WILD AQUATIC BIRD AVIAN INFLUENZA VIRUS INFECTIONS IN MAMMALS: A POTENTIAL ZOONOTIC THREAT?

by

ELIZABETH ANN DRISKELL

(Under the Direction of Elizabeth Howerth)

ABSTRACT

Numerous cases of direct transmission of avian H5, H7, and H9 influenza viruses from birds to humans have stimulated concern regarding a potential avian influenza pandemic. The purpose of this research is to evaluate the potential of avian influenza viruses (AIVs) from wild aquatic birds to replicate in mammals with the greater goal of assessing indirect or direct AIV transmission risk to humans. Four hundred nineteen AIV isolates from the reservoir hosts, waterfowl and shorebirds, were examined in vitro via plaquing in Madin-Darby canine kidney (MDCK) cells. Of these isolates, 93% replicated in MDCK cells and 28% of these isolates plaqued without the addition of trypsin. Twenty eight of these isolates were further examined in vivo in a BALB/c mouse model. The ability to replicate in the lower respiratory tract (lungs) of mice was highly variable and isolate dependent; however, many isolates were able to replicate to high titers in the mouse lung and induce pulmonary pathology in the absence of overt clinical disease. Two isolates that exhibited robust replication in the mouse lung were further characterized in ferrets. Both of these isolates resulted in upper and lower respiratory tract infection with nasal shedding, respiratory pathology, and seroconversion with minimal clinical disease. One of these isolates was also able to transmit from infected to naïve ferrets via direct

contact. The two isolates examined in ferrets were also examined in domestic cats. Experimental infection of domestic cats with these two different shorebird AIVs did not result in disease, led to variable pharyngeal viral shedding with one of the viruses, and resulted in low levels of seroconversion of all cats. Taken together, these results indicate that wild bird influenza viruses have the ability to infect mammals without adaptation in domestic birds or other mammals; although the ability to infect mammals appears to be extremely variable based on the isolate and productive infections and transmission would likely be dependent on the magnitude of viral exposure and resultant shedding. Not only was the capacity for direct transmission demonstrated, but such events in domestic cats could lead to adaptation to or reassortment in this species.

INDEX WORDS: Avian influenza virus, Cat, Ferret, Infectivity, Mammals, Mouse, Pathogenicity, Wild bird

WILD AQUATIC BIRD AVIAN INFLUENZA VIRUS INFECTIONS IN MAMMALS: A POTENTIAL ZOONOTIC THREAT?

by

ELIZABETH ANN DRISKELL

BS, Truman State University, 2001

DVM, Iowa State University, 2005

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

WILD AQUATIC BIRD AVIAN INFLUENZA VIRUS INFECTIONS IN MAMMALS: A POTENTIAL ZOONOTIC THREAT?

by

ELIZABETH ANN DRISKELL

Major Professor:	Elizabeth Howerth
Committee:	S. Mark Tompkins
	David Stallknecht
	Elizabeth Uhl
	David Swayne

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2011

DEDICATION

For Jeremy. My husband, my best friend, my rock. All of my crazy ideas seem possible with you.

ACKNOWLEDGEMENTS

Summing up thanks for years and years of support and inspiration in education in a few pages is pretty impossible. But here it goes…

Thanks to my graduate committee. To Dr. Buffy Howerth, first and foremost, who everyone, including me, is in complete awe of. And rightfully so. You are an amazing pathologist, scientist, and mentor. The most amazing thing about you is your unending selfless support of us. Thanks for the open door; weekdays, weekends, and evenings. You have mastered more pathology than I can even dream to see. To Dr. Betsy Uhl, who shared great wisdom with me on a practical approach to balancing diagnostics, research, and teaching without compromising a gold standard. Thanks for the open door when I was frustrated or to just chat about art and life! To Dr. Mark Tompkins, who always had confidence in me and was brave enough to let me take the reins but always had an open chair on the other side of his desk for me to discuss plans and ideas. To Drs. David Stallknecht and David Swayne, who provided me with insight on the nature of avian influenza in wild birds and poultry and always asked me questions that got me thinking.

Heartfelt thanks to all the residents of the UGA Pathology Department, past and present. No one can survive a combined pathology residency and PhD without co-suffering friends for support. To Drs. Holly and Justin Brown, great friends who were there when I needed a shoulder to cry on or an escape to the beach! To Dr. Julie Webb who helped me find humor in everything. Special emotional thanks to Drs. Melinda Camus, Rita McManamon, and Janildo Reis who helped me survive studying for boards. Melinda, I couldn't have made it through gen path

without you. Rita, your insight and optimism was crucial to prevent a breakdown. Finally, Janildo, I simply wouldn't have passed without those countless hours we put in at Jittery Joe's as I forced us through vet path and you quizzed me on gross path. Thanks for helping me to stay on task, keeping me from going crazy, and for introducing me to caipirinha! To all the other residents old and new…Raquel, Tomislav, Tim, Parag, Susan, Dave, Daniel, Sasmita, Leo, Shawn, Tory, Bala, Nicole, Monique, Karelma, Paola, Sabrina...though an ever-changing group, it has always been a great group of supportive friends and amazing people.

Thanks to the entire UGA Pathology Department and Athens Veterinary Diagnostic Lab. From pathologists to support staff, I am the pathologist, scientist, and teacher today because I have been able to learn valuable things from all of you. Especially thanks to Dr. Harris, who is a magnificent leader and supporter, accessible to all no matter where you rank in the department. Big thanks to Glen and Bryan, necropsy couldn't run without you and is a great place to be for residents and students because of you…thanks for always being willing to go bucket diving for me! Also special thanks to the histopathology laboratory, always willing to go the extra step with a smile so we have perfect slides.

Thanks to Ginger and Becky at SCWDS who helped me master the egg. To animal resources who gave the countless mice, ferrets, and cats I used top notch care and kindness. To Merial for funding this position in the pathology department; it has been one of the best experiences of my life made possible by you. Also thanks to the CDC for funding this fantastic research project.

Thanks to all my previous mentors, especially Dr. Janick-Buckner at Truman State University, had I not wanted to be a veterinarian I would have wanted to be an immunologist because of you. A big thanks to Dr. Julia Ridpath at the NADC who convinced me that I should

do research; you are the most warm, inspiring, graceful mentor I have encountered, I would like to be just like you when I grow up!

Thanks to my family, who are a never ending source of support no matter what I choose for my life. Mom and Dad, you help me ponder the right questions so I can find my path. Kelley, you help me escape the grind. To Meme and Papa, my surrogate Mom and Dad, who are a never-ending source of love and support. To my in-laws, Dan, Gerry, and Blake, how did I get so lucky to have a second set of parents and a brother that are so supportive and proud?

To my son, Liam. Thanks for sharing your mom's time with her work. You are a daily reminder of what's important in life; your delight in living is a fresh breath of air when coming home from a day of frantic diagnostic work or unsuccessful experiments.

To Jeremy, who I'd say was as close to a saint as is possible. When I decided to not only do a residency but also a PhD nearly right after vet school, you simply said "OK, if this is what you need to do." Your patience, love and support sustain me. Thanks may cover the time you spent editing my papers, serving as my sounding board for work, covering at home when I studied for boards and even pipetting in the hood for me. But it doesn't even come close for everything else. Don't worry, I think I've run out of degrees to earn. I'm not interested in law school. Yet.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Direct transmission of avian influenza viruses (AIVs) from birds to mammals was a largely unexplored topic until the recent direct transmission of highly pathogenic avian influenza virus H5N1 subtype (HPAI H5N1) from poultry to humans drew attention to the pandemic risk associated with a human population naïve to these isolates. Additionally, other H7 and H9 AIV subtypes have been transmitted directly to humans, leading to disease [2,6]. The pandemic potential of these emerging avian isolates that can directly infect humans is troubling, and this combined with the severity of disease induced by HPAI H5N1 could be disastrous for the human population [1]. Isolates that potentially transmit to humans, whether or not they directly cause disease, could also contribute to reassortment with other mammalian isolates that could lead to the emergence of a pandemic strain. At this point, AIVs that have directly caused disease in humans, such as HPAI H5N1, seem to have a low transmissibility as they are not well host adapted [4,9,13]. In this research, the potential for AIVs to directly infect mammals is explored through two different classic mammalian influenza models, the mouse and the ferret. Additionally, the cat is used as a third experimental model, as domestic cats are a potential factor for human exposure, given their susceptibility to HPAI H5N1[8].

Human infections with AIVs resulting in disease have been limited to poultry isolates, however, HPAI H5N1 infections in cats have been attributed to exposure to both infected poultry and wild birds [11]. Shorebirds and waterfowl are the natural reservoir of influenza A viruses; the potential for these strains to replicate and induce disease in mammals or transmit between

mammals has not been adequately evaluated. Experimentally, we will answer four main questions: 1) Which AIV isolates from wild aquatic birds have the ability to directly transmit and replicate in mammals? 2) What is the pathogenicity/pathogenesis of wild aquatic bird AIVs that replicate in mammals? 3) What is the variability between mammalian species susceptibility to AIV isolates from wild aquatic birds? 4) What is the potential for cats to serve as a vector for transmission of AIVs to humans? Better understanding of the potential for these isolates to directly infect mammals will help shape biosecurity and surveillance efforts for AIVs.

These four questions were addressed through experiments to investigate five main objectives. These objectives are:

1. To identify AIV isolates that replicate in a mammalian cell line, specifically isolates that exhibit trypsin independent replication.
2. To determine if AIVs can replicate in the lung of BALB/c mice or if there is viral replication in other tissues.
3. To further characterize AIV pathogenicity in BALB/c mice via determining lung viral titers and examining histopathology over the time course of infection.
4. To determine if selected AIVs that can replicate in the lung of BALB/c mice can replicate and transmit between ferrets.
5. To determine if selected AIVs that can replicate in the lung of BALB/c mice can replicate in domestic cats.

In vitro examination of AIV isolates (objective 1) was performed with 419 AIV isolates from Southeastern Cooperative Wildlife Disease Study, utilizing plaquing assays in Madin-Darby canine kidney cells. These isolates are from 14 different species of waterfowl and shorebirds, and included 11 of the 16 hemagglutinin subtypes and were isolated between 1998 through 2006.

This *in vitro* examination allowed for selection of isolates that replicated well in a mammalian cell line and that replicated without the addition of trypsin, a characteristic associated with increased pathogenicity, with the goal of selecting optimal isolates for mammalian *in vivo* studies[12,14,15]. The first *in vivo* model (objectives 2 and 3) utilized BALB/c mice. The BALB/c mouse model has been widely utilized as an influenza model, and in this research the mouse was used as an *in vivo* screening tool for further selection of isolates to examine using *in vivo* models, as well as a means of comparing replication ability, kinetics, route of infection and pathology induced by a large number of AIV isolates [7,19]. The second *in vivo* model (objective 4) was not only to further characterize the kinetics and pathology of infection, but to examine capacity for mammal to mammal transmission of AIV isolates. Ferrets are naturally susceptible to influenza infections and have been used as models for not only human influenza infections, but also in HPAI H5N1 infections and other subtypes of avian influenza viruses that are of zoonotic concern; this species is a particularly good model for transmission of influenza virus[3,5,10,16-18]. The final *in vivo* model (objective 5), provided insight into the capacity for domestic cats to be infected with these AIV isolates, allowing assessment of the potential zoonotic concern of AIV infection in this species.

LITERATURE CITED

1 Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM: Update on avian influenza A (H5N1) virus infection in humans. The New England Journal of Medicine **358:** 261-273, 2008

2 Belser JA, Bridges CB, Katz JM, Tumpey TM: Past, present, and possible future human infection with influenza virus A subtype H7. Emerging Infectious Diseases **15:** 859-865, 2009

3 Belser JA, Lu X, Maines TR, Smith C, Li Y, Donis RO, Katz JM, Tumpey TM: Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. Journal of Virology **81:** 11139-11147, 2007

4 Gambotto A, Barratt-Boyes SM, de Jong MD, Neumann G, Kawaoka Y: Human infection with highly pathogenic H5N1 influenza virus. Lancet **371:** 1464-1475, 2008

5 Gillim-Ross L, Santos C, Chen Z, Aspelund A, Yang CF, Ye D, Jin H, Kemble G, Subbarao K: Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. Journal of Virology **82:** 10854-10863, 2008

6 Gillim-Ross L, Subbarao K: Emerging respiratory viruses: challenges and vaccine strategies. Clinical Microbiology Reviews **19:** 614-636, 2006

7 Guarner J, Falcón-Escobedo R: Comparison of the pathology caused by H1N1, H5N1, and H3N2 influenza viruses. Archives of Medical Research **40:** 655-661, 2009

8 Harder TC, Vahlenkamp TW: Influenza virus infections in dogs and cats. Veterinary Immunology and Immunopathology **134:** 54-60, 2010

9 Kumlin U, Olofsson S, Dimock K, Arnberg N: Sialic acid tissue distribution and influenza virus tropism. Influenza and Other Respiratory Viruses **2:** 147-154, 2008

10 Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, Gauger PC, Patnayak DP, Webby RJ, Richt JA: Identification of H2N3 influenza A viruses from

swine in the United States. Proceedings of the National Academy of Sciences of the United States of America **104:** 20949-20954, 2007

11 Marschall J, Hartmann K: Avian influenza A H5N1 infections in cats. Journal of Feline Medicine and Surgery **10:** 359-365, 2008

12 Rott R: The pathogenic determinant of influenza virus. Veterinary Microbiology **33:** 303-310, 1992

13 Salomon R, Webster RG: The influenza virus enigma. Cell **136:** 402-410, 2009

14 Steinhauer DA: Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:** 1-20, 1999

15 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A: Characterization of the Reconstructed 1918 Spanish Influenza Pandemic Virus. Science (New York, N Y) **310:** 77-80, 2005

16 Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A: A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science (New York, N Y) **315:** 655-659, 2007

17 van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, Munster V, Bestebroer T, Fouchier RA, Kuiken T, Osterhaus AD: Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. The Journal of Infectious Diseases **201:** 993-999, 2010

18 Wan H, Sorrell EM, Song H, Hossain MJ, Ramirez-Nieto G, Monne I, Stevens J, Cattoli G, Capua I, Chen LM, Donis RO, Busch J, Paulson JC, Brockwell C, Webby R, Blanco J,

Al-Natour MQ, Perez DR: Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One **3:** e2923, 2008

19 Ward AC: Virulence of influenza A virus for mouse lung. Virus Genes **14:** 187-194, 1997

CHAPTER 2

LITERATURE REVIEW

INFLUENZA A: A VIRUS LOOKING FOR OPPORTUNITY

Influenza is a virus with several characteristics that have allowed it to be a continuous force of disease including 1) a swift ability to mutate and recombine resulting in shift and drift and 2) a wide range of host species. Influenza is a negative sense segmented RNA enveloped virus with three outer envelope proteins, including hemagglutinin (HA), neuraminidase (NA), and matrix (M2) proteins. The segmentation of the genome and the lack of proofreading capability both contribute to functional and antigenic alterations that provide opportunity for the virus to continue to infect and induce disease in numerous species.

Shorebirds and waterfowl are the natural reservoir of all 16 HA and 9 NA subtypes of influenza A viruses. Infection in these reservoir species is typically subclinical and limited to replication in the intestine. Domestic poultry are susceptible to influenza A, in some cases resulting in respiratory disease and decreased egg production following infection with low pathogenic avian influenza viruses (LPAI), while highly pathogenic avian influenza viruses (HPAI) cause systemic disease with high mortality[167]. HA subtypes 1-13 have been found infecting domestic poultry[166]. Numerous mammals, including domestic species (humans, swine, horses, ferrets, cats, dogs, mice, and Syrian hamsters) and wildlife species (various marine mammals, tigers, and non-human primates) are susceptible to infection with influenza A viruses as demonstrated by natural or experimental infections[3,40,53,65,70-72,108,120,130,131,138,145,179]. Although many mammals have been demonstrated to be susceptible to influenza infections, swine,

humans, horses, and dogs have active cycling influenza infections. This demonstrates the ability of influenza viruses to adapt to their host and become endemic, and these viruses have become more adapted to their host species[64,125,136].

Although we have been studying influenza for centuries, predication of its threat as a pathogen often eludes us[191]. Endemic circulation with occasional human pandemics from large shifts due to reassortment had been the trend with influenza. However, starting in 1997, instances of direct transmission from birds to humans occurred, as poultry HPAI H5N1 influenza virus infection was documented in humans. Currently, infections with H5, H7, and H9 AIV subtypes have been reported in humans[2,7,16,23,36,73,128]. Additionally, canine influenza H3N8 led to fatal outbreaks in greyhounds, jumping from horses to dogs in 2004 and has since established endemic circulation[29,52,122]. Most recently, the 2009 pandemic H1N1 has further emphasized the importance of influenza research to better understand the viral mechanisms and that it is critical to have a good surveillance and risk assessment system.

Poultry avian influenza viruses are categorized into two pathotypes, LPAI and HPAI. These pathotypes are applicable only to chickens. The classification of isolates into pathotypes is done by determining the mortality rate after experimental intravenous infection in chickens. HPAI also has the ability to plaque without the addition of trypsin to cell culture[161]. Another methodology for classifying an AIV as an HPAI is by determining via sequencing if the HA has a polybasic cleavage site (discussed more in depth in the "Determinants of Host Pathogenicity and Range" section). Although these pathotypes do not apply to mammals, the most severe human disease has been with HPAI H5N1 and some HPAI H7 viruses, but human and mammalian infection with AIVs has not been limited to HPAI isolates.

Our previous dogma regarding influenza host range, transmission, and pathogenicity has now been challenged. There are currently intense research efforts into the public health risks of AIVs from poultry, with the focus especially on HPAI H5N1. One important focus has been the investigation of the possible role of wild birds in the spread of HPAI H5N1. Other public health risks, such as current circulating AIVs from the wild bird reservoir or environmental AIVs, must also be assessed. Through these efforts, we will develop a better understanding of the mechanisms and risks of AIVs.

THE ECOLOGY OF AVIAN INFLUENZA

Wild birds across the world serve as reservoirs for the 16 HA and 9 NA subtypes of AIVs. AIVs have been isolated from over 100 different avian species, but are most prevalent in the Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shorebirds) orders[154]. Replication of these AIVs in wild birds occurs preferentially in the intestine, with fecal-oral transmission serving as the primary route of AIV infection, with contamination of surface and ground water also potentially contributing to viral maintenance[13,37,119]. Different AIV isolates appear to readily transmit between wild bird species[22]. The limited studies examining experimental and natural infections of these circulating AIVs in the wild bird host indicate these infections are subclinical and do not result in lesions, although recent studies have demonstrated possible loss of body condition[63,67,78,182].

AIV transmission from the wild bird reservoir into poultry and domestic waterfowl is centered around man-made systems; free living outdoor birds are exposed either via direct contact with wild birds carrying LPAIs or through environmental exposure from contaminated pond and lake water[166]. These AIVs must adapt to poultry to result in efficient transmission and clinical disease. Additionally, co-mingling of species, such as poultry and domestic waterfowl,

also increases chances of LPAI transmission, as AIVs are more adapted to the domestic waterfowl. Indeed, many AIV outbreaks and AIV reassortment are reported from countries with high numbers of outdoor poultry rearing systems and mingling of poultry and waterfowl in live bird markets[68,84,192]. Prior to the transmission of HPAI H5N1 directly from poultry to humans, swine were thought to be the critical mixing vessel for introduction of novel AIV components into humans. It is now apparent that these poultry systems also pose risk to public health, but there are still many questions about the risks regarding the vast number of poultry and wild bird isolates that circulate.

It was previously thought that AIVs from poultry did not readapt back to the wild bird host, however, HPAI H5N1 infections have not followed this rule[83,87,146,188]. Wild birds have even been implicated in the transmission of HPAI H5N1, although it does not seem established in the wild bird population[66]. In contrast to LPAIs in wild birds, cases of HPAI infections have been associated with respiratory (oropharnygeal) rather than alimentary (cloacal) viral shedding[14,160]. Clearly, wild birds pose a potential, albeit improbable, risk of direct transmission of AIV to humans and influenza viruses have the potential to contribute to reassortants of risk[133,186,192]. To underscore this point, the 2009 H1N1 pandemic was a triple reassortant virus, containing avian, human, and swine components[20,140]. More importantly, surveillance to monitor AIVs in the wild bird population combined with a better understanding of populations that have isolates of risk, especially regarding transmission to domestic species that may serve as an interface (indirect transmission route) between transmission of wild bird AIVs and humans, is of utmost importance.

