Bridges, C.B., Cox, N.J., Mak, K.H., Fukuda, K., 2002. Lack of evidence for human-to-human transmission of avian influenza A (H9N2) viruses in Hong Kong, China 1999. Emerg Infect Dis 8, 154-159.

Uyeki, T.M., Nguyen, D.C., Rowe, T., Lu, X., Hu-Primmer, J., Huynh, L.P., Hang, N.L., Katz, J.M., 2012. Seroprevalence of antibodies to avian influenza A (H5) and A (H9) viruses among market poultry workers, Hanoi, Vietnam, 2001. PLoS One 7, e43948.

Varga, Z.T., Ramos, I., Hai, R., Schmolke, M., Garcia-Sastre, A., Fernandez-Sesma, A., Palese, P., 2011. The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS Pathog 7, e1002067.

Vijaykrishna, D., Poon, L.L., Zhu, H.C., Ma, K., Li, O.T.W., Cheung, C.L., Smith, G.J.D., Peiris, J.S., Guan, Y., 2010. Reassortment of Pandemic H1N1/2009 Influenza A Virus in Swine. Science 328, 1529.

Vincent AL, M.W., Lager KM, Janke BH, Richt JA, 2008. Swine influenza viruses a North American perspective. Adv Virus Res 72, 127-154.

Waddell, G.H., Teigland, M.B., Sigel, M.M., 1963. A New Influenza Virus Associated with Equine Respiratory Disease. J Am Vet Med Assoc 143, 587-590.

Wagner, R., Matrosovich, M., Klenk, H.D., 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol 12, 159-166.

Wan, H., Perez, D.R., 2006. Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 346, 278-286.

Wan, H., Perez, D.R., 2007. Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J Virol 81, 5181-5191.

Wan, H., Sorrell, E.M., Song, H., Hossain, M.J., Ramirez-Nieto, G., Monne, I., Stevens, J., Cattoli, G., Capua, I., Chen, L.M., Donis, R.O., Busch, J., Paulson, J.C., Brockwell, C., Webby, R., Blanco, J., Al-Natour, M.Q., Perez, D.R., 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One 3, e2923.

Wang, B., Chen, Q., Chen, Z., 2012. Complete genome sequence of an H9N2 avian influenza virus isolated from egret in Lake Dongting wetland. J Virol 86, 11939.

Wang, M., Fu, C.-X., Zheng, B.-J., 2009. Antibodies against H5 and H9 Avian Influenza among Poultry Workers in China. New England Journal of Medicine 360, 2583-2584.

Wang, P., Palese, P., O'Neill, R.E., 1997. The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal. J Virol 71, 1850-1856.

Watanabe, T., Watanabe, S., Noda, T., Fujii, Y., Kawaoka, Y., 2003. Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes. J Virol 77, 10575-10583.

Webster, R.G., Laver, W.G., Kilbourne, E.D., 1968. Reactions of antibodies with surface antigens of influenza virus. J Gen Virol 3, 315-326.

Wilson Smith, C. H. Andrewes, P. P. Laidlaw, Timbury, M.C., 1933. A virus obtained from influenza patients. Lancet, 66-68.

Wise, H.M., Foeglein, A., Sun, J., Dalton, R.M., Patel, S., Howard, W., Anderson, E.C., Barclay, W.S., Digard, P., 2009. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol 83, 8021-8031.

Wu, W., Air, G.M., 2004. Binding of influenza viruses to sialic acids: reassortant viruses with A/NWS/33 hemagglutinin bind to alpha2,8-linked sialic acid. Virology 325, 11.

Xie, Q., Yan, Z., Ji, J., Zhang, H., Liu, J., Sun, Y., Li, G., Chen, F., Xue, C., Ma, J., Bee, Y., 2012. Complete Genome Sequence of a Novel H9N2 Subtype Influenza Virus FJG9 Strain in China Reveals a Natural Reassortant Event. J Virol 86, 10240-10241.

Xu, C., Fan, W., Wei, R., Zhao, H., 2004. Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003 (H9N2) virus. Microbes Infect 6, 919-925.

Xu, K., Ferreri, L., Rimondi, A., Olivera, V., Romano, M., Ferreyra, H., Rago, V., Uhart, M., Chen, H., Sutton, T., Pereda, A., Perez, D.R., 2012. Isolation and characterization of an H9N2 influenza virus isolated in Argentina. Virus Res 168, 41-47.

Xu, X.J., Xu, G.Y., Zhou, H.B., Yu, Z.J., Zhang, A.D., Song, Y.F., Jin, M.L., Chen, H.C., 2008. Evolutionary characterization of influenza virus A/duck/Hubei/W1/2004 (H9N2) isolated from central China. Virus Genes 36, 79-83.

Ye, Z., Liu, T., Offringa, D.P., McInnis, J., Levandowski, R.A., 1999. Association of influenza virus matrix protein with ribonucleoproteins. J Virol 73, 7467-7473.

Yen, H.L., Liang, C.H., Wu, C.Y., Forrest, H.L., Ferguson, A., Choy, K.T., Jones, J., Wong, D.D., Cheung, P.P., Hsu, C.H., Li, O.T., Yuen, K.M., Chan, R.W., Poon, L.L., Chan, M.C., Nicholls, J.M., Krauss, S., Wong, C.H., Guan, Y., Webster, R.G., Webby, R.J., Peiris, M., 2011. Hemagglutinin-neuraminidase balance

confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proc Natl Acad Sci U S A 108, 14264-14269.

Yu, H., Hua, R.H., Wei, T.C., Zhou, Y.J., Tian, Z.J., Li, G.X., Liu, T.Q., Tong, G.Z., 2008. Isolation and genetic characterization of avian origin H9N2 influenza viruses from pigs in China. Vet Microbiol 131, 82-92.

Yu, H., Zhou, Y.J., Li, G.X., Ma, J.H., Yan, L.P., Wang, B., Yang, F.R., Huang, M., Tong, G.Z., 2011. Genetic diversity of H9N2 influenza viruses from pigs in China: a potential threat to human health? Vet Microbiol 149, 254-261.

Zhou, J.P., Ge, F.F., Liu, J., Ju, H.B., Yang, D.Q., Wang, J., Zhang, W.Y., Liu, P.H., 2012. Epidemiological survey and genetic evolution of H9 subtype influenza viruses in Shanghai, China, from 2006 to 2010. Arch Virol 157, 1193-1198.

Zhu, X., Yang, H., Guo, Z., Yu, W., Carney, P.J., Li, Y., Chen, L.M., Paulson, J.C., Donis, R.O., Tong, S., Stevens, J., Wilson, I.A., 2012. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. Proc Natl Acad Sci U S A 109, 18903-18908.