THE EPIDEMIOLOGY OF INFLUENZA IN HUMANS

Influenza is one of the most clinically important infectious diseases of humans, with approximately 200,000 hospitalizations and 41,000 deaths in the United States annually just due to seasonal influenza infections[32,172]. The human population's susceptibility to influenza is most frequently a result of genetic drift in the hemagglutinin gene but major pandemics and avian influenza outbreaks in humans are associated with major shifts in the HA and NA glycoproteins[42,91]. Humoral immunity is a major factor of protection against influenza infection and HA and NA surface glycoproteins are the main antigens that protective antibodies are directed against. Therefore, the human population is immunologically naïve to any additional HA subtypes that can potentially infect humans, such as H5 that caused recent infections in humans, and these HA subtypes are the ones of highest concern. Direct transmission of avian H5N1, H7N2, H7N3, H7N7, and H9N2 influenza from birds to humans has drawn attention to these particular HA subtypes, because of the pandemic potential of these viruses[28].

Poultry-human interaction has been the source of these outbreaks of avian influenza in humans, and these outbreaks show that these avian origin influenza viruses have the ability to bind human cell receptors with successful replication. Although the 2009 pandemic H1N1 was a surprise, there remains a high possibility that the next pandemic influenza will be avian in origin and that our influenza monitoring efforts should be focused on these avian-origin influenza strains. Numerous studies have already demonstrated mixing of AIV and human influenza viruses in vitro and in vivo, demonstrating compatibility between even several avian subtype isolates and pandemic H1N1 isolates, resulting in some that had increased virulence compared to parent strains[21,60,118,163].

DETERMINANTS OF INFLUENZA PATHOGENICITY AND HOST RANGE

AIV outbreaks in humans have stimulated the intense investigation into two critical questions: What drives the expansion of host range? What drives the alteration of pathogenicity? Answering these questions are not purely academic exercises, but can aid in prevention of the next outbreak and provide us with good parameters for surveying circulating avian influenza viruses for potential threatening strains. Studies often reveal that changes in pathogenicity are related to mutations that can be isolate dependent; frequently multiple concurrent factors appear to affect pathogenicity[94]. For example, recombinant studies of the 1918 influenza demonstrated that to retain the original pathogenicity, all 8 original segments of the genome were required[173]. The segmented genome of influenza codes for at least 11 proteins including HA, NA, matrix proteins (M1 and M2), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), nonstructural proteins (NS1 and NEP), nucleocapsid protein (NP), and more recently discovered PB1-F2. Nearly all of these genes have been implicated in contributing to host range and pathogenicity of individual isolates, but mutations in the hemagglutinin and the polymerase have been most implicated in the adaptation of many avian isolates to mammals and increasing virulence of these infections in mammals[127].

Hemagglutinin (HA)

The HA has the critical role of both binding the host cell as well as inducing release of the viral genome into the host cell. Regarding host range restriction, HA receptor specificity is thought to be a large contributor to host range, however the polymerase also appears to play an important role in host range[114]. The function of the HA as a ligand for the host cell receptors seems to be a key factor due to receptor preference differences of AIVs versus human influenza viruses. Avian influenza strains bind to cell glycoproteins/glycolipids that have terminal sialyl-

galactosyl residues with a 2-3 linkage [Neu5Ac(α2-3)Gal] and human influenza strains bind to terminal 2-6 linked glycoproteins/glycolipids [Neu5Ac(α2-6)Gal]. The occurrence of direct avian to human infection with avian influenza shows that these cell surface binding restrictions are not absolute protection from avian strain infection. Further investigation into receptors on human respiratory cells has shed light on this matter, as both types of linkages are present in human respiratory tracts. However, 2-6 linked receptors are mainly on the nonciliated cells and somewhat on the ciliated cells of the upper respiratory tract and 2-3 linked receptors are expressed in a high enough level on the ciliated cells of the respiratory tract and type II pneumocytes to allow binding and entry of avian influenza viruses[76,101,102,171]. Additionally, distribution of cells expressing either 2,3 linked sialyl-galactosyl residues or 2,6 linked residues have differences in the distribution in the human respiratory tract, with 2,3 expression throughout the upper (nasopharynx and trachea) and lower (bronchi, bronchioles, and alveoli) respiratory tract and 2,6 expression on cells in the upper respiratory tract[76]. Lower expression of the appropriate receptors in readily exposed regions (upper respiratory tract) has been hypothesized to serve as a barrier of influenza infections between species (avian to human) as well as within a species (human to human). This indeed seems true for current human HPAI H5N1 infections, as human to human transmission has been suspected but has not been definitively documented at this point[195]. Therefore, it is implied that mutation in HA binding preference, such as a change that would switch binding preference from a 2,3 linkage to a 2,6 linkage in an avian influenza virus, would result in emergence of an avian influenza virus that readily infects humans and potentially is transmitted between humans.

Several experiments have demonstrated that expression of 2,3 linkages versus 2,6 linkages on cells vary in prevalence and distribution not only between cell types but also

between species[26,58,123,132]. *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) plant lectins can detect distribution of these types of linkages in different tissues and are used experimentally to demonstrate these different glycans[185]. Researchers have used both human airway epithelial cell cultures and fixed pulmonary tissues to differentiate receptor expression using SNAs and MAAs [57,69,85,102,116,141,164,171,193]. These lectins can be useful, however, they have limitations as they may not distinguish other modifications in glycan residues that may be important in binding[30]. Glycan microarrays have been developed to distinguish more subtle binding differences in influenza isolates as a complement to pathogencity studies[159].

More compelling is the direct demonstration of binding patterns of avian influenza in different species. These studies demonstrate viral presence in fixed cell cultures via immunostaining and microscopy[24,102,171,184]. An extensive study was performed by Van Riel et al. that demonstrated differences in attachment patterns of human versus avian influenza viruses in human respiratory tracts as well as identifying attachment patterns of these viruses in other species including the mouse, pig, ferret, and macaque[184]. These patterns have further supported HPAI H5N1 attachment is most prominent in the lower respiratory tract (type I and type II pneumocytes of the alveoli and alveolar macrophages) and human influenza most prominent in the upper respiratory tract (ciliated and noncilated cells of the nasal mucosa and ciliated epithelial cells of the trachea and bronchi, less in the bronchioles)[57,115,178,184]. This study also demonstrated that the attachment pattern of AIVs in human respiratory tissues was most like that of ferrets, pigs, and cats. Another interesting outcome of this study was that LPAIs had the same attachment pattern as HPAI H5N1, suggesting that differences in HA attachment doesn't explain the severity of disease in HPAI H5N1 infections.

Animal studies have built upon knowledge and hypotheses from in vitro studies regarding avian influenza binding in the respiratory tract. Two different ferret transmission studies utilizing H9 and H7 AIVs have supported that when there is glycan binding preference for 2, 6 linked SA (the typical human influenza binding pattern) rather than 2,3 linked SA (the typical AIV binding pattern) there is efficient transmission between ferrets, demonstrating a loss of species barrier[6,153,187]. Along the same lines, experiments with H5N1 transmission in ferrets demonstrate that reassortant viruses of H5N1 with human H3 and N2 surface proteins efficiently transmit via aerosol between ferrets, but human influenza viruses that exhibits avian H5 and N1 do not transmit via aerosol[96]. Additionally, Tumpey's examination of the 1918 influenza revealed that switching the 1918 influenza's binding preference from 2,6 linkages to 2,3 linkages abolished transmission in ferrets[175]. Matrosovich et al. demonstrated that HA mutations that alter receptor binding may be a requirement for development of endemic mammalian influenza from avian strains[100].

The increased pathogenicity of avian influenza viruses (development of HPAI viruses), at least for poultry, is related to HA cleavage[56,81,135,158]. The HA of influenza must be cleaved for viable infectivity. All HPAI viruses share the ability to be cleaved intracellularly by ubiquitous endoproteases (furin-like or subtilisin-like), where LPAI isolates can only be cleaved by extracellular trypsin-like proteases at mucosal surfaces[114,135,158]. Genomic changes in the HA that manifest as polybasic regions within the cleavage site of the HA allow the HA to be cleaved by ubiquitous proteases that are located intracellularly in many tissues. Therefore, the virus is able to replicate in numerous other tissues rather than remaining restricted to the respiratory or gastrointestinal tract. Polybasic insertions in the HA cleavage site have been shown to alter pathogenicity for chickens—but what about the effects on pathogencity in mammals? The

property of ubiquitous HA cleavage of HPAIs has been explored as a reason for systemic infection of poultry, mice, ferrets and cats and experiments have reproduced the results of systemic infection[45,62,93,131]. Some studies, however, have indicated that a polybasic cleavage site may not necessarily lead to systemic infection in mammals[8,137]. Indeed, even experimental adaptations of LPAIs to generate polybasic cleavage sites and strains that replicate in cell culture without the addition of trypsin do not necessarily meet the in vivo HPAI criterion in chickens[155].

HPAI H5N1 infections in humans appear to share the commonality of an increased pathogenicity in vivo, although severe pathogenic affects in humans appear to be mostly limited to the lung[178]. Whether this increased pathogenicity is at all related to HA cleavage as in systemic infections in other species remains to be definitively determined; additional studies have suggested that stimulation of cytokines by HPAI H5N1 may be responsible for the increased pathogenicity in human lung[17,19,82,126,168].

Neuraminidase (NA)

The NA participates in efficient viral movement from the cell surface after budding by cleaving sialic acids so it doesn't self-bind. Therefore, the NA must have a balanced relationship with the HA for productive influenza virus infections. Binding and sequestering plasminogen to increase HA cleavage has been thought to possibly be a property of the NA that increases pathogenicity[43,44]. Modification of the NA by glycosylation was shown to have an effect on pathogenicity in chickens and mice[56,104]. Deletions within the stalk portion of the NA is potentially associated with adaptation from quail to chickens and adaptation from waterfowl to chickens, and stalk length alterations affect pathogenicity in mice[55,104,111,152].

Polymerase proteins (PB1, PB2, PA)

Three polymerase proteins, PB1, PB2, and PA play a role in the production of mRNA and viral RNA during replication. Essentially, the more efficiently the polymerase proteins work in an infected host, the higher titer the virus can grow in the tissues. PB2 is currently an intensely implicated component in the ability of influenza to infect different species as well as modulate the pathogenicity of the virus. Host range effects have been demonstrated by plaquing viruses with mutations in their polymerases in avian and human cells[38,39,77,156]. Differences between replication ability between species are suggested to be due to different polymerase forms tolerating different temperatures[12,77]. In vivo experiments have further supported that the polymerase has an important role in altered pathogenicity including mouse, ferret, and guinea pig experiments with H5, H7, and H9 AIVs[38,55,88,190]. Again, some mutations appear isolate dependent in importance. For example, Lys at the 627 position of PB2 in many HPAI H5N1 isolates and in the human outbreak of HPAI H7N7 in the Netherlands has been consistently demonstrated as increasing virulence but changing this amino acid in 2009 pandemic H1N1 does not have the same effect[8,35,38,49,50,80,88,98,99,113,142,156,162,183,201]. Also, other experiments have demonstrated increased pathogenicity with different mutations in PB1, PB2, and PA[134,149].

Nonstructural proteins: Nuclear export protein (NEP) and nonstructural protein 1 (NS1)

Both nonstructural proteins participate as regulatory proteins that also affect the replication efficiency of influenza and can therefore have a dramatic effect on viral titer. NEP (also known as nonstructural protein 2-NS2) has a role of moving viral proteins to and from the nucleus. NS1 protein is only found in low amounts in virions, but is in high amounts in host cells. NS1 halts the production of beta interferon mRNA. Lack of this function is associated with increased interferon mRNA production and decreased virus replication[157]. Therefore, mutations

that allow high activity of the NS1 gene resulting in interference with beta interferon production is theorized to result in high viral replication and increased pathogenicity[177]. This is supported by experiments that demonstrate attenuation in activity of NS1 that alter pathogenicity in chickens and mice for not only HPAI H5N1 but also for multiple other subtypes of influenza[61,89]. Other experiments have shown that alterations in NS1 in other isolates do not affect replication, pathogencity, or transmission in vitro or in vivo[47].

Matrix proteins (M1 and M2), nucleoprotein (NP), and PB1-F2

The M1, like NEP, also has a role of moving viral proteins to and from the nucleus. Additionally, M1 assists in virion assembly and has a structural role by bridging the membrane and the core of the virons. The M2 protein is an ion channel that allows acidification of the viron to promote the HA's fusion activity for viral genome release and participates in assembly of virons. A few studies on alterations in the M2 have indicated a possible role in contribution to virulence[46]. The NP has a role in nuclear localization of the genome and polymerases. Finally, some influenza A viruses contain PB1-F2—this protein is currently being investigated for playing a role in apoptosis of infected host cells. Some studies have implicated PB1-F2 as a contributing factor to increased virulence, but others have in demonstrated that PB1-F2 does not have an effect on pathogenicity for a variety of influenza virus isolates[25,106,198].

COMPARISON OF DISEASE AND PATHOLOGY FOR AVIAN INFLUENZA INFECTIONS IN DIFFERENT SPECIES

Influenza presents as a spectrum of disease in humans, depending on the isolate and the immune status of the human host. The pathology described for human cases of influenza are somewhat skewed and difficult to compare, as the most intensely examined cases are from pandemic outbreaks that resulted in many deaths (1918, 1957, 1968, and 2009) and from

interpandemic cases that were severe and also resulted in death. Additionally, secondary bacterial infections are a frequent fatal complication, further complicating the lesions observed. Clinically, seasonal influenza typically affects the healthy human individual as an upper respiratory disease with peak replication about 48 hours after infection that correlates with onset of acute respiratory illness, with little shedding after 4 days post infection[79]. Within the trachea and bronchi of these cases, there is necrosis of the epithelium, edema and hyperemia of the lamina propria with a very mild mononuclear leukocyte infiltration[75]. Obviously, pulmonary lesions are not described in these types of cases, as they are not investigated. However, there are cells that influenza can bind and infect throughout the entire human respiratory tract as discussed in the previous section; therefore, seasonal influenza also has the capacity to induce a severe pneumonia. With more severe cases of human influenza that lead to pneumonia, necrosis and flattening of the bronchiolar epithelium is present with interstitial congestion, edema, and inflammation. Alveolar walls are thickened by hyperemia and have increased leukocytes. Bronchiolar and alveolar lumens may exhibit hyaline membranes and can contain edema and neutrophils, although a heavy influx of neutrophils is not a characteristic. Vascular changes can also be present, with capillary thrombosis resulting in alveolar necrosis and hemorrhage. As these lesions progress, there can be squamous metaplasia, epithelial regeneration with numerous mitotic figures, type II pneumocyte hyperplasia in the alveoli, alveolar wall fibrosis and interstitial infiltration of mononuclear leukocytes[169].

The lesions of seasonal influenza pneumonia are similar to the severe lesions observed with fatal cases of pandemic influenza including 1918 and the 2009 pandemic influenzas[18,105,139,169]. Although the lesions were similar, influenza viral antigen was present in tracheal, bronchial, and bronchiolar cells in interpandemic influenza but not in alveolar

epithelium or alveolar macrophages, while in cases from the 1918 pandemic, viral antigen was also present in these deeper cells[169]. Pandemic 1918 was also remarkable due to the high mortality was in young adults, in contrast to seasonal influenza that causes more severe disease in the young and elderly. Acute alveolar edema was a prominent feature compared to its absence in other pandemic influenza suggesting and increased pathogenicity[169]. Atypical severe disease in young adults also occurred with the 2009 pandemic[124].

From December 2003 to August 2005, there were 112 reported and confirmed cases of human H5N1 influenza with an alarming 57 of these cases resulting in death[5]. HPAI H5N1 has also been clinically unique in its own respect, producing a large number of cases of severe pneumonia with hyaline membranes and hemorrhage. Hemophagocytosis has also been frequently described in HPAI H5N1 cases in multiple organs[75]. Additionally, clinical signs may also include diarrhea, rather than being limited to the respiratory tract, which has left many pondering the possibility of gastrointestinal infection[5]. Although lesions of seasonal influenza pneumonia, various pandemic human influenza pneumonias, and HPAI H5N1 pneumonia may be similar, viral attachment patterns between the human influenza viruses and HPAI H5N1 are very different, as previously discussed in "Determinants of Influenza Host Range and Pathogenicity" section. This again highlights the multifactorial contributions of the virus isolate and the host in influenza pathogenesis.

Viremia has been demonstrated in some human influenza infections but hasn't been established as typical. Systemic dissemination of human influenza is controversial. Virus has been detected in the ependyma, neurons of the pontine nuclei and Purkinje cells in the brain by immunofluorescence or immunohistochemistry in some cases of neurological disease associated with seasonal influenza and was also demonstrated by these methods in one case of

myocarditis[75]. Systemic dissemination of HPAI H5N1 in humans has been demonstrated in some cases, usually as positive immunohistochemistry, with no or minimal lesions including in the brain (neurons), intestine (epithelial and mononuclear cells), liver (Kupffer cells), and lymph nodes (lymphocytes)[75,178,199].

Human infections with subtypes of AIVs other than HPAI H5N1 have also rarely caused fatal pneumonia (one case of H7 infection), but more consistently induce less severe disease[73]. Transmission of H7 AIVs to humans has resulted predominantly in conjunctivitis, most notably in the 2003 outbreak of HPAI H7N7 in the Netherlands, with fewer cases of respiratory disease[7,36]. Rare cases of H9 AIV transmission to humans have all resulted in mild to moderate respiratory disease with recovery[23]. An experimental study was performed by Beare and Webster using a variety of duck and turkey AIVs including H1, H3, H4, H6, H9, and H10 subtypes to infect humans. Some of the inoculated subjects became infected and shed virus but did not seroconvert[4]. Fortunately at this point, these subtypes have rarely induced severe disease and do not appear to be readily transmissible; therefore the data that exists regarding pathology and pathogenesis of these infections in humans is limited.

Natural infections in other mammals

Swine and horses, also naturally susceptible to their own endemic influenza viruses, demonstrate clinical respiratory signs and lesions similar to human infections with a self-limiting necrotizing bronchitis and bronchiolitis that can in rare cases lead to a severe bronchointerstitial pneumonia[109]. Ferrets are also naturally susceptible to human influenza, again resulting in a range of clinical disease and lesions. An outbreak of a swine H1N1 strain in a ferret colony with sneezing, dyspnea, nasal discharge and coughing in the animals resulted in marked pulmonary lesions of bronchiolar necrosis and bronchioles and alveoli filled with macrophages, neutrophils,

and cell debris with alveolar septal thickening[121]. Finally, the emergence of a canine adapted H3N8 from horses has induced influenza disease in dogs, a species previously only experimentally susceptible to influenza, resulting in high morbidity. Most frequently these cases have only moderate clinical signs with rare cases of pneumonia that are typically complicated by concurrent bacterial infection[29,52,197].

Natural outbreaks of HPAI H5N1 infections in other animals have been most clinically severe in large zoo felids (tigers) and domestic cats, causing pneumonia and in some cases and systemic dissemination in domestic cats[65,71,151,196]. Yet there is certainly a spectrum of disease that occurs, demonstrated by serosurveys of domestic cats[86]. HPAI H5N1 has also naturally infected dogs, raccoons, pikas, and a stone marten, demonstrated mostly by seroconversion but occasionally inducing disease[54,72,150,200]. Severe disease in dogs and cats infected by HPAI H5N1 is presumed to be due to ingestion of infected birds.

As we continue to search for influenza in mammalian species not traditionally thought to be susceptible to influenza, we are finding many have the capacity to be naturally infected, as determined by seroconversion of these species to a variety of human influenza and AIV subtypes[48,147]. Notably, the recent 2009 pandemic influenza has induced severe pneumonia in a number of domestic cats and seroconversion has been found in domestic cats (21% of surveyed animals) and a number of wildlife species[108,117,138]. Additionally, a recent survey of cats showed a 41% seroconversion against seasonal human H1N1 influenza and a 25% seroconversion against seasonal human H3N2 influenza[108].

Experimental models: Mice

Mice have been used as an experimental model of influenza for decades, despite the fact that they are not naturally susceptible to influenza; effective infection is typically accomplished

through anesthesia[27,144]. Experimental infections are productive although there is minimal clinical disease and pathology unless the strain is mouse adapted[144,189]. Different strains of mice have different susceptibility to influenza, which not only has implications for selection in animal models but also demonstrates the importance of the host genetic makeup in the virus-host interaction[10,11,176]. Pulmonary lesions that develop in influenza infected mice are similar to humans, although there is typically a lack of lesions in the nasal cavity and rare mild lesions in the trachea of infected mice, possibly due to paucity of sialic acid expression on cells in these regions[116]. Despite the absence of lesions in the upper respiratory tract of mice, virus is often isolated and titratable from the nasal turbinates and trachea of mice infected with a variety of influenza isolates, though replication is much better in the lung[59]. Pulmonary lesions in mice infected with non-adapted human or swine strains of influenza include a necrotizing bronchitis and bronchiolitis, peaking at day 3 to 4 post inoculation. Neutrophils predominate early in infection with progression to interstitial mononuclear leukocytes on days 5 through 8 post inoculation and marked bronchiolar regeneration beginning day 4 post inoculation[31]. Peribronchiolar and perivascular cuffing with mononuclear leukocytes is often observed[15]. A few swine isolates have induced more severe pulmonary lesions in mice without adaptation[95,110]. When mouse adapted human strains are inoculated into mice, pulmonary lesions are similar to unadapted strains but lesions are more severe and deeper in the respiratory tract, including damage to alveolar pneumocytes, similar to cases of influenza induced pneumonia in humans[10,15,34,127]. Positive immunoreactivity for influenza has been correlated in the cytoplasm and nucleus in epithelial cells of the affected airways as well as within intralesional macrophages in these studies. Hyaline membranes are not observed in mice infected with these viruses. Depending on the virulence of the isolate for mice, there may be minimal clinical signs or

anorexia, decreased activity, and ruffled fur that correlate with peak viral shed and peak severity of epithelial necrosis in airways.

The reconstructed 1918 pandemic influenza also induces similar pulmonary pathology with the addition of striking lesions of alveolar edema and hemorrhage and high infiltration of neutrophils[173]. A swine H1N1 from 1931 that is a descendant of the 1918 human influenza also induces similar severe lesions in mice and is accompanied by severe clinical disease[110]. Infectivity of 2009 pandemic influenza in mice is strain dependent, with some reporting a high MID_{50} required for infection with immunoreactivity for virus primarily restricted to bronchi and larger bronchioles and other reports of a high LD_{50} but a low MID_{50}[97,201].

In stark contrast to influenza infections in mice with human or swine strains that cause minimal disease and lesions, mouse infections with a variety of HPAI H5N1 isolates from human cases are often lethal, although severity of disease is also isolate dependent[33,93,98,174,194]. Studies have shown a slightly variable clinical course of HPAI H5N1 in mice between isolates, with clinical disease (anorexia, weight loss, ruffled fur, huddling) beginning 2 to 4 days post inoculation, high titers of virus in the lung between days 4 and 6 post inoculation, and death (if it occurs) ranging from 6 to 9 days post inoculation[93,174,194]. Lesions and viral antigen are not only in the lungs but can also be found in the nasal cavity and trachea in some cases[33]. Pulmonary lesions include a severe necrotizing bronchointerstitial pneumonia, but with a distinguishing exudation of fibrin and edema for some isolates, demonstrated on days 4 up to day 8 post inoculation[33,93,194]. A leukopenia due to profound lymphopenia has also been consistently demonstrated[98,174,194].

A handful of different AIVs have been examined in mice, again inducing a variety of clinical outcomes and lesions. Both HPAI and LPAI H7 isolates from humans and poultry

demonstrated low MID_{50} for mice without adaptation, little is reported on lesions and clinical disease progression induced by these isolates but it appears to be similar to other influenza infections in mice[8,62,129]. HPAI H7 AIVs isolated from the 2003 human outbreak in the Netherlands induced clinical disease in mice, sometimes severe. Some of these isolates replicated to titers as high as some HPAI H5N1 isolates in the lungs. In contrast, LPAI H7 isolates from poultry and North American H7 AIVs that caused conjunctivitis in humans did not cause significant morbidity in mice[8]. Many of these H7 isolates also replicated in ocular tissue in mice, though without clinical ocular disease[9]. A few wild bird H6 AIVs were shown to infect mice and cause disease, and sometimes was lethal if a high $TCID_{50}$ was used, but did not show systemic spread[41]. Finally, a chicken H9 AIV had demonstrated pulmonary infection in mice resulting in lesions of mild bronchiolar necrosis[163].

Systemic infection in mice have been demonstrated with a variety of AIVs[8,93]. Neurotropism is often reported, likely due to extension from the olfactory and/or vagus nerves during intranasal infection[33,51,93,103]. Mice infected with HPAI H5N1 had systemic spread due to viremia in the thymus, liver, spleen, kidney and heart detected not only by virus titration but also by immunohistochemistry; however, not all isolates demonstrated systemic spread[33,93,98]. Viremia and systemic spread to organs has been detected at day 4 pi[93]. Current studies show no evidence of systemic spread in mice with 1918 pandemic or 2009 pandemic H1N1 influenza, though RT-PCR in organs other than the respiratory tract has been positive in studies of mouse adapted influenza infections without presence of lesions[34].

Experimental models: Ferrets

Ferrets have been used as an experimental model for influenza as long as mice have been; they are naturally susceptible to influenza and serve as an excellent model for transmission[143].

Lesions mimic human patterns, with increasing virulence of virus leading to more severe pneumonia with alveoli more prominently affected and more widespread lesions throughout the lung[98]. A time course study of A/Brisbane/59/2007 (human H1N1) in ferrets resulted in mild clinical disease (sneezing and nasal discharge) with a range of EID_{50} (from 3.8 up to 7.8) and nasal shedding of virus from days 1 through 5 post inoculation, with peak shedding in the lower EID_{50} groups at day 3 post inoculation. Microscopic lung lesions, albeit minimal, were observed in all animals (days 3 and 7 post inoculation) and included scattered macrophages and neutrophils in airways, bronchiolar epithelial regeneration, and bronchial gland inflammation; viral titers were obtained from lung day 3 post inoculation only in animals inoculated with a higher EID_{50}[107]. Although experimental infections of ferrets are typically performed under anesthesia, which likely increases the virus delivery to the lung and may lead to some pulmonary lesions that may not occur in a natural situation, the clinical outcome of this study is probably the more common presentation of natural influenza infection in ferrets rather than outbreaks that have resulted in severe pneumonia. Infection in ferrets with other human H1N1 and H3N2 isolates resulted in nasal shedding with similar mild pulmonary lesions, characterized by mild bronchial epithelial necrosis and small numbers intraluminal inflammatory cells; gross lesions in some animals were more striking[110,165]. Localization of antigen in this study noted that positive cells were not correlated with areas of microscopic lesions.

The recovered 1918 influenza induces severe clinical disease and death in ferrets. There is good viral replication in both the upper and lower respiratory tract, though Tumpey demonstrated inefficient ferret to ferret aerosol transmission[175]. Severe pulmonary lesions develop in ferrets deep in the respiratory tract, including bronchiolar epithelial necrosis and alveolar edema and hemorrhage, with localization of antigen in the bronchial, bronchiolar, and

alveolar epithelium[110,175]. A closely related 1931 swine H1N1 induced similar clinical disease and lesions in ferrets as the 1918 influenza[110]. Several studies have been done with 2009 pandemic H1N1 in ferrets, supporting an increased pathogenicity compared to seasonal H1N1 viruses. Studies have demonstrated slightly higher to equivalent upper respiratory tract replication compared to a seasonal H1N1, detected as shedding on day 3. But the 2009 pandemic H1N1 replicated to higher titers than seasonal H1N1 in the trachea and lung and induced lesions in the lung, with viral antigen presence in peribronchial glands and alveoli, where the seasonal H1N1 produced minimal pulmonary lesions with minimal or no viral antigen in the lung[59,112,180]. Pulmonary lesions observed with pandemic H1N1 experiments include bronchial and bronchiolar epithelial necrosis with luminal inflammatory cells and edema that is more severe than seasonal H1N1 lesions, alveolar septal thickening, alveolar filling with fibrin, edema, neutrophils, and macrophages, where no alveolar lesions are observed with seasonal H1N1[112,180]. These findings were accompanied by mild to minimal clinical disease for both the pandemic and seasonal H1N1 groups, though one study showed delayed clinical recovery with the pandemic H1N1 group.

Many HPAI H5N1 isolates, human or poultry in origin, induce severe clinical signs in ferrets (dyspnea, lethargy, nasal discharge, diarrhea) with some isolates causing neurological signs, a high mortality rate day 7 to 9 post inoculation, and severe respiratory lesions including bronchial and bronchiolar necrosis, septal thickening by mononuclear leukocytes, alveolar edema, and viral antigen in the bronchiolar epithelium[45,98]. Again, repair after damage is evident by bronchiolar epithelial hyperplasia and alveolar type II pneumocyte hyperplasia. Viral nasal shedding has similar kinetics to seasonal influenzas in ferrets, occurring for up to 7 days post inoculation with peak shedding around day 3, but lower levels of virus are shed nasally in some

cases compared to seasonal influenza[96,98,202]. Pulmonary lesions have been demonstrated to be more severe than lesions induced with 2009 pandemic H1N1virus and the clinical course of disease is significantly more severe. Interestingly, both viruses replicate in the alveolar epithelium in contrast to seasonal influenza infections in ferrets, but studies have shown significantly more antigen present in alveolar epithelium in HPAI H5N1 infections[180]. A marked lymphopenia on days 3 and 5 post inoculation has been demonstrated in ferrets infected with HPAI H5N1[202].

Experimental infections exploring virulence of H7 AIVs in ferrets also have accurately mirrored virulence in human disease. The fatal case of the Netherlands HPAI H7N7 resulted in severe disease and death in ferrets, where the conjunctivitis cases led to moderate disease and LPAI H7 human and poultry isolates induced mild disease[8]. High titers of virus were shed from the nasal cavity as well as high titers of virus were present in the lung for the HPAI H7 isolates. Ferrets have also been infected with HPAI H7 isolates from poultry with nasal shedding peaking at day 3 post inoculation, though clinical disease and pathologic changes have not been described[1]. A mallard H7N3 isolate was examined in ferrets, resulting in minimal clinical disease with typical influenza viral shedding kinetics and moderate interstitial pneumonia in the lung[148]. A spectrum of poultry and wild bird H6 AIVs have also been examined in ferrets, resulting in variable ability to infect ferrets with minimal clinical disease[41]. Some isolates replicated to higher titers in the lung compared to the nasal cavity, and an H6 mallard isolate appeared to replicate less efficiently. Similarly, a few H9 poultry AIV isolates also had the ability to infect ferrets with nasal shedding[187]. Clearly, though these studies demonstrate the capacity of a few avian origin influenza viruses to infect ferrets with shedding, they reflect many gaps in the knowledge of the pathogenesis of this enormous group of AIVs.

Extrarespiratory lesions in ferrets infected with HPAI H5N1 influenza include encephalitis with neuronal degeneration, neuronophagia with or without glial nodule formation and hepatic necrosis with hepatitis in addition to pulmonary lesions. Viremia and systemic spread with HPAI H5N1 has been demonstrated by viral titration in blood and organs including spleen, liver, heart, intestine, and brain, although, as in mice, neurologic infections are suspected to be due to local spread[45,98,202]. No systemic spread has been noted with 2009 pandemic H1N1 experimental infections in ferrets[112].

Experimental models: Cats

Although previously thought resistant to influenza infection, the domestic cat has proved to be naturally susceptible to infection and disease with not only HPAI H5N1 but also 2009 pandemic H1N1 influenza viruses. Cats naturally infected with the 2009 pandemic H1N1 influenza had severe clinical disease in some cases, with marked pneumonia resulting in dyspnea; however, experimentally infected cats had only mild to minimal signs of disease (lethargy, anorexia) despite significant pulmonary lesions. Lesions in natural infections of 2009 pandemic H1N1 in cats that result in severe pneumonia are similar to experimental infections and prominently affect the lower respiratory tract. Lesions include bronchiolar necrosis with intraluminal fibrin, macrophages and edema, hyaline membranes, sloughed pneumocytes, macrophages filling alveoli, type II pneumoncyte hyperplasia and relatively little inflammation in the bronchi[92,181]. Antigen is localized in the nucleus and cytoplasm of the bronchiolar epithelium, type I pneumocytes, type II pneumocytes, alveolar macrophages and rare bronchial glandular epithelium by immunohistochemistry; with stronger staining day 4 post inoculation compared to day 7.

Natural infection of HPAI H5N1 in domestic cats has proven fatal and experimental infections have produced clinical disease, with fever, lethargy, and dyspnea[131,196]. Naturally and experimentally infected cats had similar respiratory lesions that are deep in the lung, consisting of bronchiolar necrosis and alveoli filled with edema, hemorrhage, and necrotic cells and some cases with hyaline membranes in alveoli and type II pneumocyte hyperplasia[71,74,131]. Immunohistochemistry shows influenza antigen in bronchiolar and alveolar epithelium (type I and II pneumocytes) and macrophages. Interestingly, striking systemic spread has occurred in some cats, with lesions of encephalitis, hepatic necrosis, adrenocortical necrosis, tubulonephritis, myocardial necrosis and lymphoid depletion or necrosis in the spleen or Peyer's patches with immunohistochemical localization of virus in some of these organs[71,131,151] . Lesions in large zoo felids with HPAI H5N1 infections are similar to findings in domestic cats regarding appearance of pneumonia and systemic spread[65,170].

Few other influenza strains have been explored in cats. A few experiments have demonstrated shedding of human and a single avian isolate in cats with no clinical signs, lesions were not explored[53,120]. It is important to note that for cats, ingestion with pharyngeal exposure to high titers of virus and extensional spread to the upper respiratory tract is the suspected mechanism of pulmonary infection, and this has been further demonstrated in ferret studies[90].

LITERATURE CITED

1 Aamir UB, Naeem K, Ahmed Z, Obert CA, Franks J, Krauss S, Seiler P, Webster RG: Zoonotic potential of highly pathogenic avian H7N3 influenza viruses from Pakistan. Virology **390:** 212-220, 2009

2 Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM: Update on avian influenza A

(H5N1) virus infection in humans. The New England Journal of Medicine **358:** 261-273, 2008

3 Baskin CR, Garcia-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villan E, Katze MG: Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (Macaca nemestrina). Journal of Virology **78:** 10420-10432, 2004

4 Beare AS, Webster RG: Replication of avian influenza viruses in humans. Archives of Virology **119:** 37-42, 1991

5 Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, Lochindarat S, Nguyen TK, Nguyen TH, Tran TH, Nicoll A, Touch S, Yuen KY: Avian influenza A (H5N1) infection in humans. The New England Journal of Medicine **353:** 1374-1385, 2005

6 Belser JA, Blixt O, Chen LM, Pappas C, Maines TR, Van Hoeven N, Donis R, Busch J, McBride R, Paulson JC, Katz JM, Tumpey TM: Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. Proceedings of the National Academy of Sciences of the United States of America **105:** 7558-7563, 2008

7 Belser JA, Bridges CB, Katz JM, Tumpey TM: Past, present, and possible future human infection with influenza virus A subtype H7. Emerging Infectious Diseases **15:** 859-865, 2009

8 Belser JA, Lu X, Maines TR, Smith C, Li Y, Donis RO, Katz JM, Tumpey TM: Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. Journal of Virology **81:** 11139-11147, 2007

9 Belser JA, Wadford DA, Xu J, Katz JM, Tumpey TM: Ocular infection of mice with influenza A (H7) viruses: A site of primary replication and spread to the respiratory tract. Journal of Virology **83:** 10, 2009

10 Blazejewska P, Koscinski L, Viegas N, Anhlan D, Ludwig S, Schughart K: Pathogenicity of different PR8 influenza A virus variants in mice is determined by both viral and host factors. Virology **412:** 10, 2011

11 Boon AC, deBeauchamp J, Hollmann A, Luke J, Kotb M, Rowe S, Finkelstein D, Neale G, Lu L, Williams RW, Webby RJ: Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. Journal of Virology **83:** 10417-10426, 2009

12 Bradel-Tretheway BG, Kelley Z, Chakraborty-Sett S, Takimoto T, Kim B, Dewhurst S: The human H5N1 influenza A virus polymerase complex is active in vitro over a broad range of temperatures, in contrast to the WSN complex, and this property can be attributed to the PB2 subunit. The Journal of General Virology **89:** 2923-2932, 2008

13 Breban R, Drake JM, Stallknecht DE, Rohani P: The role of environmental transmission in recurrent avian influenza epidemics. Plos Computational Biology **5:** e1000346, 2009

14 Brown J, Stallknecht D, Swayne DE: Experimental infections of herring gulls (Larus argentatus) with H5N1 highly pathogenic avian influenza viruses by intranasal inoculation of virus and ingestion of virus-infected chicken meat. Avian Pathology **37:** 393-397, 2008

15 Buchweitz JP, Karmaus PW, Harkema: Time-Dependent Airway Epithelial and Inflammatory Cell Responses Induced by Influenza Virus A/PR/8/34 in C57BL/6 Mice. Toxicologic Pathology **35:** 424-435, 2007

16 Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JS, Guan Y: Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. Journal of Clinical Microbiology **43:** 5760-5767, 2005

17 Cameron CM, Cameron MJ, Bermejo-Martin JF, Ran L, Xu L, Turner PV, Ran R, Danesh A, Fang Y, Chan P-KM, Mytle N, Sullivan TJ, Collins TL, Johnson MG, Medina JC, Rowe T, Kelvin DJ: Gene Expression Analysis of Host Innate Immune Responses during Lethal H5N1 Infection in Ferrets. Journal of Virology **82:** 11308-11317, 2008

18 Capelozzi VL, Parra ER, Ximenes M, Bammann RH, Barbas CS, Duarte MI: Pathological and ultrastructural analysis of surgical lung biopsies in patients with swine-origin influenza type A/H1N1 and acute respiratory failure. Clinics (Sao Paulo) **65:** 1229-1237, 2010

19 Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS: Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respiratory Research **6:** doi:10.1186/1465-9921-1186-1135, 2005

20 Chen GW, Shih SR: Genomic signatures of influenza A pandemic (H1N1) 2009 virus. Emerging Infectious Diseases **15:** 1897-1903, 2009

21 Chen LM, Davis CT, Zhou H, Cox NJ, Donis RO: Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. PLoS Pathogens **4:** e1000072, 2008

22 Chen R, Holmes EC: Frequent inter-species transmission and geographic subdivision in avian influenza viruses from wild birds. Virology **383:** 156-161, 2009

23 Cheng VC, Chan JF, Wen X, Wu WL, Que TL, Chen H, Chan KH, Yuen KY: Infection of immunocompromised patients by avian H9N2 influenza A virus. Journal of Infection **doi:10.1016/j.inf.2011.02.007**, 2011

24 Chutinimitkul S, van Riel D, Munster VJ, van den Brand JM, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA, de Wit E: In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. Journal of Virology **84:** 6825-6833, 2010

25 Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P: A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog **3:** 1414-1421, 2007

26 Connor RJ, Kawaoka Y, Webster RG, Paulson JC: Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:** 17-23, 1994

27 Cottey R, Rowe CA, Bender BS: Influenza virus. Curr Protoc Immunol **Chapter 19:** Unit 19.11, 2001

28 Cox N, Uyeki TM: Public Health Implications of Avian Influenza Viruses. *In*: Avian Influenza, ed. Swayne D, pp. 453-484. Blackwell, Ames, IA, 2008

29 Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO: Transmission of equine influenza virus to dogs. Science (New York, N Y) **310:** 482-485, 2005

30 de Vries RP, de Vries E, Bosch BJ, de Groot RJ, Rottier PJ, de Haan CA: The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity. Virology **403:** 17-25, 2010

31 Dubin IN: A Pathological Study of Mice Infected with the Virus of Swine Influenza. The American Journal of Pathology **21:** 1121-1141, 1945

32 Dushoff J, Plotkin JB, Viboud C, Earn DJ, Simonsen L: Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. American Journal of Epidemiology **163:** 181-187, 2006

33 Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML: Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. Journal of Virology **74:** 1443-1450, 2000

34 Fislova T, Gocnik M, Sladkova T, Durmanova V, Rajcani J, Vareckova E, Mucha V, Kostolansky F: Multiorgan distribution of human influenza A virus strains observed in a mouse model. Archives of Virology **154:** 409-419, 2009

35 Fornek JL, Gillim-Ross L, Santos C, Carter V, Ward JM, Cheng LI, Proll S, Katze MG, Subbarao K: A single-amino-acid substitution in a polymerase protein of an H5N1 influenza virus is associated with systemic infection and impaired T-cell activation in mice. Journal of Virology **83:** 11102-11115, 2009

36 Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD: Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proceedings of the National Academy of Sciences of the United States of America **101:** 1356-1361, 2004

37 Fouchier RAM, Munster VJ, Keawcharoen J, Osterhaus ADME, Kuiken T: Virology of avian influenza in relation to wild birds. Journal of Wildlife Diseases **43:** 8, 2007

38 Gabriel G, Abram M, Keiner B, Wagner R, Klenk HD, Stech J: Differential polymerase activity in avian and mammalian cells determines host range of influenza virus. Journal of Virology **81:** 9601-9604, 2007

39 Gabriel G, Herwig A, Klenk HD: Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathogens **4:** e11, 2008

40 Geraci JR, St Aubin DJ, Barker IK, Webster RG, Hinshaw VS, Bean WJ, Ruhnke HL, Prescott JH, Early G, Baker AS, Madoff S, Schooley RT: Mass mortality of harbor seals: pneumonia associated with influenza A virus. Science (New York, NY) **215:** 1129-1131, 1982

41 Gillim-Ross L, Santos C, Chen Z, Aspelund A, Yang CF, Ye D, Jin H, Kemble G, Subbarao K: Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. Journal of Virology **82:** 10854-10863, 2008

42 Gillim-Ross L, Subbarao K: Emerging respiratory viruses: challenges and vaccine strategies. Clinical Microbiology Reviews **19:** 614-636, 2006

43 Goto H, Kawaoka Y: A novel mechanism for the acquisition of virulence by a human influenza A virus. Proceedings of the National Academy of Sciences of the United States of America **95:** 10224-10228, 1998

44 Goto H, Wells K, Takada A, Kawaoka Y: Plasminogen-binding activity of neuraminidase determines the pathogenicity of influenza A virus. Journal of Virology **75:** 9297-9301, 2001

45 Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E: Lethality to

ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. Journal of Virology **79:** 2191-2198, 2005

46 Grantham ML, Wu WH, Lalime EN, Lorenzo ME, Klein SL, Pekosz A: Palmitoylation of the influenza A virus M2 protein is not required for virus replication in vitro but contributes to virus virulence. Journal of Virology **83:** 8655-8661, 2009

47 Hale BG, Steel J, Manicassamy B, Medina RA, Ye J, Hickman D, Lowen AC, Perez DR, Garcia-Sastre A: Mutations in the NS1 C-terminal tail do not enhance replication or virulence of the 2009 pandemic H1N1 influenza A virus. The Journal of General Virology **91:** 1737-1742, 2010

48 Hall JS, Bentler KT, Landolt G, Elmore SA, Minnis RB, Campbell TA, Barras SC, Root JJ, Pilon J, Pabilonia K, Driscoll C, Slate D, Sullivan H, McLean RG: Influenza infection in wild raccoons. Emerging Infectious Diseases **14:** 1842-1848, 2008

49 Hatta M, Gao P, Halfmann P, Kawaoka Y: Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science (New York, N Y) **293:** 1840-1842, 2001

50 Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y: Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathogens **3:** 1374-1379, 2007

51 Hayashi T, Chaichoune K, Patchimasiri T, Hiromoto Y, Kawasaki Y, Wiriyarat W, Chakritbudsabong W, Prayoonwong N, Chaisilp N, Parchariyanon S, Ratanakorn P, Uchida Y, Tsuda T, Saito T: Differential host gene responses in mice infected with two highly pathogenic avian influenza viruses of subtype H5N1 isolated from wild birds in Thailand. Virology **412:** 9, 2011

52 Hayward JJ, Dubovi EJ, Scarlett JM, Janeczko S, Holmes EC, Parrish CR: Microevolution of canine influenza virus in shelters and its molecular epidemiology in the United States. Journal of Virology **84:** 12636-12645, 2010

53 Hinshaw VS, Webster RG, Easterday BC, Bean WJ, Jr.: Replication of avian influenza A viruses in mammals. Infection and Immunity **34:** 354-361, 1981

54 Horimoto T, Maeda K, Murakami S, Kiso M, Iwatsuki-Horimoto K, Shashika M, Ito T, Suzuki K, Yokoyama M, Kawaoka Y: Highly pathogenic avian influenza virus infection in feral racoons, Japan. Emerging Infectious Diseases **17:** 714-717, 2011

55 Hossain MJ, Hickman D, Perez DR: Evidence of expanded host range and mammalian-associated genetic changes in a duck H9N2 influenza virus following adaptation in quail and chickens. PLoS One **3:** e3170, 2008

56 Hulse DJ, Webster RG, Russell RJ, Perez DR: Molecular determinants within the surface proteins involved in the pathogenicity of H5N1 influenza viruses in chickens. Journal of Virology **78:** 9954-9964, 2004

57 Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, Holtzman MJ, Brody SL: Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. Journal of Virology **80:** 7469-7480, 2006

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

59 Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, Sakabe S, Imai M, Hatta Y, Watanabe S, Li C, Yamada S, Fujii K, Murakami S, Imai H, Kakugawa S, Ito M, Takano R, Iwatsuki-Horimoto K, Shimojima

M, Horimoto T, Goto H, Takahashi K, Makino A, Ishigaki H, Nakayama M, Okamatsu M, Warshauer D, Shult PA, Saito R, Suzuki H, Furuta Y, Yamashita M, Mitamura K, Nakano K, Nakamura M, Brockman-Schneider R, Mitamura H, Yamazaki M, Sugaya N, Suresh M, Ozawa M, Neumann G, Gern J, Kida H, Ogasawara K, Kawaoka Y: In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature **460:** 1021-1025, 2009

60 Jackson S, Van Hoeven N, Chen LM, Maines TR, Cox NJ, Katz JM, Donis RO: Reassortment between avian H5N1 and human H3N2 influenza viruses in ferrets: a public health risk assessment. Journal of Virology **83:** 8131-8140, 2009

61 Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, Liu W, Bu Z, Kawaoka Y, Chen H: A Single-Amino-Acid Substitution in the NS1 Protein Changes the Pathogenicity of H5N1 Avian Influenza Viruses in Mice. Journal of Virology **82:** 1146-1154, 2008

62 Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K: Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. Journal of Virology **81:** 10558-10566, 2007

63 Jourdain E, Gunnarsson G, Wahlgren J, Latorre-Margalef N, Brojer C, Sahlin S, Svensson L, Waldenstrom J, Lundkvist A, Olsen B: Influenza virus in a natural host, the mallard: experimental infection data. PLoS One **5:** e8935, 2010

64 Kalthoff D, Grund C, Harder TC, Lange E, Vahlenkamp TW, Mettenleiter TC, Beer M: Limited susceptibility of chickens, turkeys, and mice to pandemic (H1N1) 2009 virus. Emerging Infectious Diseases **16:** 703-705, 2010

65 Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theambooniers A, Tantilertcharoen R, Pattanarangsan

R, Arya N, Ratanakorn P, Osterhaus DM, Poovorawan Y: Avian influenza H5N1 in tigers and leopards. Emerging Infectious Diseases **10:** 2189-2191, 2004

66 Keawcharoen J, van Riel D, van Amerongen G, Bestebroer T, Beyer WE, van Lavieren R, Osterhaus AD, Fouchier RA, Kuiken T: Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). Emerging Infectious Diseases **14:** 600-607, 2008

67 Kida H, Yanagawa R, Matsuoka Y: Duck influenza lacking evidence of disease signs and immune response. Infection and Immunity **30:** 547-553, 1980

68 Kim HR, Park CK, Oem JK, Bae YC, Choi JG, Lee OS, Lee YJ: Characterization of H5N2 influenza viruses isolated in South Korea and their influence on the emergence of a novel H9N2 influenza virus. The Journal of General Virology **91:** 1978-1983, 2010

69 Kirkeby S, Martel CJ, Aasted B: Infection with human H1N1 influenza virus affects the expression of sialic acids of metaplastic mucous cells in the ferret airways. Virus Research **144:** 225-232, 2009

70 Klingeborn B, Englund L, Rott R, Juntti N, Rockborn G: An avian influenza A virus killing a mammalian species--the mink. Archives of Virology **86:** 347-351, 1985

71 Klopfleisch R, Wolf PU, Uhl W, Gerst S, Harder T, Starick E, Vahlenkamp TW, Mettenleiter TC, Teifke JP: Distribution of lesions and antigen of highly pathogenic avian influenza virus A/Swan/Germany/R65/06 (H5N1) in domestic cats after presumptive infection by wild birds. Veterinary Pathology **44:** 261-268, 2007

72 Klopfleisch R, Wolf PU, Wolf C, Harder T, Starick E, Niebuhr M, Mettenleiter TC, Teifke JP: Encephalitis in a stone marten (Martes foina) after natural infection with highly

pathogenic avian influenza virus subtype H5N1. Journal of Comparative Pathology **137:** 155-159, 2007

73 Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, Meijer A, van Steenbergen J, Fouchier R, Osterhaus A, Bosman A: Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet **363:** 587-593, 2004

74 Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, Osterhaus A: Avian H5N1 influenza in cats. Science (New York, N Y) **306:** 241, 2004

75 Kuiken T, Taubenberger JK: Pathology of human influenza revisited. Vaccine **26 Suppl 4:** D59-66, 2008

76 Kumlin U, Olofsson S, Dimock K, Arnberg N: Sialic acid tissue distribution and influenza virus tropism. Influenza and Other Respiratory Viruses **2:** 147-154, 2008

77 Labadie K, Dos Santos Afonso E, Rameix-Welti MA, van der Werf S, Naffakh N: Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. Virology **362:** 271-282, 2007

78 Latorre-Margalef N, Gunnarsson G, Munster VJ, Fouchier RA, Osterhaus AD, Elmberg J, Olsen B, Wallensten A, Haemig PD, Fransson T, Brudin L, Waldenstrom J: Effects of influenza A virus infection on migrating mallard ducks. Proceedings of the Royal Society Biological Sciences **276:** 1029-1036, 2009

79 Lau LL, Cowling BJ, Fang VJ, Chan KH, Lau EH, Lipsitch M, Cheng CK, Houck PM, Uyeki TM, Peiris JS, Leung GM: Viral shedding and clinical illness in naturally acquired influenza virus infections. The Journal of Infectious Diseases **201:** 1509-1516, 2010

80 Le QM, Sakai-Tagawa Y, Ozawa M, Ito M, Kawaoka Y: Selection of H5N1 influenza virus PB2 during replication in humans. Journal of Virology **83:** 5278-5281, 2009

81 Lee CW, Lee YJ, Senne DA, Suarez DL: Pathogenic potential of North American H7N2 avian influenza virus: a mutagenesis study using reverse genetics. Virology **353:** 388-395, 2006

82 Lee SM, Cheung CY, Nicholls JM, Hui KP, Leung CY, Uiprasertkul M, Tipoe GL, Lau YL, Poon LL, Ip NY, Guan Y, Peiris JS: Hyperinduction of cyclooxygenase-2-mediated proinflammatory cascade: a mechanism for the pathogenesis of avian influenza H5N1 infection. The Journal of Infectious Diseases **198:** 525-535, 2008

83 Lee YJ, Choi YK, Kim YJ, Song MS, Jeong OM, Lee EK, Jeon WJ, Jeong W, Joh SJ, Choi KS, Her M, Kim MC, Kim A, Kim MJ, Ho Lee E, Oh TG, Moon HJ, Yoo DW, Kim JH, Sung MH, Poo H, Kwon JH, Kim CJ: Highly pathogenic avian influenza virus (H5N1) in domestic poultry and relationship with migratory birds, South Korea. Emerging Infectious Diseases **14:** 487-490, 2008

84 Lee YJ, Shin JY, Song MS, Lee YM, Choi JG, Lee EK, Jeong OM, Sung HW, Kim JH, Kwon YK, Kwon JH, Kim CJ, Webby RJ, Webster RG, Choi YK: Continuing evolution of H9 influenza viruses in Korean poultry. Virology **359:** 313-323, 2007

85 Leigh MW, Connor RJ, Kelm S, Baum LG, Paulson JC: Receptor specificity of influenza virus influences severity of illness in ferrets. Vaccine **13:** 1468-1473, 1995

86 Leschnik M, Weikel J, Möstl K, Revilla-Fernández S, Wodak E, Bagó Z, Vanek E, Benetka V, Hess M, Thalhammer JG: Subclinical infection with avian influenza A (H5N1) virus in cats. Emerging Infectious Diseases **13:** 243-247, 2007

87 Li Y, Shi J, Qi Q, Deng G, Tian G, Zhao H, Zhong G, Chen H: H5N1 avian influenza outbreak in wild bird in Western China in 2005. Journal of Wildlife Diseases **43:** 1, 2007

88 Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K: Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. Journal of Virology **79:** 12058-12064, 2005

89 Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H: The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. Journal of Virology **80:** 11115-11123, 2006

90 Lipatov AS, Kwon YK, Pantin-Jackwood MJ, Swayne DE: Pathogenesis of H5N1 influenza virus infections in mice and ferret models differs according to respiratory tract or digestive system exposure. The Journal of Infectious Diseases **199:** 717-725, 2009

91 Lofgren E, Fefferman NH, Naumov YN, Gorski J, Naumova EN: Influenza Seasonality: Underlying Causes and Modeling Theories. Journal of Virology **81:** 5429-5436, 2007

92 Löhr CV, DeBess EE, Baker RJ, Hiett SL, Hoffman KA, Murdoch VJ, Fischer KA, Mulrooney DM, Selman RL, Hammill-Black WM: Pathology and viral antigen distribution of lethal pneumonia in domestic cats due to pandemic (H1N1) 2009 influenza A virus. Veterinary Pathology **47:** 378-386, 2010

93 Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM: A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. Journal of Virology **73:** 5903-5911, 1999

94 Lycett SJ, Ward MJ, Lewis FI, Poon AF, Kosakovsky Pond SL, Brown AJ: Detection of mammalian virulence determinants in highly pathogenic avian influenza H5N1 viruses: multivariate analysis of published data. Journal of Virology **83:** 9901-9910, 2009

95 Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, Gauger PC, Patnayak DP, Webby RJ, Richt JA: Identification of H2N3 influenza A viruses from swine in the United States. Proceedings of the National Academy of Sciences of the United States of America **104:** 20949-20954, 2007

96 Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, Ortin J, Falcon A, Nguyen TH, Mai le Q, Sedyaningsih ER, Harun S, Tumpey TM, Donis RO, Cox NJ, Subbarao K, Katz JM: Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proceedings of the National Academy of Sciences of the United States of America **103:** 12121-12126, 2006

97 Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, Zeng H, Gustin KM, Pearce MB, Viswanathan K, Shriver ZH, Raman R, Cox NJ, Sasisekharan R, Katz JM, Tumpey TM: Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science (New York, NY) **325:** 484-487, 2009

98 Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P, Chittaganpitch M, Waicharoen S, Nguyen DT, Nguyen T, Nguyen HH, Kim JH, Hoang LT, Kang C, Phuong LS, Lim W, Zaki S, Donis RO, Cox NJ, Katz JM, Tumpey TM: Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. Journal of Virology **79:** 11788-11800, 2005

99 Mase M, Tanimura N, Imada T, Okamatsu M, Tsukamoto K, Yamaguchi S: Recent H5N1 avian influenza A virus increases rapidly in virulence to mice after a single passage in mice. The Journal of General Virology **87:** 3655-3659, 2006

100 Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y: Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. Journal of Virology **74:** 8502-8512, 2000

101 Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Karlsson KA: Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology **233:** 224-234, 1997

102 Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD: Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proceedings of the National Academy of Sciences of the United States of America **101:** 4620-4624, 2004

103 Matsuda K, Park CH, Sunden Y, Kimura T, Ochiai K, Kida H, Umemura T: The vagus nerve is one route of transneural invasion for intranasally inoculated influenza a virus in mice. Veterinary Pathology **41:** 101-107, 2004

104 Matsuoka Y, Swayne DE, Thomas C, Rameix-Welti MA, Naffakh N, Warnes C, Altholtz M, Donis R, Subbarao K: Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. Journal of Virology **83:** 4704-4708, 2009

105 Mauad T, Hajjar LA, Callegari GD, da Silva LF, Schout D, Galas FR, Alves VA, Malheiros DM, Auler JO, Jr., Ferreira AF, Borsato MR, Bezerra SM, Gutierrez PS, Caldini ET, Pasqualucci CA, Dolhnikoff M, Saldiva PH: Lung pathology in fatal novel human

influenza A (H1N1) infection. American Journal of Respiratory and Critical Care Medicine **181:** 72-79, 2010

106 McAuley JL, Zhang K, McCullers JA: The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. Journal of Virology **84:** 558-564, 2009

107 McBrayer A, Camp JV, Tapp R, Yamshchikov V, Grimes S, Noah DL, Jonsson CB, Bruder CE: Course of seasonal influenza A/Brisbane/59/07 H1N1 infection in the ferret. Virology Journal **7:** 5, 2010

108 McCullers JA, Van De Velde L, Schultz RD, Mitchell CG, Halford CR, Boyd K, Schultz-Cherry S: Seroprevalence of seasonal and pandemic influenza A viruses in domestic cats. Archives of Virology **156:** 117-120, 2011

109 McGavin DM, Zachary JF: Pathologic Basis of Veterinary Disease, 4 ed. Mosby, St. Louis, 2007

110 Memoli MJ, Tumpey TM, Jagger BW, Dugan VG, Sheng ZM, Qi L, Kash JC, Taubenberger JK: An early 'classical' swine H1N1 influenza virus shows similar pathogenicity to the 1918 pandemic virus in ferrets and mice. Virology **393:** 338-345, 2009

111 Munier S, Larcher T, Cormier-Aline F, Soubieux D, Su B, Guigand L, Labrosse B, Cherel Y, Quere P, Marc D, Naffakh N: A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. Journal of Virology **84:** 940-952, 2010

112 Munster VJ, de Wit 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: Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science (New York, NY) **325:** 481-483, 2009

113 Munster VJ, de Wit E, van Riel D, Beyer WE, Rimmelzwaan GF, Osterhaus AD, Kuiken T, Fouchier RA: The molecular basis of the pathogenicity of the Dutch highly pathogenic human influenza A H7N7 viruses. The Journal of Infectious Diseases **196:** 258-265, 2007

114 Neumann G, Kawaoka Y: Host range restriction and pathogenicity in the context of influenza pandemic. Emerging Infectious Diseases **12:** 881-886, 2006

115 Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL, Wong MP, Chui WH, Poon LL, Tsao SW, Guan Y, Peiris JS: Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nature Medicine **13:** 147-149, 2007

116 Ning Z, Luo M, Qi W, Yu B, Jiao P, Liao M: Detection of expression of influenza virus receptors in tissues of BALB/c mice by histochemistry. Veterinary Research Communications **33:** 895-903, 2009

117 Nofs S, Abd-Eldaim M, Thomas KV, Toplon D, Rouse D, Kennedy M: Influenza virus A (H1N1) in giant anteaters (Myrmecophaga tridactyla). Emerging Infectious Diseases **15:** 1081-1083, 2009

118 Octaviani CP, Ozawa M, Yamada S, Goto H, Kawaoka Y: High level of genetic compatibility between swine-origin H1N1 and highly pathogenic avian H5N1 influenza viruses. Journal of Virology **84:** 10918-10922, 2010

119 Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA: Global patterns of influenza a virus in wild birds. Science (New York, N Y) **312:** 384-388, 2006

120 Paniker CK, Nair CM: Experimental infection of animals with influenzavirus types A and B. Bulletin of the World Health Organization **47:** 461-463, 1972

121 Patterson AR, Cooper VL, Yoon KJ, Janke BH, Gauger PC: Naturally occurring influenza infection in a ferret (Mustela putorius furo) colony. Journal of Veterinary Diagnostic Investigation **21:** 527-530, 2009

122 Payungporn S, Crawford PC, Kouo TS, Chen LM, Pompey J, Castleman WL, Dubovi EJ, Katz JM, Donis RO: Influenza A virus (H3N8) in dogs with respiratory disease, Florida. Emerging Infectious Diseases **14:** 902-908, 2008

123 Pekosz A, Newby C, Bose PS, Lutz A: Sialic acid recognition is a key determinant of influenza A virus tropism in murine trachea epithelial cell cultures. Virology **386:** 61-67, 2009

124 Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, Hernandez M, Quinones-Falconi F, Bautista E, Ramirez-Venegas A, Rojas-Serrano J, Ormsby CE, Corrales A, Higuera A, Mondragon E, Cordova-Villalobos JA: Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. The New England Journal of Medicine **361:** 680-689, 2009

125 Perez DR, Webby RJ, Hoffmann E, Webster RG: Land-based birds as potential disseminators of avian mammalian reassortant influenza A viruses. Avian Diseases **47:** 1114-1117, 2003

126 Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM: H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog **4:** e1000115, 2008

127 Ping J, Dankar SK, Forbes NE, Keleta L, Zhou Y, Tyler S, Brown EG: PB2 and hemagglutinin mutations are major determinants of host range and virulence in mouse-adapted influenza A virus. Journal of Virology **84:** 10606-10618, 2010

128 Puzelli S, Di Trani L, Fabiani C, Campitelli L, De Marco MA, Capua I, Aguilera JF, Zambon M, Donatelli I: Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. The Journal of Infectious Diseases **192:** 1318-1322, 2005

129 Rigoni M, Shinya K, Toffan A, Milani A, Bettini F, Kawaoka Y, Cattoli G, Capua I: Pneumo- and neurotropism of avian origin Italian highly pathogenic avian influenza H7N1 isolates in experimentally infected mice. Virology **364:** 28-35, 2007

130 Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD: A primate model to study the pathogenesis of influenza A (H5N1) virus infection. Avian Diseases **47:** 931-933, 2003

131 Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, Osterhaus AD, Kuiken T: Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. The American Journal of Pathology **168:** 176-183, 2006

132 Rogers GN, Paulson JC: Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology **127:** 361-373, 1983

133 Rohani P, Breban R, Stallknecht DE, Drake JM: Environmental transmission of low pathogenicity avian influenza viruses and its implications for pathogen invasion. Proceedings of the National Academy of Science of the United States of America **106:** 10365-10369, 2009

134 Rolling T, Koerner I, Zimmermann P, Holz K, Haller O, Staeheli P, Kochs G: Adaptive mutations resulting in enhanced polymerase activity contribute to high virulence of influenza A virus in mice. Journal of Virology **83:** 6673-6680, 2009

135 Rott R: The pathogenic determinant of influenza virus. Veterinary Microbiology **33:** 303-310, 1992

136 Russell C, Hanna A, Barrass L, Matrosovich M, Nunez A, Brown IH, Choudhury B, Banks J: Experimental infection of turkeys with pandemic (H1N1) 2009 influenza virus (A/H1N1/09v). Journal of Virology **83:** 13046-13047, 2009

137 Schrauwen EJ, Bestebroer TM, Munster VJ, De Wit E, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA: Insertion of a multi basic cleavage site in the hemagglutinin of a human influenza H3N2 virus does not increase pathogenicity in ferrets. The Journal of General Virology **doi:10.1099/vir.0.030379-0**, 2011

138 Schrenzel M, more: Pandemic (H1N1) 2009 virus in 3 wildlife species, San Diego, California, USA. Emerging Infectious Diseases **14:** 747-749, 2011

139 Shieh WJ, Blau DM, Denison AM, Deleon-Carnes M, Adem P, Bhatnagar J, Sumner J, Liu L, Patel M, Batten B, Greer P, Jones T, Smith C, Bartlett J, Montague J, White E, Rollin D, Gao R, Seales C, Jost H, Metcalfe M, Goldsmith CS, Humphrey C, Schmitz A, Drew C, Paddock C, Uyeki TM, Zaki SR: 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. The American Journal of Pathology **177:** 166-175, 2010

140 Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, Lindstrom S, Gubareva LV, Deyde V, Garten RJ, Harris M, Gerber S, Vagasky S, Smith F, Pascoe N, Martin K, Dufficy D, Ritger K, Conover C, Quinlisk P, Klimov A, Bresee JS, Finelli L: Triple-

reassortant swine influenza A (H1) in humans in the United States, 2005-2009. The New England Journal of Medicine **360:** 2616-2625, 2009

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

142 Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y: PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology **320:** 258-266, 2004

143 Shope RE: The Infection of Ferrets with Swine Influenza Virus. The Journal of Experimental Medicine **60:** 49-61, 1934

144 Shope RE: The Infection of Mice with Swine Influenza Virus. The Journal of Experimental Medicine **62:** 561-572, 1935

145 Shope RE: Swine Influenza : I. Experimental Transmission and Pathology. The Journal of Experimental Medicine **54:** 349-359, 1931

146 Smith GJ, Vijaykrishna D, Ellis TM, Dyrting KC, Leung YH, Bahl J, Wong CW, Kai H, Chow MK, Duan L, Chan AS, Zhang LJ, Chen H, Luk GS, Peiris JS, Guan Y: Characterization of avian influenza viruses A (H5N1) from wild birds, Hong Kong, 2004-2008. Emerging Infectious Diseases **15:** 402-407, 2009

147 Song D, Kang B, Lee C, Jung K, Ha G, Kang D, Park S, Park B, Oh J: Transmission of avian influenza virus (H3N2) to dogs. Emerging Infectious Diseases **14:** 741-746, 2008

148 Song H, Wan H, Araya Y, Perez DR: Partial direct contact transmission in ferrets of a mallard H7N3 influenza virus with typical avian-like receptor specificity. Virology Journal **6:** 12, 2009

149 Song MS, Pascua PN, Lee JH, Baek YH, Lee OJ, Kim CJ, Kim H, Webby RJ, Webster RG, Choi YK: The polymerase acidic protein gene of influenza a virus contributes to pathogenicity in a mouse model. Journal of Virology **83:** 12325-12335, 2009

150 Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Pariyothorn N, Payungporn S, Theamboonlers A, Chutinimitkul S, Thanawongnuwech R, Poovorawan Y: Fatal avian influenza A H5N1 in a dog. Emerging Infectious Diseases **12:** 1744-1747, 2006

151 Songsermn T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, Poovorawan Y: Avian influenza H5N1 in naturally infected domestic cat. Emerging Infectious Diseases **12:** 681-683, 2006

152 Sorrell EM, Song H, Pena L, Perez DR: A 27-amino-acid deletion in the neuraminidase stalk supports replication of an avian H2N2 influenza A virus in the respiratory tract of chickens. Journal of Virology **84:** 11831-11840, 2010

153 Sorrell EM, Wan H, Araya Y, Song H, Perez DR: Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proceedings of the National Academy of Sciences of the United States of America **106:** 7565-7570, 2009

154 Stallknecht DE, Brown JD: Ecology of Avian Influenza in Wild Birds. *In*: Avian Influenza, ed. Swayne D, pp. 43-58. Blackwell, Ames, IA, 2008

155 Stech O, Veits J, Weber S, Deckers D, Schroer D, Vahlenkamp TW, Breithaupt A, Teifke J, Mettenleiter TC, Stech J: Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain. Journal of Virology **83:** 5864-5868, 2009

156 Steel J, Lowen AC, Mubareka S, Palese P: Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathogens **5:** e1000252, 2009

157 Steel J, Lowen AC, Pena L, Angel M, Solorzano A, Albrecht R, Perez DR, Garcia-Sastre A, Palese P: Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. Journal of Virology **83:** 1742-1753, 2009

158 Steinhauer DA: Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:** 1-20, 1999

159 Stevens J, Blixt O, Paulson JC, Wilson IA: Glycan microarray technologies: tools to survey host specificity of influenza viruses. Nature Reviews Microbiology **4:** 857-864, 2006

160 Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG: Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? Journal of Virology **79:** 11269-11279, 2005

161 Suarez DL: Influenza A Virus. *In*: Avian Influenza, ed. Swayne D, pp. 3-22. Blackwell, Ames, IA, 2008

162 Subbarao EK, London W, Murphy BR: A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. Journal of Virology **67:** 1761-1764, 1993

163 Sun Y, Qin K, Wang J, Pu J, Tang Q, Hu Y, Bi Y, Zhao X, Yang H, Shu Y, Liu J: High genetic compatibility and increased pathogenicity of reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza viruses. Proceedings of the National Academy of Sciences of the United States of America **108:** 4164-4169, 2011

164 Suzuki Y, Ito T, Suzuki T, Holland RE, Jr., Chambers TM, Kiso M, Ishida H, Kawaoka Y: Sialic acid species as a determinant of the host range of influenza A viruses. Journal of Virology **74:** 11825-11831, 2000

165 Svitek N, Rudd PA, Obojes K, Pillet S, von Messling V: Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. Virology **376:** 53-59, 2008

166 Swayne D: Epidemiology of Avian Influenza in Agriculture and Other Man-Made Systems. *In*: Avian Influenza, ed. Swayne D, pp. 59-86. Blackwell, Ames, IA, 2008

167 Swayne D, Pantin-Jackwood M: Pathobiology of Avian Influenza Virus Infections in Birds and Mammals. *In*: Avian Influenza, ed. Swayne D, pp. 87-122. Blackwell, Ames, IA, 2008

168 Szretter KJ, Gangappa S, Lu X, Smith C, Shieh WJ, Zaki SR, Sambhara S, Tumpey TM, Katz JM: Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. Journal of Virology **81:** 2736-2744, 2007

169 Taubenberger JK, Morens DM: The pathology of influenza virus infections. Annual Review of Pathology: Mechanisms of Disease **3:** 499-522, 2008

170 Thanawongnuwech R, Amonsin A, Tantilertcharoen R, Damrongwatanapokin S, Theamboonlers A, Payungporn S, Nanthapornphiphat K, Ratanamungklanon S, Tunak E, Songserm T, Vivatthanavanich V, Lekdumrongsak T, Kesdangsakonwut S, Tunhikorn S, Poovorawan Y: Probable tiger-to-tiger transmission of avian influenza H5N1. Emerging Infectious Diseases **11:** 699-701, 2005

171 Thompson CI, Barclay WS, Zambon MC, Pickles RJ: Infection of human airway epithelium by human and avian strains of influenza a virus. Journal of Virology **80:** 8060-8068, 2006

172 Thompson WW, Comanor L, Shay DK: Epidemiology of seasonal influenza: use of surveillance data and statistical models to estimate the burden of disease. The Journal of Infectious Diseases **194 Suppl 2:** S82-91, 2006

173 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A: Characterization of the Reconstructed 1918 Spanish Influenza Pandemic Virus. Science (New York, N Y) **310:** 77-80, 2005

174 Tumpey TM, Lu X, Morken T, Zaki SR, Katz JM: Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. Journal of Virology **74:** 6105-6116, 2000

175 Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A: A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science (New York, NY) **315:** 655-659, 2007

176 Tumpey TM, Szretter KJ, Van Hoeven N, Katz JM, Kochs G, Haller O, Garcia-Sastre A, Staeheli P: The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. Journal of Virology **81:** 10818-10821, 2007

177 Twu KY, Kuo RL, Marklund J, Krug RM: The H5N1 Influenza Virus NS Genes Selected after 1998 Enhance Virus Replication in Mammalian Cells. Journal of Virology **81:** 8112-8121, 2007

178 Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, Nicholls JM, Chokephaibulkit K, Vanprapar N, Auewarakul P: Influenza A H5N1 replication sites in humans. Emerging Infectious Diseases **11:** 1036-1041, 2005

179 Vahlenkamp TW, Harder TC: Influenza virus infections in mammals. Berliner Und Munchener Tierarztliche Wochenschrift **119:** 123-131, 2006

180 van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, Munster V, Bestebroer T, Fouchier RA, Kuiken T, Osterhaus AD: Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. The Journal of Infectious Diseases **201:** 993-999, 2010

181 van den Brand JM, Stittelaar KJ, van Amerongen G, van de Bildt MW, Leijten LM, Kuiken T, Osterhaus AD: Experimental pandemic (H1N1) 2009 virus infection of cats. Emerging Infectious Diseases **16:** 1745-1747, 2010

182 van Gils JA, Munster VJ, Radersma R, Liefhebber D, Fouchier RA, Klaassen M: Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. PLoS One **2:** e184, 2007

183 Van Hoeven N, Pappas C, Belser JA, Maines TR, Zeng H, García-Sastre A, Sasisekharan R, Katz JM, Tumpey TM: Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proceedings of The National Academy of Sciences of the United States of America **106:** 3366-3371, 2009

184 van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T: Human and Avian Influenza Viruses Target Different Cells in the Lower Respiratory Tract of Humans and Other Mammals. The American Journal of Pathology **171:** 1215-1223, 2007

185 Varki NM, Varki A: Diversity in cell surface sialic acid presentations: implications for biology and disease. Laboratory Investigation **87:** 851-857, 2007

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

187 Wan H, Sorrell EM, Song H, Hossain MJ, Ramirez-Nieto G, Monne I, Stevens J, Cattoli G, Capua I, Chen LM, Donis RO, Busch J, Paulson JC, Brockwell C, Webby R, Blanco J, Al-Natour MQ, Perez DR: Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One **3:** e2923, 2008

188 Wang G, Zhan D, Li L, Lei F, Liu B, Liu D, Xiao H, Feng Y, Li J, Yang B, Yin Z, Song X, Zhu X, Cong Y, Pu J, Wang J, Liu J, Gao GF, Zhu Q: H5N1 avian influenza re-emergence of Lake Qinghai: phylogenetic and antigenic analyses of the newly isolated viruses and roles of migratory birds in virus circulation. The Journal of General Virology **89:** 697-702, 2008

189 Ward AC: Virulence of influenza A virus for mouse lung. Virus Genes **14:** 187-194, 1997

190 Watanabe T, Watanabe S, Shinya K, Kim JH, Hatta M, Kawaoka Y: Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets. Proceedings of the National Academy of Sciences of the United States of America **106:** 588-592, 2009

191 Webster R, Walker E: Influenza. American Scientist **91:** 8, 2003

192 Xu KM, Smith GJD, Bahl J, Duan L, Tai H, Vijaykrishna D, Wang J, Zhang JX, Li KS, Fan XH, Webster RG, Chen H, Peiris JSM, Guan Y: The Genesis and Evolution of H9N2 Influenza Viruses in Poultry from Southern China, 2000 to 2005. Journal of Virology **81:** 10389-10401, 2007

193 Xu Q, Wang W, Cheng X, Zengel J, Jin H: Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. Journal of Virology **84:** 4936-4945, 2010

194 Xu T, Qiao J, Zhao L, Wang G, He G, Li K, Tian Y, Gao M, Wang J, Wang H, Dong C: Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. American Journal of Respiratory and Critical Care Medicine **174:** 1011-1017, 2006

195 Yang Y, Halloran ME, Sugimoto JD, Longini IMJ: Detecting Human-to-Human Transmission of Avian Influenza A (H5N1). Emerging Infectious Diseases **13:** 1348-1358, 2007

196 Yingst SL, Saad MD, Felt SA: Qinghai-like H5N1 from domestic cats, northern Iraq. Emerging Infectious Diseases **12:** 1295-1297, 2006

197 Yoon KJ, Cooper VL, Schwartz KJ, Harmon KM, Kim WI, Janke BH, Strohbehn J, Butts D, Troutman J: Influenza virus infection in racing greyhounds. Emerging Infectious Diseases **11:** 1974-1976, 2005

198 Zamarin D, Ortigoza MB, Palese P: Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. Journal of Virology **80:** 7976-7983, 2006

199 Zhang Z, Zhang J, Huang K, Li KS, Yuen KY, Guan Y, Chen H, Ng WF: Systemic infection of avian influenza A virus H5N1 subtype in humans. Human Pathology **40:** 735-739, 2009

200 Zhou J, Sun W, Wang J, Guo J, Yin W, Wu N, Li L, Yan Y, Liao M, Huang Y, Luo K, Jiang X, Chen H: Characterization of the H5N1 highly pathogenic avian influenza virus derived from wild pikas in China. Journal of Virology **83:** 8957-8964, 2009

201 Zhu H, Wang J, Wang P, Song W, Zheng Z, Chen R, Guo K, Zhang T, Peiris JS, Chen H, Guan Y: Substitution of lysine at 627 position in PB2 protein does not change virulence of the 2009 pandemic H1N1 virus in mice. Virology **401:** 1-5, 2010

202 Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM: Pathogenesis of avian influenza A (H5N1) viruses in ferrets. Journal of Virology **76:** 4420-4429, 2002

CHAPTER 3

AVIAN INFLUENZA ISOLATES FROM WILD BIRDS REPLICATE AND CAUSE DISEASE IN A MOUSE MODEL OF INFECTION[1]

[1]Driskell, E.A., C.A. Jones, D.E. Stallknecht, E.W. Howerth, and S.M. Tompkins. 2010. *Virology* 399: 280-289. Reprinted here with permission of publisher.

ABSTRACT

The direct transmission of highly pathogenic avian influenza (HPAI) viruses to humans in Eurasia and subsequent disease has sparked research efforts leading to better understanding of HPAI virus transmission and pathogenicity in mammals. There has been minimal focus on examining the capacity of circulating low pathogenic wild bird avian influenza viruses to infect mammals. We have utilized a mouse model for influenza virus infection to examine 28 North American wild bird avian influenza virus isolates that include the hemagglutinin subtypes H2, H3, H4, H6, H7, and H11. We demonstrate that many wild bird avian influenza viruses of several different hemagglutinin types replicate in this mouse model without adaptation and induce histopathologic lesions similar to other influenza virus infections, but cause minimal morbidity. These findings demonstrate the potential of wild avian influenza viruses to directly infect mice without prior adaptation and support their potential role in emergence of pandemic influenza.

Keywords: Avian influenza; mammals; mice; pathogenicity

INTRODUCTION

The 1997 outbreak of highly pathogenic H5N1 avian influenza (HPAI) virus in humans marked the beginning of intense investigation into the pathogenesis of human infections with avian influenza viruses (AIV) (Subbarao et al., 1998). These "novel" AIVs are of concern not only because of the severity of disease observed but also because they have a pandemic potential (Li et al., 2004; Maines et al., 2005). Since the 1997 H5N1 outbreak, many other examples of human infections with AIVs have occurred. These outbreaks have also demonstrated that other hemagglutinin subtypes of AIVs are capable of direct human infection, such as H7 and H9, with varying morbidity and mortality (Belser et al., 2009; Butt et al., 2005; Fouchier et al., 2004; Guo,

Li, and Cheng, 1999; Koopmans et al., 2004). In cases of H5N1 infections, disease in humans has been geographically and temporally associated with a HPAI outbreak in poultry (Beigel et al., 2005). Human infections with H7 and H9 AIV subtypes have also most frequently been attributed to transmission from poultry (Belser et al., 2009; Butt et al., 2005). The mechanism of how AIVs are capable of directly infecting humans and other mammals is multifactorial, including differences in hemagglutinin receptor specificity and polymerase activity, and is still being elucidated (Gabriel et al., 2007; Gambaryan et al., 2006; Labadie et al., 2007; Li et al., 2005; Rogers and Paulson, 1983; Thompson et al., 2006; van Riel et al., 2007). These studies have primarily focused on H5N1 virus isolates, and there is minimal information available on the abundant other AIVs that exist in nature.

Direct transmission of AIV from birds is not unique to humans but also has been observed in other mammals not traditionally considered susceptible to influenza, such as domestic cats and dogs, wild cats, and seals (Geraci et al., 1982; Hinshaw et al., 1984; Keawcharoen et al., 2004; Klingeborn et al., 1985; Klopfleisch et al., 2007; Song et al., 2008; Songserm et al., 2006a; Songserm et al., 2006b). Again, a range of avian influenza hemagglutinin subtypes have been demonstrated to infect other mammals, including H4, H5, H6, H7, H9 and H10, by natural or experimental infection. Investigators are finding increasingly more avian influenza subtypes that can productively infect mammals resulting in varied morbidity (Belser et al., 2007; Dybing et al., 2000; Gillim-Ross et al., 2008; Hinshaw et al., 1981; Joseph et al., 2007; Rigoni et al., 2007; Wan et al., 2008). The mouse model for influenza infection has been widely used in studies of human and avian influenza to elucidate virulence and pathogenesis of these viruses (Fislova et al., 2009; Lu et al., 1999; Tumpey et al., 2007; Ward, 1997). Pathogenicity studies have primarily focused on HPAI isolates that have been

involved in human infections, with only a small number of studies examining low pathogenic avian influenza (LPAI) isolates from poultry and even fewer isolates examined from wild birds (Gillim-Ross et al., 2008; Henzler et al., 2003; Joseph et al., 2007; Wan et al., 2008). Wild birds are the reservoirs for all influenza subtypes and recently have been implicated in the spread of HPAI H5N1(Cattoli and Capua, 2007; Keawcharoen et al., 2008; Li et al., 2004; Olsen et al., 2006; Stallknecht and Brown, 2007). Examining the behavior of a large variety of wild bird avian influenza isolates in the mouse model is a step towards deeper understanding of the risks and mechanisms of avian influenza infections in mammals.

All pandemic influenza viruses since 1918, including the 2009 H1N1 virus have at least an AIV component (Glaser et al., 2005; Matrosovich et al., 2000; Stevens et al., 2006). There is a possibility that the next pandemic influenza precursor is circulating in the wild bird population. In this study, the mouse model is utilized for influenza to examine a large variety of AIVs isolated from wild birds from influenza surveillance in the United States. Results demonstrate that many of these isolates can replicate in the lung of mice and induce pulmonary lesions with minimal morbidity. For some isolates, virus was localized with immunohistochemistry and demonstrated robust replication in epithelial tissues. Serology after infection showed that immunogenicity was variable and unrelated to replication or pathology. These data support the idea that humans and other mammals may be directly infected with wild bird AIVs, in some cases subclinically and without seroconversion, providing a potential avenue for emergence of influenza viruses with pandemic potential via mutation and/or reassortment.

METHODS

Viruses

Avian influenza viruses used were cloacal swab isolates from wild birds in the United States acquired from Southeastern Cooperative Wildlife Disease Study at the University of Georgia collected between 1998 and 2006. Viruses used in this study are shown in Table 3.1. Isolates used for in vitro screening were isolated from cloacal swabs in 9 day old ECE at 37°C for 72 hours and then minimally passaged (3 or fewer passages) in ECEs. Approximately 400 influenza virus isolates were examined in vitro. Viruses that exhibited plaquing on Madin-Darby canine kidney (MDCK) cells without the addition of trypsin were selected for additional in vivo selection in mice, as this phenotype is suggestive of enhanced pathogenicity of influenza viruses. The original low passage isolates, once selected by in vitro screening methods, were grown once more in 9 to 10 day old ECE to generate a stock of the virus. Allantoic fluid pooled from eggs was frozen in aliquots at -80°C. Virus stocks were plaqued on MDCK cells to elucidate a PFU/mL titer.

Mouse experiments

Female 6 to 8 week old BALB/c mice (Harlan Laboratories, Indianapolis, IN) were anesthetized with intraperitoneal injection of 2,2,2-tribromoethanol in *tert*-amyl alcohol and inoculated intranasally with 50 µL of diluted virus in sterile phosphate buffered saline (PBS). A screen to determine the replication capacity of selected AIV isolates in mice was performed by harvesting lungs of mice inoculated with a 1:10 dilution of virus stock (3 to 4 mice per virus) or a 1:100 dilution of virus stock (3 to 4 mice per virus) on day 4 post-inoculation. A group of mice was mock infected with PBS and another group was infected with X31 to serve as controls. Mouse experiments were performed in enhanced BSL2 facilities in HEPA filtered isolators.

Studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia. Clarified lung homogenate and fixed lung tissue from selected influenza inoculated mice (RT/625) exhibiting histopathologic lesions were negative for *Mycoplasma pulmonis* via PCR.

The MID_{50} was determined as previously described (Cottey, Rowe, and Bender, 2001). Briefly, mice were infected with 10-fold dilutions of each virus. Five mice per group were euthanized on day 4 p.i. and clarified lung homogenate was serially titrated in MDCK cells to determine the MID_{50} calculated by the method of Reed and Muench (Reed and Muench, 1938). The pathogenesis of RT/625, RT/645, and RK/470 was determined by inoculation of groups of mice with $20MID_{50}$ of virus and harvesting lungs on days 1, 3, 5, 7, and 14 p.i. and harvesting spleen, liver, kidney and brain on day 5 p.i (5 mice per virus per day). Tissues were homogenized in 1 mL PBS, clarified by centrifugation, and frozen at -80°C for later titration. Clarified lung homogenate was titrated in MDCK cells starting at a 1:10 dilution with a limit of detection at $10^{2.4}$ $TCID_{50}$/gram. Clarified organ homogenate (spleen, liver, kidney, and brain) was initially titrated in MDCK cells starting at a 1:10 dilution. Samples were further selected for virus isolation in 9 to 10 day old ECE. Real time RT-PCR was performed on hemagglutination assay positive samples from virus isolation. Briefly, viral RNA was extracted from allantoic fluid by using RNeasy mini kit (Qiagen, Valencia, CA) and the Quiagen one-step RT-PCR kit was used for RRT-PCR with a Stratagene MX300P/3005P thermocyler and Mx Pro QPCR software (La Jolla, CA). Reaction mixture and PCR cycling protocol is available upon request. An influenza virus matrix gene specific primer and probe set were used as follows: primer M+25, sequence AGA TGA GTC TTC TAA CCG AGG TCG; primer M-124, sequence TGC AAA

AAC ATC TTC AAG TCT CTG; and probe M+64, sequence FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA (Biosearch Technologies, Novato, CA).

For morbidity and seroconversion studies, five mice per group for viruses RT/625, RT/645, RK/470, and a mock infected PBS group were inoculated with $20MID_{50}$ of virus and weighed daily for 11 days and then every other day for an additional 4 days. Serum was collected from these mice on day 23 p.i. to assay serum antibody response to the AIVs. Statistical significance of weight loss between groups of mice was determined using Student's *t* test.

Histopathology and immunohistochemistry

Tissues from infected and control mice were examined by histopathology and immunohistochemistry. Mice were inoculated with $20MID_{50}$ virus and lung, trachea, thymus, thyroid, esophagus, heart, spleen, liver, stomach, intestine, pancreas, kidneys, adrenal gland, ovary, uterus, bladder, brain, and nasal turbinates were collected on days 1, 3, 5, 7, 14, and 28 p.i. (three mice per virus per day). The lungs were inflated with 10% neutral buffered formalin and all tissues were preserved in 10% neutral buffered formalin. Additional groups of mice were inoculated with allantoic fluid in sterile PBS (3 mice) or with X31 (9 mice) to serve as controls and organs were collected on day 5 p.i (PBS) or days 3, 5, and 14 p.i. (X31). Five equal transverse sections were made through the entire lungs. Mouse skulls were decalcified and four transverse sections were made through the nasal cavity to examine the nasal turbinates. Tissues were routinely processed, embedded and stained with hematoxylin and eosin. The severity of inflammation in lungs of infected mice was calculated by finding the average percent area of lung affected by inflammation per day p.i. for each virus using Image Pro Plus software vs. 4.5.1 (MediaCybernetics, Bethesda, MD).

Immunohistochemical staining was performed on lung tissue (days 1, 3, 5, and 7 p.i. for 3 mice per day) or on nasal turbinates (days 3 and 5 p.i. for 2 mice per day) and all other organs (day 5 p.i. for 2 mice per day) in mice infected with RT/625, RT/645, and RK/470. Immunohistochemistry on mouse lung tissue was performed using a commercially available mouse monoclonal antibody to the nucleoprotein of influenza A virus at a 1:200 dilution (Biodesign International, Sako, Maine) or on mouse nasal turbinates and organs other than lung using a commercially available goat polyclonal antibody to the nucleoprotein of influenza A virus at a 1:10,000 dilution (Biodesign International, Sako, Maine), as excessive background staining was observed in nasal turbinates and organs using the mouse monoclonal. Tissues were deparaffinized and blocked with a commercial protein blocking agent (Dako Cytomation, Carpinteria, CA) and a linked strepavidin-biotin immunoperoxidase system was used for immunolabeling. The reaction was visualized with 3, 3'-diaminobenzidine substrate (Dako Cytomation, Carpinteria, CA).

Serum ELISA assay

Sera from individual mice were assayed via ELISA against each whole influenza virus (RT/625, RT/645, or RK/470) inoculated in the individual mouse. Additionally, the same sera were assayed against allantoic fluid to account for any antibodies generated against components other than the virus. Virus or allantoic fluid was coated 100uL per well on 96 well Immulon 2HB microtiter plates and incubated 24 hours at 4°C. Twofold dilutions of sera were applied to pre-absorbed plates, starting with a dilution of 1:20 and virus specific antibodies were measured using alkaline phosphatase labeled goat anti mouse IgG(H+L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The p-nitrophenyl phosphate substrate (Kiregaard and Perry Laboratories, Gaithersburg, MD) was added and absorbance measured at 405 nm on a 96 well

format plate reader (BioTek, Winooski, VT). Absorbance readings for sera against allantoic fluid were subtracted from absorbance readings for sera against virus and plotted.

RESULTS

Wild bird avian influenza viruses replicate robustly in mice

Wild bird isolates selected for *in vivo* replication analysis covered six hemagglutinin (HA) subtypes and four known avian species (Table 3.1). Replication of each isolate in mice was categorized as: "No replication" (no virus was detected by $TCID_{50}$ assay from lung samples), "poor replication" (virus detected from lung samples was low titer and/or only present in mice inoculated with a high concentration inoculum), and "efficient replication" (virus detected from lung samples was high titer and present in mice inoculated with both concentrations of virus). Many of the isolates (71%) were able to replicate in the mouse lung when assessed on day four post-inoculation (p.i.) by $TCID_{50}$ assay, where 29% of these isolates showed efficient replication (Table 3.1). Lung titers in individual mice from isolates that demonstrated efficient replication are shown in Figure 3.1. Inoculum concentration was not standardized as viruses only grew to a moderate titer and repeated passage of virus in embryonated chicken eggs (ECE) to derive a high-titer stock was specifically avoided. Despite this lack of standardization, the wide range of inoculum concentrations (as low as 5.5E+02 PFU/mouse and as high as 3.5E+05 PFU/mouse) between isolates that had efficient replication in the mouse lung did not appear to be associated with the magnitude of $TCID_{50}$ virus titer from the lung on day four p.i. (Figure 3.1). Virus was not detected in mock infected mice via $TCID_{50}$ assay.

Three virus isolates that exhibited efficient replication in the mouse lung were further selected for detailed pathogenesis studies (viruses RT/645, RT/625, and RK/470). These particular isolates were selected to diversify the HA subtypes examined. A 50% mouse infectious

dose (MID_{50}) was elucidated for each of these three selected isolates. The MID50 was similar for RT/645 (2.5E+03 PFU/mouse) and RT/625 (1.2E+03 PFU/mouse) but much lower for RK/470 (3.0E+01 PFU/mouse).

Wild bird avian influenza virus infection exhibits low pathogenicity in mice despite robust replication in mouse lung

Clinical signs in mice inoculated with $20MID_{50}$ of RT/645, RT/625, or RK/470 were mild and included slightly ruffled fur on days 1 and 3 post-inoculation. Similar clinical signs were observed on day 1 p.i. in mock infected mice; these clinical signs were attributed to anesthetic recovery. Clinical signs in X31 inoculated mice included ruffled fur and lethargy on days 3 and 5 post-inoculation. Mice infected with RT/645 and RK/470 exhibited more weight loss than mice infected with RT/625 and mock infected control mice on day 2 p.i. and weight gain remained poor in mice infected with RK/470 on day 3 p.i. (Figure 3.2). Despite these differences, mice infected with RT/625, RT/645 and RK/470 exhibited overall minimal weight loss early p.i. that was not significantly different from mock infected control mice by a Student's *t* test. In comparison, mice infected intranasally with X31 and monitored for weight loss for other studies in our lab exhibit approximately 15-20% peak weight loss on day 5 p.i. One PBS inoculated mouse was found to be an outlier by Dixon's Q test based on weight loss data and dropped from the group.

Virus replication in the mouse lung was further examined over time by infecting mice with $20MID_{50}$ of each of the selected isolates (RT/645, RT/625, and RK/470) and euthanizing mice to determine virus titer in the lung at multiple time points (Figure 3.3). All viruses reached a similar peak titer in mouse lungs (2.4E +07 to 7.7E+07 $TCID_{50}$/gram). Mice infected with RT/625 and RK/470 both had a pattern of peak virus in the lung at day 5 p.i. but RT/645 had a

less clear time point of peak virus, with highest titers around days 1 and 3 p.i. Virus was present in mouse lung up to day 7 p.i. for all three viruses and cleared by day 14 p.i.

The spleen, liver, and kidney of mice infected with RT/625 and RT/645 (day 5 p.i.) and the brain of mice infected with RT/625 (day 5 p.i.) was examined for presence of virus via titration on MDCK cells. A subset of mice from both RT/625 and RT/645 had evidence of low titers of virus in the spleen and liver and these samples were further evaluated via virus isolation in ECE. Virus isolation in eggs indicated that a single mouse infected with RT/645 had influenza virus present in the liver, which was confirmed by real-time PCR for influenza (data not shown).

Wild bird avian influenza virus infection induces pulmonary lesions in mice

The lungs of mice infected with RT/645, RT/625, and RK/470 exhibited similar histopathologic lesions as well as a similar resolution of lesions; however, development of lesions differed with RT/625 lagging slightly behind RT/645 and RK/470. No significant histopathologic lesions were present in RT/645, RT/625, or RK/470 infected mice day 1 post-inoculation. By day 3 p.i., mice infected with RT/645 and RK/470 had a necrotizing bronchiolitis in many bronchioles and peribronchiolar inflammation, but mice infected with RT/625 exhibited only rare scattered areas of peribronchiolar inflammation (Figure 3.4). A similar average percentage of lung was affected by inflammation on day 3 p.i. for all viruses (1.5% for RT/625, 2.7% for RT/645, and 2.0% for RK/470). Peribronchiolar inflammation present in mice infected with RT/625 at day 3 p.i. was primarily lymphocytes, with fewer macrophages and neutrophils present in adjacent alveoli, where in RT/645 and RK/470 infected mice the composition of inflammatory cells was more neutrophilic. Mild tracheal inflammation, characterized by small numbers of lymphocytes within the tracheal submucosa was present in some RT/625 and RK/470 infected mice. Many apoptotic tracheal epithelial cells were observed only in RK/470

infected mice at day 3 p.i. In X31 infected mice on day 3 p.i., there was a striking necrotizing bronchitis and bronchiolitis with loss of epithelium and peribronchiolar cell debris with some inflammatory cells.

Histopathology in mice on day 5 p.i. again showed that lesions in mice infected with RT/645 and RK/470 were ahead of mice infected with RT/625. Mice infected with RT/645 and RK/470 had continued peribronchiolar pneumonia and necrotizing bronchiolitis, however, the necrotizing bronchiolitis was resolving by this point in time and the bronchiolar epithelium was beginning to become hyperplastic (Figure 3.4). Conversely, mice infected with RT/625 had continued peribronchiolar pneumonia that increased in severity and also a necrotizing bronchitis and bronchiolitis appeared at this time point. Mice infected with RT/625 exhibited prominent lymphocytic perivascular cuffing. The degree of inflammation present in the lung was slightly higher on day 5 p.i. compared to day 3 p.i. but similar between all viruses examined (8.6% for RT/625, 5.7% for RT/645, and 3.5% for RK/470). No lesions were present in the lungs of mock infected mice (Figure 3.4). Mice infected with X31 had continued lesions of necrotizing bronchitis and bronchiolitis with peribronchiolar inflammation day 5 p.i., but many areas of bronchiolar epithelium were hyperplastic at this time point.

Mice infected with RT/625 had continued peribronchiolar pneumonia and necrotizing bronchiolitis at day 7 p.i., however, the pneumonia was more widespread (Figure 3.4). Mice infected with RT/645 and RK/470 had similar peribronchiolar pneumonia day 7 p.i. as described for day 5 p.i., however, the degree of inflammation was much less in severity than RT/625 (24.9% lung affected for RT/625, 3.6% for RT/645, and 5.6% for RK/470) and there was complete resolution of bronchiolar necrosis. Additionally, there was prominent type II

pneumocyte hyperplasia observed in some mice infected with RT/645 beginning at day 7 postinoculation.

Examination of lungs in infected mice on days 14 and 28 p.i. revealed similar resolution of mice infected with RT/625, RT/645, and RK/470. Peribronchiolar inflammation in all of these mice was primarily consolidated areas of lymphocytes and there were many peribronchiolar areas of type II pneumocyte hyperplasia. Lesions progressed to scattered nodular collections of peribronchiolar lymphocytes by day 28 p.i. in all virus infected mice. Mice infected with X31 had similar histopathologic changes of lymphocytic inflammation and type II pnuemocyte hyperplasia on day 14 as the 3 AIVs that were examined.

Immunohistochemistry (IHC) for the nucleoprotein of influenza A on the lungs of mice infected with RT/625, RT/645, and RK/470 all exhibited strong positive intranuclear staining of tracheal, bronchiolar, and/or alveolar epithelium in areas of histopathologic lesions between days 3 and 5 p.i. (Figure 3.5). Mice infected with RT/625 had very minimal positive staining on day 3 p.i. but staining became more prominent by day 5 p.i., versus RT/645 and RK/470, which both had more prominent positive staining on day 3 p.i. and magnitude of positive staining was lessened by day 5 p.i. These observations are in concord with histopathology that indicates earlier productive infection in mice infected with RT/645 and RK/470 versus mice infected with RT/625. Additionally, no positive staining of tracheal epithelium was observed in mice infected with RT/625, but there was good staining of the tracheal epithelium on day 3 p.i. for mice infected with RT/645 and RK/470. There was no positive staining in the lung of mock infected mice examined on day 5 post-inoculation.

Mice infected with RT/625 and RT/645 did not have any histopathologic lesions in the nasal turbinates at any time point (days 1, 3, 5, 7, and 14 p.i.). Two mice that were infected with

RK/470 (day 5 p.i.) and one mouse that was infected with RK/470 (day 7 p.i.) had a single area in the nasal cavity that contained mixed mucus and neutrophils, but there were no lesions in the nasal epithelium in any of the RK/470 infected mice. Although epithelial lesions were not observed, there was positive intranuclear staining in very rare ciliated epithelial cells of the paranasal sinuses in mice infected with RT/645 and in mice infected with RK/470. There was no positive staining for influenza in other organs examined on day 5 p.i. for any of the 3 viruses.

There was only a single extrapulmonary lesion observed in mice infected with the 3 AIVs, this was thymic atrophy with lymphocyte depletion present in a mouse infected with RT/645 on day 3 p.i. Thymic atrophy has been associated with influenza infection in mice, however, we did not consistently observe this lesion in mice infected with this virus and observation of thymic atrophy in a different study occurred much later in infection (Fislova et al., 2009). No other lesions were observed in any of the organs examined for mice infected with RT/625, RT/645 or RK/470 on days 1,3,5,7, or 14 p.i.

Wild bird avian influenza viruses exhibit differences in immunogenicity in mice

A serum ELISA was performed on sera collected from mice infected with RT/625, RT/645, or RK/470 on day 23 p.i. to assess seroconversion after AIV infection. All five mice infected with RT/645 or RK/470 had a robust IgG response to the specific AIV with a prominent signal from serum dilutions up to 1:320 (Figure 3.6). In contrast, mice infected with RT/625 had a poor IgG response to the viral infection, with little difference in signal from serum dilutions when compared to mock infected mice (Figure 3.6).

DISCUSSION

The perspectives regarding the host range and mechanisms of influenza infections are ever broadening to a view of a more promiscuous virus than previously thought. Through natural

and experimental infections, the list of mammals that are susceptible to AIV infection is rapidly increasing along with the scope of organ tropism of these viruses. These findings apply not only to highly pathogenic AIVs, but also to low pathogenic viruses from poultry (Belser et al., 2007; Gillim-Ross et al., 2008; Joseph et al., 2007; Wan et al., 2008). These evolving viewpoints are further supported with this data that includes a large scope of avian influenza isolates from wild birds, demonstrating that many AIVs from wild birds can replicate in the BALB/c mouse model without adaptation and that hemagglutinin subtype was not a barrier to infection (Table 3.1). Methodology in screening the isolates for replication in the mouse lung was limited by the titer of the virus stock, as the virus isolates were minimally passaged to avoid mutations associated with egg adaptation. For the purpose of these studies it was critical to keep these isolates as close as possible to the original sample from the wild bird, to better assess replication of the original isolate. This resulted in many isolates that were only of moderate titer. The inoculum concentration for the primary screening experiment could not be standardized, as lowering the inoculum concentration for some isolates to normalize the concentration could have resulted in missing some isolates that were able to replicate. This method enabled identification of the maximum number of isolates that replicated in the mice and did not have any effect on peak viral titer for isolates that exhibited efficient replication (Figure 3.2), although some concentration effect was observed on isolates that exhibited poor replication (data not shown).

Mice did not require a high concentration inoculum for infection with RT/625, RT/645, or RK/470, as evident in the MID_{50} for each virus. Studies with LPAI H7 viruses have also shown that low concentrations of virus are adequate for infection of mice (Belser et al., 2007). RK/470 had a very low MID_{50} in mice, however, when mice were inoculated with $20MID_{50}$ for pathogenicity studies; several mice did not become infected. Therefore, it is possible that a

higher concentration of inoculum was required for consistent infection of all these mice. Despite these observations, it is also interesting to note that all H7 subtype viruses examined in this screen replicated to a high titer despite a low inoculum concentration, suggesting there is a lower threshold for infection in the H7 subtypes (Figure 3.1). Additionally, although there was not an equal number of hemagglutinin subtypes represented, all viruses of the H7 subtype exhibited efficient replication compared to other subtypes that exhibited varied ability to replicate in mouse lung (Table 3.1). The H7 subtype of avian influenza is of particular concern in human infections (Belser et al., 2009; Gillim-Ross and Subbarao, 2006). The data shown here is suggestive of an increased ability of the H7 viruses to replicate in the mouse model and further investigation to examine more isolates of this subtype may provide insight to mechanisms of mammalian infection. While previous studies focused on LPAI and HPAI H7 influenza viruses isolated from humans and poultry, the viruses tested here were exclusively isolates from North American shore birds. This presents additional opportunities for transmission; whether through contact with infected wild birds or environmental exposure in the birds' habitats (Rohani et al., 2009).

Infections in these mice were very similar to infections with human or other LPAIVs in BALB/c mice regarding pathogenesis and lesion development (Buchweitz, Karmaus, and Harkema, 2007). Peak viral titers in the lung for other LPAIVs are generally at day 4 but can occur as early as day 2 with virus present in the lung through day 7 (Gillim-Ross et al., 2008; Joseph et al., 2007). In this study, there is a similar peak of infection based on virus titers from the lung as RT/625 and RT/470 had peak titers in most mice on day 5 p.i. and RT/645 had peak titers in most mice at days 1 and 3 p.i. (Figure 3.3). Previous immunohistochemistry studies on influenza infected mice also demonstrate similar strong intranuclear staining of bronchiolar

epithelium (Fislova et al., 2009; Rigoni et al., 2007). Interestingly, all of the viruses we examined demonstrated positive viral antigen staining of not only bronchiolar epithelial cells but also alveolar epithelial cells, but in the X31 infected mice viral antigen staining was restricted to bronchiolar epithelium. Similar findings were present in a study of mouse adapted influenza viruses, where IHC staining for influenza was only observed in bronchiolar epithelial cells (Fislova et al., 2009). Moreover, there was prominent type II pneumocyte hyperplasia present in mice infected with viruses RT/625, RT/645, and RK/470 by day 14 p.i., also supporting that there was significant alveolar epithelial damage. HA receptor specificity is thought to be a large contributor to host range, as avian influenza strains preferentially bind to cell glycoproteins/glycolipids that have terminal sialyl-galactosyl (SA) residues with a 2-3 linkage [Neu5Ac(α2-3)Gal] and human influenza strains preferentially bind to terminal 2-6 linked glycoproteins/glycolipids [Neu5Ac(α2-6)Gal] (Connor et al., 1994; Mansfield, 2007). Additionally, the distribution of these receptors on cells of the respiratory tract is demonstrated to vary between mammalian species and mice have been shown to have primarily the 2-3 linked SA receptor in ciliated epithelial cells in large airways and in type II pneumocytes (Ibricevic et al., 2006; van Riel et al., 2007). Indeed, many studies of HPAI H5N1 have demonstrated enhanced alveolar damage in humans and mice, suggesting increased affinity for binding of these cells compared to human strains of influenza (Abdel-Ghafar et al., 2008; Dybing et al., 2000). This affinity for binding cells of the lower respiratory tract was also demonstrated with some LPAI isolates(van Riel et al., 2007). Our results are also supportive that these wild bird AIVs have an increased binding affinity for receptors present on alveolar epithelial cells compared to human strains. In mice infected with RT/645 and RK/470 there was minimal presence of viral antigen in the nasal cavity via IHC with no antigen present in RT/625. Other influenza isolates,

particularly human origin, typically exhibit replication in this area of the upper respiratory tract. The minimal presence of antigen in the nasal turbinates despite efficient replication and lesions in the lung supports that these isolates, similar to other avian isolates, have a preference for replication in the lower respiratory tract. There is recent evidence that the regional replication preferences of avian influenza in mammals are in part due to mutations in PB2 that change temperature and cell type replication preferences in the virus (Hatta et al., 2007). Further investigation into the molecular aspects of these wild type avian viruses may provide insight into common molecular mechanisms of host range and pathogenicity in mammals.

It has been shown that many HPAI viruses have extrapulmonary spread, with virus presence and lesions most frequently in the brain and spleen of infected mice (Belser et al., 2007; Joseph et al., 2007; Maines et al., 2005; Rigoni et al., 2007). Examination of LPAIVs has demonstrated that some of these viruses that exhibit pulmonary replication also have the capacity for extrapulmonary spread, while others do not (Gillim-Ross et al., 2008; Joseph et al., 2007). Interestingly, mouse adapted influenza viruses have also been shown to have extrapulmonary spread to numerous organs with possible viremia, regardless of virulence (Fislova et al., 2009). Additionally, a case study of a human infection with HPAI H5N1 demonstrated viral antigen in extrapulmonary sites without lesions in these sites (Zhang et al., 2009). We also have evidence of inconsistent extrapulmonary spread of one of the viruses examined (RT/645) without significant morbidity or extrapulmonary lesions in these mice. This may be further supportive evidence that viremia and extrapulmonary spread is not as uncommon as previously believed and conclusions of virulence of an influenza isolate must be evaluated not only in context of viral presence, but also with consideration of morbidity and severity of lesions in the mouse model.

In depth studies of isolates RT/625, RT/645, and RK/470 revealed very similar peak titers of virus, pulmonary lesions, and location of viral antigen. However, slight differences were present between these viruses, most notably in RT/625. This isolate exhibited slower progression of viral infection, characterized by a later peak of pulmonary lesions and viral antigen compared to RT/645 and RK/470 (Figure 3.4). Additionally, the amount of peribronchiolar inflammation in RT/625 was greater in magnitude than RT/645 and RK/470 on day 5 post-inoculation. Viral titer in the lungs corresponded with the progression of lesions in RT/625 (peak virus at day 5) and RT/645 (peak virus at days 1 and 3) but not as well with RK/470 (apparent peak virus at day 5) regardless of inclusion or exclusion of the several mice of RK/470 that did not become infected (Figure 3.3). Interestingly, there was a good antibody response in mice infected with RT/645 and RK/470 but a poor response in mice infected with RT/625. In addition, RT/625 infection caused marked lymphocytic infiltrates early in the lung, whereas RT/645 and RK/470 infection resulted in primarily neutrophilic infiltrates and followed a more typical inflammatory pattern (Buchweitz, Karmaus, and Harkema, 2007). It is possible that the differences in inflammation with RT/625 infection may be related to an altered immune response or poor specific immune response to the virus. Virus was present in all RT/625 infected mice through day 7 p.i.; additional sampling days between day 7 and day 14 p.i., in addition to analysis of the cytokine and chemokines expressed during infection would be useful to determine if there was an altered immune response influencing viral clearance from the lung and seroconversion compared to RT/645 and RK/470 infected mice.

Many inbred mouse strains including BALB/c are more susceptible to influenza infection because of defective alleles in the Mx1 gene (Staeheli et al., 1988). Studies have shown that mice with a wild type Mx1 gene are resistant to infection with LPAI and have lower viral replication

with HPAI H5N1 infections than BALB/c mice (Henzler et al., 2003; Tumpey et al., 2007). Therefore, interpretation of the potential of mammalian and human infection with these wild bird AIVs must be carefully evaluated in additional models. Nonetheless, the evaluation of these wild AIVs in the BALB/c mouse model are invaluable for selection of isolates for additional molecular and *in vivo* studies using other animal models. Many of the isolates in this study should be further explored to better understand mechanisms of AIV infection in mammals. From this study, we conclude that numerous wild bird AIVs replicate without adaptation and to high titer in the mouse model. While all viruses induced lung pathology, the extent, type and time course varied. Moreover, virus titer and lung pathology were not necessarily associated with seroconversion. Deductions about the behavior of influenza in BALB/c mice and mammals in general should not simply be based on magnitude of replication of the virus or seroconversion, but also correlated with clinical signs, evidence of morbidity, and severity of lesions. Avian influenza viruses from wild bird populations could be crossing species barriers and causing sub-clinical disease with limited seroconversion; providing unknown opportunities for mutation and emergence of novel influenza viruses.

ACKNOWLEDGMENTS

The authors wish to thank the Animal Resources personnel at the College of Veterinary Medicine, University of Georgia for excellent animal husbandry. We would also like to thank histotechnicians in the Veterinary Pathology Department at University of Georgia for their assistance, especially Abbie Butler for her outstanding immunohistochemistry support. Additional thanks go to Raydel Mair, James Gordy, Ginger Goekjian, and Becky Poulson at the College of Veterinary Medicine, University of Georgia for excellent technical support. This work was supported by a grant from the CDC: 5U19Cl000401-02.

REFERENCES

Abdel-Ghafar, A. N., Chotpitayasunondh, T., Gao, Z., Hayden, F. G., Nguyen, D. H., de Jong, M. D., Naghdaliyev, A., Peiris, J. S., Shindo, N., Soeroso, S., and Uyeki, T. M. (2008). Update on avian influenza A (H5N1) virus infection in humans. *The New England Journal Of Medicine* **358**(3)**,** 261-73.

Beigel, J. H., Farrar, J., Han, A. M., Hayden, F. G., Hyer, R., de Jong, M. D., Lochindarat, S., Nguyen, T. K., Nguyen, T. H., Tran, T. H., Nicoll, A., Touch, S., and Yuen, K. Y. (2005). Avian influenza A (H5N1) infection in humans. *The New England Journal Of Medicine* **353**(13)**,** 1374-85.

Belser, J. A., Bridges, C. B., Katz, J. M., and Tumpey, T. M. (2009). Past, present, and possible future human infection with influenza virus A subtype H7. *Emerging Infectious Diseases* **15**(6)**,** 859-65.

Belser, J. A., Lu, X., Maines, T. R., Smith, C., Li, Y., Donis, R. O., Katz, J. M., and Tumpey, T. M. (2007). Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. *Journal Of Virology* **81**(20)**,** 11139-47.

Buchweitz, J. P., Karmaus, P. W., and Harkema (2007). Time-Dependent Airway Epithelial and Inflammatory Cell Responses Induced by Influenza Virus A/PR/8/34 in C57BL/6 Mice. *Toxicologic Pathology* **35,** 12.

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., and Guan, Y. (2005). Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. *Journal Of Clinical Microbiology* **43**(11)**,** 5760-7.

Cattoli, G., and Capua, I. (2007). Diagnosing Avian Influenza in the Framework of Wild Surveillance Efforts and Environmental Samples. *Journal Of Wildlife Diseases* **43**(3)**,** S5-S9.

Connor, R. J., Kawaoka, Y., Webster, R. G., and Paulson, J. C. (1994). Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* **205**(1)**,** 17-23.

Cottey, R., Rowe, C. A., and Bender, B. S. (2001). Influenza virus. *Curr Protoc Immunol* **Chapter 19,** Unit 19.11.

Dybing, J. K., Schultz-Cherry, S., Swayne, D. E., Suarez, D. L., and Perdue, M. L. (2000). Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *Journal Of Virology* **74**(3)**,** 1443-50.

Fislova, T., Gocnik, M., Sladkova, T., Durmanova, V., Rajcani, J., Vareckova, E., Mucha, V., and Kostolansky, F. (2009). Multiorgan distribution of human influenza A virus strains observed in a mouse model. *Archives Of Virology* **154**(3)**,** 409-19.

Fouchier, R. A., Schneeberger, P. M., Rozendaal, F. W., Broekman, J. M., Kemink, S. A., Munster, V., Kuiken, T., Rimmelzwaan, G. F., Schutten, M., Van Doornum, G. J., Koch, G., Bosman, A., Koopmans, M., and Osterhaus, A. D. (2004). Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci USA* **101**(5)**,** 1356-61.

Gabriel, G., Abram, M., Keiner, B., Wagner, R., Klenk, H. D., and Stech, J. (2007). Differential polymerase activity in avian and mammalian cells determines host range of influenza virus. *Journal Of Virology* **81**(17)**,** 9601-4.

Gambaryan, A., Tuzikov, A., Pazynina, G., Bovin, N., Balish, A., and Klimov, A. (2006). Evolution of the receptor binding phenotype of influenza A (H5) viruses. *Virology* **344**(2)**,** 432-8.

Geraci, J. R., St Aubin, D. J., Barker, I. K., Webster, R. G., Hinshaw, V. S., Bean, W. J., Ruhnke, H. L., Prescott, J. H., Early, G., Baker, A. S., Madoff, S., and Schooley, R. T. (1982). Mass mortality of harbor seals: pneumonia associated with influenza A virus. *Science (New York, NY)* **215**(4536)**,** 1129-31.

Gillim-Ross, L., Santos, C., Chen, Z., Aspelund, A., Yang, C. F., Ye, D., Jin, H., Kemble, G., and Subbarao, K. (2008). Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. *Journal Of Virology* **82**(21)**,** 10854-10863.

Gillim-Ross, L., and Subbarao, K. (2006). Emerging respiratory viruses: challenges and vaccine strategies. *Clinical Microbiology Reviews* **19**(4)**,** 614-36.

Glaser, L., Stevens, J., Zamarin, D., Wilson, I. A., Garcia-Sastre, A., Tumpey, T. M., Basler, C. F., Taubenberger, J. K., and Palese, P. (2005). A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. *Journal Of Virology* **79**(17)**,** 11533-6.

Guo, Y., Li, J., and Cheng, X. (1999). Discovery of men infected by avian influenza A (H9N2) virus. *Chinese Journal of Experimental and Clinical Virology* **13**(2)**,** 105-8.

Hatta, M., Hatta, Y., Kim, J. H., Watanabe, S., Shinya, K., Nguyen, T., Lien, P. S., Le, Q. M., and Kawaoka, Y. (2007). Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathogens* **3**(10)**,** 1374-9.

Henzler, D. J., Kradel, D. C., Davison, S., Ziegler, A. F., Singletary, D., DeBok, P., Castro, A. E., Lu, H., Eckroade, R., Swayne, D., Lagoda, W., Schmucker, B., and Nesselrodt, A.

(2003). Epidemiology, production losses, and control measures associated with an outbreak of avian influenza subtype H7N2 in Pennsylvania (1996-98). *Avian Diseases* **47**(3 Suppl)**,** 1022-36.

Hinshaw, V. S., Bean, W. J., Webster, R. G., Rehg, J. E., Fiorelli, P., Early, G., Geraci, J. R., and St Aubin, D. J. (1984). Are seals frequently infected with avian influenza viruses? *Journal Of Virology* **51**(3)**,** 863-5.

Hinshaw, V. S., Webster, R. G., Easterday, B. C., and Bean, W. J., Jr. (1981). Replication of avian influenza A viruses in mammals. *Infect Immun* **34**(2)**,** 354-61.

Ibricevic, A., Pekosz, A., Walter, M. J., Newby, C., Battaile, J. T., Brown, E. G., Holtzman, M. J., and Brody, S. L. (2006). Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *Journal Of Virology* **80**(15)**,** 7469-80.

Joseph, T., McAuliffe, J., Lu, B., Jin, H., Kemble, G., and Subbarao, K. (2007). Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. *Journal Of Virology* **81**(19)**,** 10558-66.

Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R. A., Amonsin, A., Payungporn, S., Noppornpanth, S., Wattanodorn, S., Theambooniers, A., Tantilertcharoen, R., Pattanarangsan, R., Arya, N., Ratanakorn, P., Osterhaus, D. M., and Poovorawan, Y. (2004). Avian influenza H5N1 in tigers and leopards. *Emerging Infectious Diseases* **10**(12)**,** 2189-91.

Keawcharoen, J., van Riel, D., van Amerongen, G., Bestebroer, T., Beyer, W. E., van Lavieren, R., Osterhaus, A. D., Fouchier, R. A., and Kuiken, T. (2008). Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). *Emerging Infectious Diseases* **14**(4)**,** 600-7.

Klingeborn, B., Englund, L., Rott, R., Juntti, N., and Rockborn, G. (1985). An avian influenza A virus killing a mammalian species--the mink. *Archives Of Virology* **86**(3-4)**,** 347-51.

Klopfleisch, R., Wolf, P. U., Wolf, C., Harder, T., Starick, E., Niebuhr, M., Mettenleiter, T. C., and Teifke, J. P. (2007). Encephalitis in a stone marten (Martes foina) after natural infection with highly pathogenic avian influenza virus subtype H5N1. *Journal Of Comparative Pathology* **137**(2-3)**,** 155-9.

Koopmans, M., Wilbrink, B., Conyn, M., Natrop, G., van der Nat, H., Vennema, H., Meijer, A., van Steenbergen, J., Fouchier, R., Osterhaus, A., and Bosman, A. (2004). Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* **363**(9409)**,** 587-93.

Labadie, K., Dos Santos Afonso, E., Rameix-Welti, M. A., van der Werf, S., and Naffakh, N. (2007). Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. *Virology* **362**(2)**,** 271-82.

Li, K. S., Guan, Y., Wang, J., Smith, G. J., Xu, K. M., Duan, L., Rahardjo, A. P., Puthavathana, P., Buranathai, C., Nguyen, T. D., Estoepangestie, A. T., Chaisingh, A., Auewarakul, P., Long, H. T., Hanh, N. T., Webby, R. J., Poon, L. L., Chen, H., Shortridge, K. F., Yuen, K. Y., Webster, R. G., and Peiris, J. S. (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**(6996)**,** 209-13.

Li, Z., Chen, H., Jiao, P., Deng, G., Tian, G., Li, Y., Hoffmann, E., Webster, R. G., Matsuoka, Y., and Yu, K. (2005). Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. *Journal Of Virology* **79**(18)**,** 12058-64.

Lu, X., Tumpey, T. M., Morken, T., Zaki, S. R., Cox, N. J., and Katz, J. M. (1999). A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *Journal Of Virology* **73**(7)**,** 5903-11.

Maines, T. R., Lu, X. H., Erb, S. M., Edwards, L., Guarner, J., Greer, P. W., Nguyen, D. C., Szretter, K. J., Chen, L. M., Thawatsupha, P., Chittaganpitch, M., Waicharoen, S., Nguyen, D. T., Nguyen, T., Nguyen, H. H., Kim, J. H., Hoang, L. T., Kang, C., Phuong, L. S., Lim, W., Zaki, S., Donis, R. O., Cox, N. J., Katz, J. M., and Tumpey, T. M. (2005). Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *Journal Of Virology* **79**(18)**,** 11788-800.

Mansfield, K. G. (2007). Viral Tropism and the Pathogenesis of Influenza in the Mammalian Host. *The American Journal Of Pathology* **171**(4)**,** 1089-92.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M. R., Donatelli, I., and Kawaoka, Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *Journal Of Virology* **74**(18)**,** 8502-12.

Olsen, B., Munster, V. J., Wallensten, A., Waldenstrom, J., Osterhaus, A. D., and Fouchier, R. A. (2006). Global patterns of influenza a virus in wild birds. *Science (New York, N Y)* **312**(5772)**,** 384-8.

Reed, L. J., and Muench, H. A. (1938). A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27,** 493-497.

Rigoni, M., Shinya, K., Toffan, A., Milani, A., Bettini, F., Kawaoka, Y., Cattoli, G., and Capua, I. (2007). Pneumo- and neurotropism of avian origin Italian highly pathogenic avian influenza H7N1 isolates in experimentally infected mice. *Virology* **364**(1)**,** 28-35.

Rogers, G. N., and Paulson, J. C. (1983). Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**(2)**,** 361-73.

Rohani, P., Breban, R., Stallknecht, D. E., and Drake, J. M. (2009). Environmental transmission of low pathogenicity avian influenza viruses and its implications for pathogen invasion. *Proc Natl Acad Sci USA* **106**(25)**,** 10365-9.

Song, D., Kang, B., Lee, C., Jung, K., Ha, G., Kang, D., Park, S., Park, B., and Oh, J. (2008). Transmission of avian influenza virus (H3N2) to dogs. *Emerging Infectious Diseases* **14**(5)**,** 741-6.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., and Poovorawan, Y. (2006a). Avian influenza H5N1 in naturally infected domestic cat. *Emerging Infectious Diseases* **12**(4)**,** 681-3.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., Chutinimitkul, S., Thanawongnuwech, R., and Poovorawan, Y. (2006b). Fatal avian influenza A H5N1 in a dog. *Emerging Infectious Diseases* **12**(11)**,** 1744-7.

Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G., and Haller, O. (1988). Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol* **8**(10)**,** 4518-23.

Stallknecht, D. E., and Brown, J. D. (2007). Wild birds and the epidemiology of avian influenza. *Journal Of Wildlife Diseases* **43**(3)**,** 6.

Stevens, J., Blixt, O., Glaser, L., Taubenberger, J. K., Palese, P., Paulson, J. C., and Wilson, I. A. (2006). Glycan microarray analysis of the hemagglutinins from modern and pandemic

influenza viruses reveals different receptor specificities. *Journal Of Molecular Biology* **355**(5)**,** 1143-55.

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., and Cox, N. (1998). Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science (New York, NY)* **279**(5349)**,** 393-6.

Thompson, C. I., Barclay, W. S., Zambon, M. C., and Pickles, R. J. (2006). Infection of human airway epithelium by human and avian strains of influenza a virus. *Journal Of Virology* **80**(16)**,** 8060-8.

Tumpey, T. M., Szretter, K. J., Van Hoeven, N., Katz, J. M., Kochs, G., Haller, O., Garcia-Sastre, A., and Staeheli, P. (2007). The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. *Journal Of Virology* **81**(19)**,** 10818-21.

van Riel, D., Munster, V. J., de Wit, E., Rimmelzwaan, G. F., Fouchier, R. A., Osterhaus, A. D., and Kuiken, T. (2007). Human and Avian Influenza Viruses Target Different Cells in the Lower Respiratory Tract of Humans and Other Mammals. *The American Journal Of Pathology* **171**(4)**,** 1215-23.

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., and Perez, D. R. (2008). Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. *PLoS One* **3**(8)**,** e2923.

Ward, A. C. (1997). Virulence of influenza A virus for mouse lung. *Virus Genes* **14**(3)**,** 187-94.

Zhang, Z., Zhang, J., Huang, K., Li, K. S., Yuen, K. Y., Guan, Y., Chen, H., and Ng, W. F. (2009). Systemic infection of avian influenza A virus H5N1 subtype in humans. *Hum Pathol* **40**(5)**,** 735-9.

Table 3.1. Study viruses and their ability to replicate in mouse lung.

Virus[a]	Name in this study	Subtype	Replication in mouse lung[b]
A/Ruddy Turnstone/NJ/650624/02	RT/624	H2N4	Poor
A/Sanderling/DE/650623/02	San/623	H2N4	Efficient
A/Ruddy Turnstone/DE/650621/02	RT/621	H2N9	No
A/Ruddy Turnstone/NJ/650638/02	RT/638	H2N9	Poor
A/Ruddy Turnstone/DE/650645/02[c]	RT/645	H2N9	Efficient
A/Ruddy Turnstone/DE/650580/02	RT/580	H2N9	Efficient
A/Ruddy Turnstone/NJ/650627	RT/627	H2N9	No
A/Mallard/MN/199105/99	Ma/105	H3N4	Poor
A/Mallard/MN/355808/00	Ma/808	H3N4/8	Poor
A/Ruddy Turnstone/NJ/1321394/05	RT/394	H3N6	No
A/Ruddy Turnstone/NJ/1321398/05	RT/398	H3N6	No
A/RuddyTurnstone/NJ/1321397/05	RT/397	H3N6	Poor
A/RuddyTurnstone/NJ/1321396/05	RT/396	H3N6	No
A/Ruddy Turnstone/NJ/1321395/05	RT/395	H3N8	Poor
A/Mallard/MN/199084/99	Ma/084	H3N8	Poor
A/Ruddy Turnstone/NJ/1321399/05	RT/399	H3N9	No
A/Mallard/MN/355807/00	Ma/807	H4N6	Poor
A/Ruddy Turnstone/DE/650625/02[c]	RT/625	H6N4	Efficient
A/RuddyTurnstone/NJ/650677/02	RT/677	H6N4	Efficient
A/Sanderling/DE/650680/02	San/680	H6N4	Poor
A/feces/DE/650574/02	Fe/574	H6N8	Poor
A/Red Knot/NJ/1523470/06[c]	RK/470	H7N3	Efficient
A/Sanderling/NJ/1523471/06	San/471	H7N3	Efficient
A/Ruddy Turnstone/1523477/06	RT/477	H7N3	Efficient
A/Ruddy Turnstone/NJ/650615/02	RT/615	H11N2	No
A/Ruddy Turnstone/NJ/650626/02	RT/626	H11N9	No
A/Mallard/MN/199116/99	Ma/116	H11N9	Poor
A/feces/DE/650619/02	Fe/619	H11N9	Poor
X31	X31	H3N2	Efficient

[a]Each of the 28 avian virus isolates listed were surveyed for ability to infect lungs of mice by infecting groups of three to four mice with two serial tenfold dilutions of the virus.
[b]Isolates were scored as: "No" which represents absence of virus in infected mice lung for either inoculum dilution on $TCID_{50}$ assay, "Poor" which represents virus presence in infected mice lung only at the higher inoculum dilution and/or low titer virus at either inoculum dilution on $TCID_{50}$ assay, and "Efficient" which represents high titer virus presence in infected mice lung at both inoculum dilutions on $TCID_{50}$ assay. The isolate X31 was used as a positive control.
[c]Indicates isolates that were selected for in depth pathogenicity studies

Figure 3.1. Wild aquatic bird influenza virus isolates replicate to high titer in mice without adaptation. Groups of two bars represent a single isolate that replicated efficiently in mice (from Table 3.1) inoculated i.n. at two dilutions (1:10 and 1:100) with lung collected and titrated at day 4 p.i. in MDCK cells. The concentration of the inoculum for the 1:10 dilution is listed after the isolate name (expressed in PFU/mouse). Each bar represents the average lung titer for a group of four mice. The dashed line is the limit of detection (2.4 $\log_{10}TCID_{50}/g$).

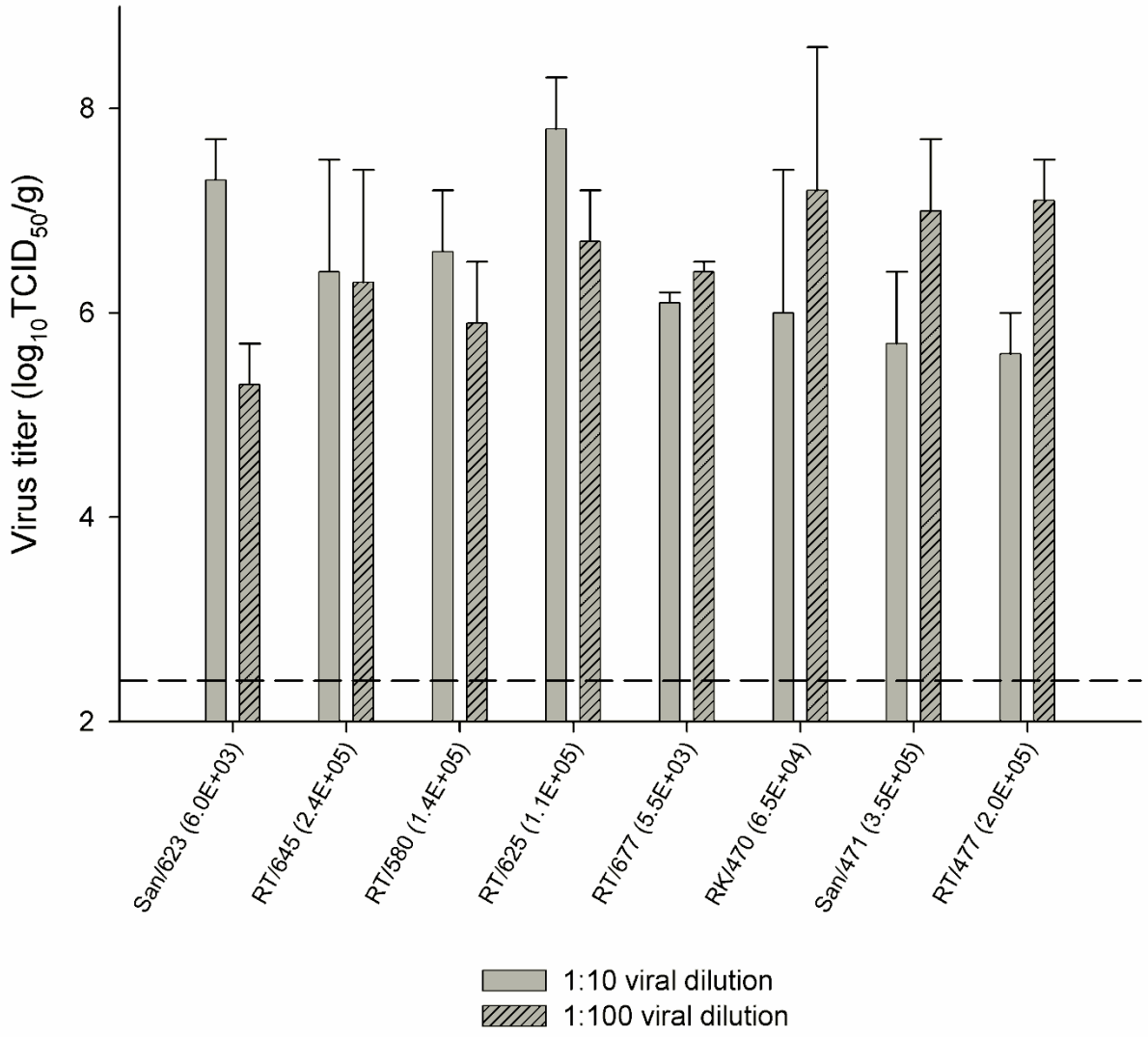


Figure 3.2. Weight loss in mice infected with selected wild aquatic bird influenza virus isolates. Groups of 5 mice were inoculated with 20MID_{50} of RT/625, RT/645, RK/470, or mock-infected with PBS only. Weights were tracked daily and percent weight lost compared to starting weight calculated. Daily weight loss was not statistically significant by Student's *t* test between virus inoculated mice and mock infected mice.

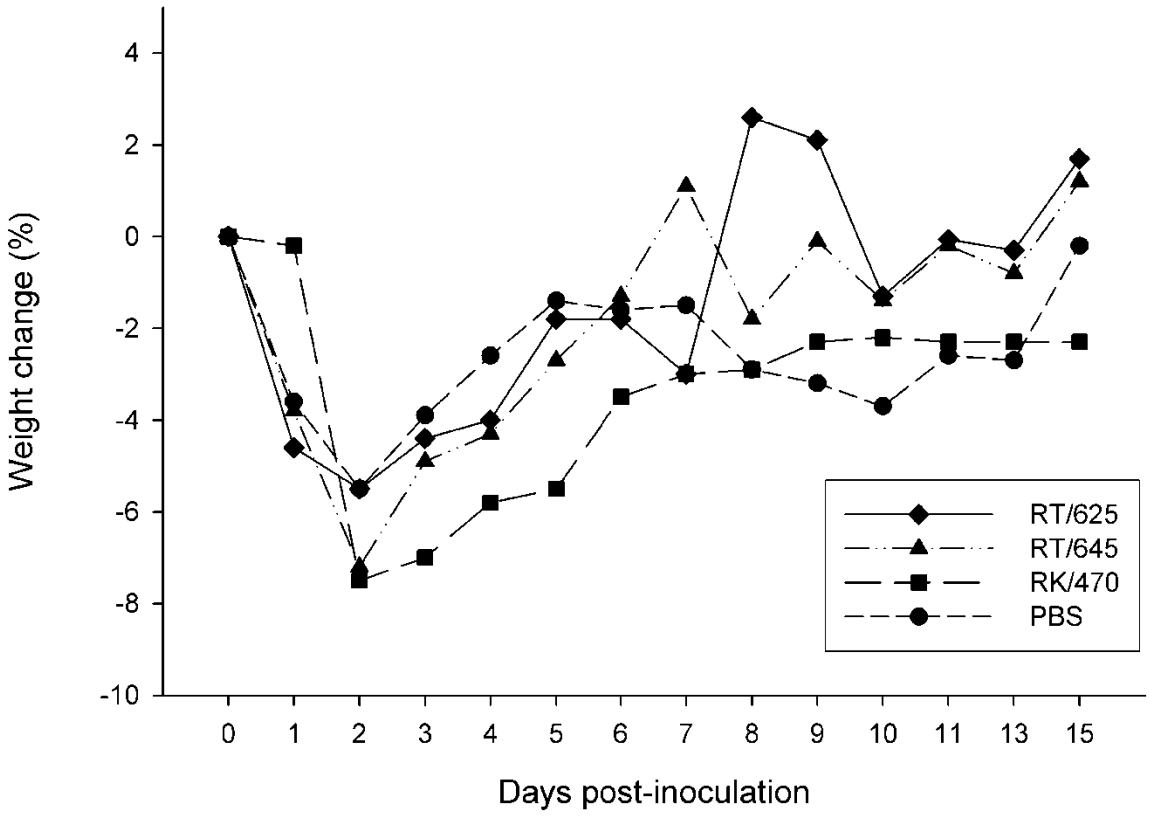


Figure 3.3. Kinetics of viral infection of RT/625, RT/645, and RK/470 in the lung of infected mice. Each bar represents the viral titer in the lung of an individual mouse infected with 20MID_{50} of virus for viruses RT/625, RT/645, and RK/470. The dashed line is the limit of detection (2.4 $\log_{10}TCID_{50}$/g).

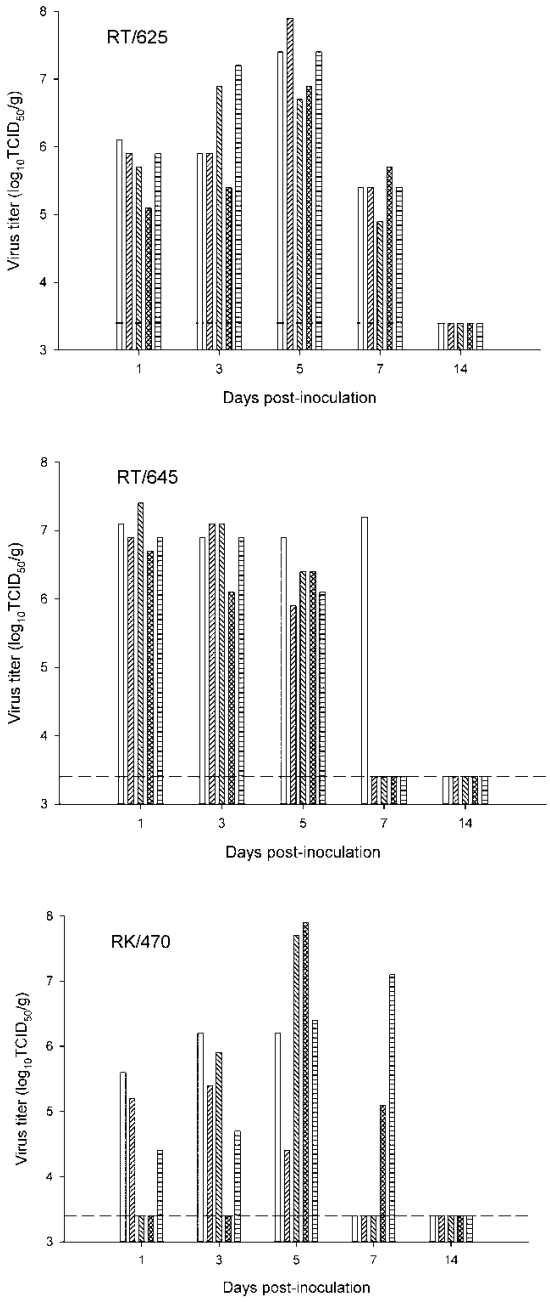


Figure 3.4. Histopathology in lung of AIV infected mice on days 3 through 28 postinoculation. Mice infected with RT/625, RT/645, and RK/470 have bronchiolar necrosis (arrow) and peribronchiolar inflammation (asterisk). Inflammation was most severe in RT/625 infected mice on day 7 p.i. Peribronchiolar inflammation was resolving as dense nodular collections of lymphocytes on days 14 and 28 postinoculation. Positive control infected mice (X31 day 3 p.i.) have similar bronchiolar necrosis as AIV inoculated mice. Mock infected mice have no lesions on histopathology.

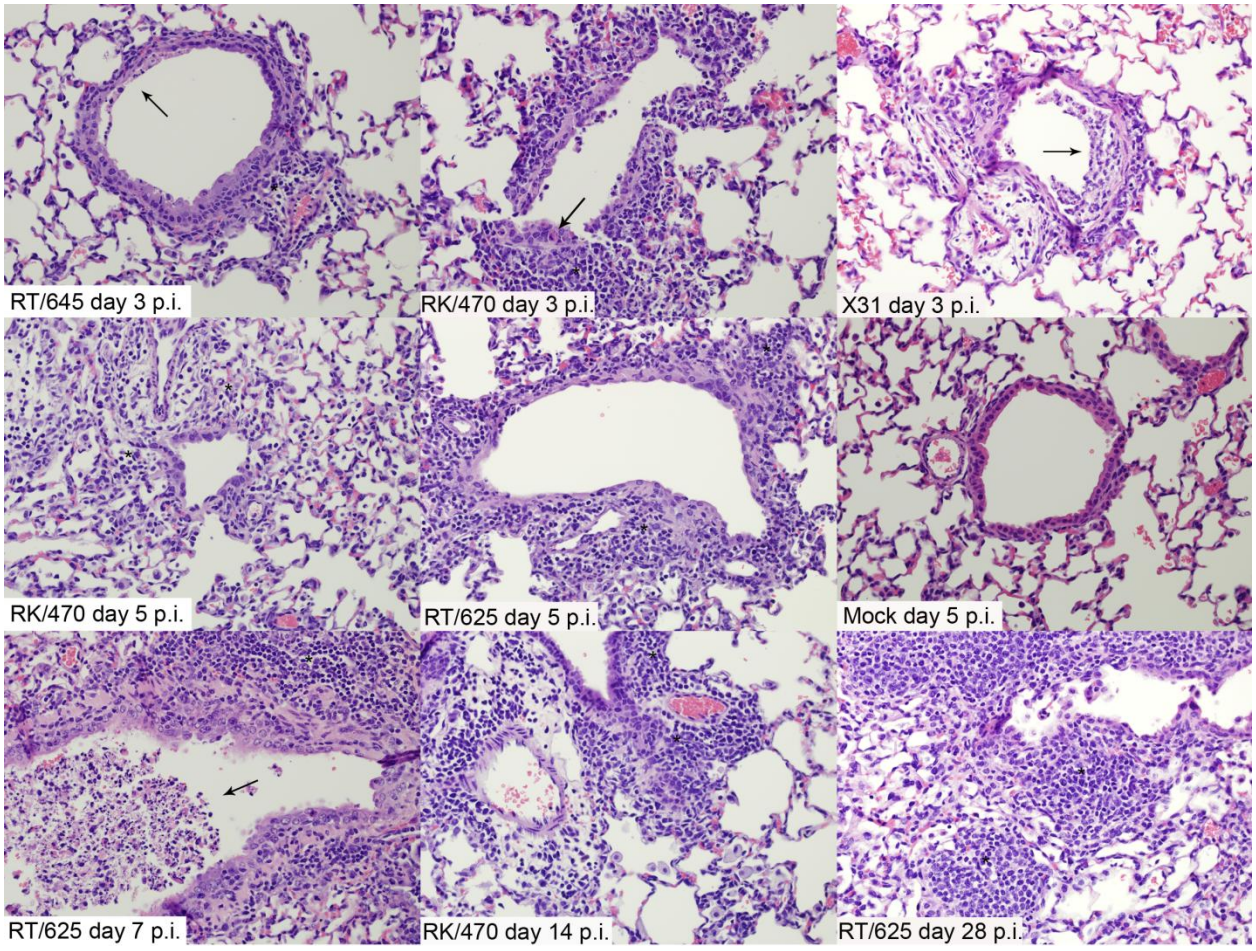


Figure 3.5. Viral antigen in the lung and nasal turbinates of AIV infected mice on days 3 and 5 postinoculation. By immunohistochemistry, there is strong positive intranuclear staining for the nucleoprotein of influenza A in tracheal epithelium (RK/470 day 3 p.i.) , bronchiolar epithelium (RT/645 day 3 p.i.), and alveolar epithelium (RT/625 day 5 p.i.) for all three AIV isolates in infected mice. There is also strong positive intranuclear staining of the nasal turbinate epithelium (RT/645 NT day 3 p.i.) in some AIV infected mice. Mock infected mice have no staining for viral antigen (Allantoic fluid day 5 p.i.). Positive control infected mice (X31 day 3 p.i.) have similar strong positive intranuclear staining of bronchiolar epithelium as AIV inoculated mice. Cellular necrosis is evident by luminal debris in the airways (arrow).

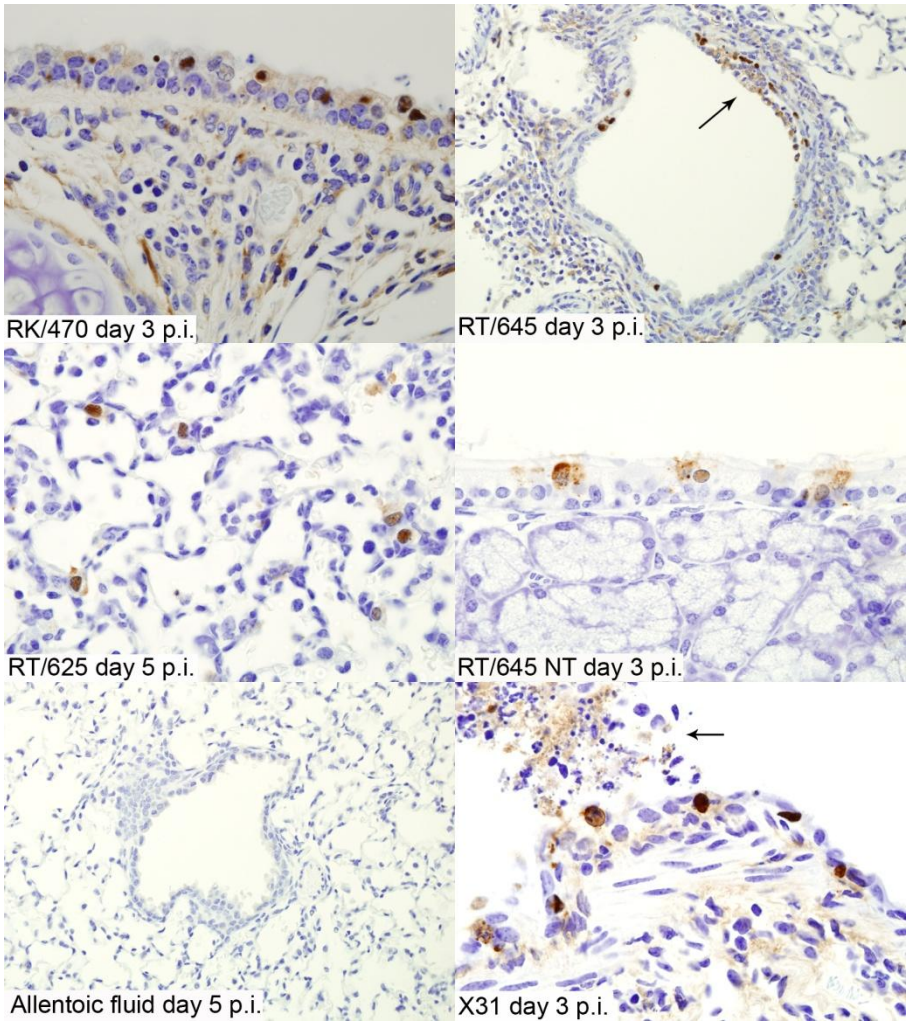
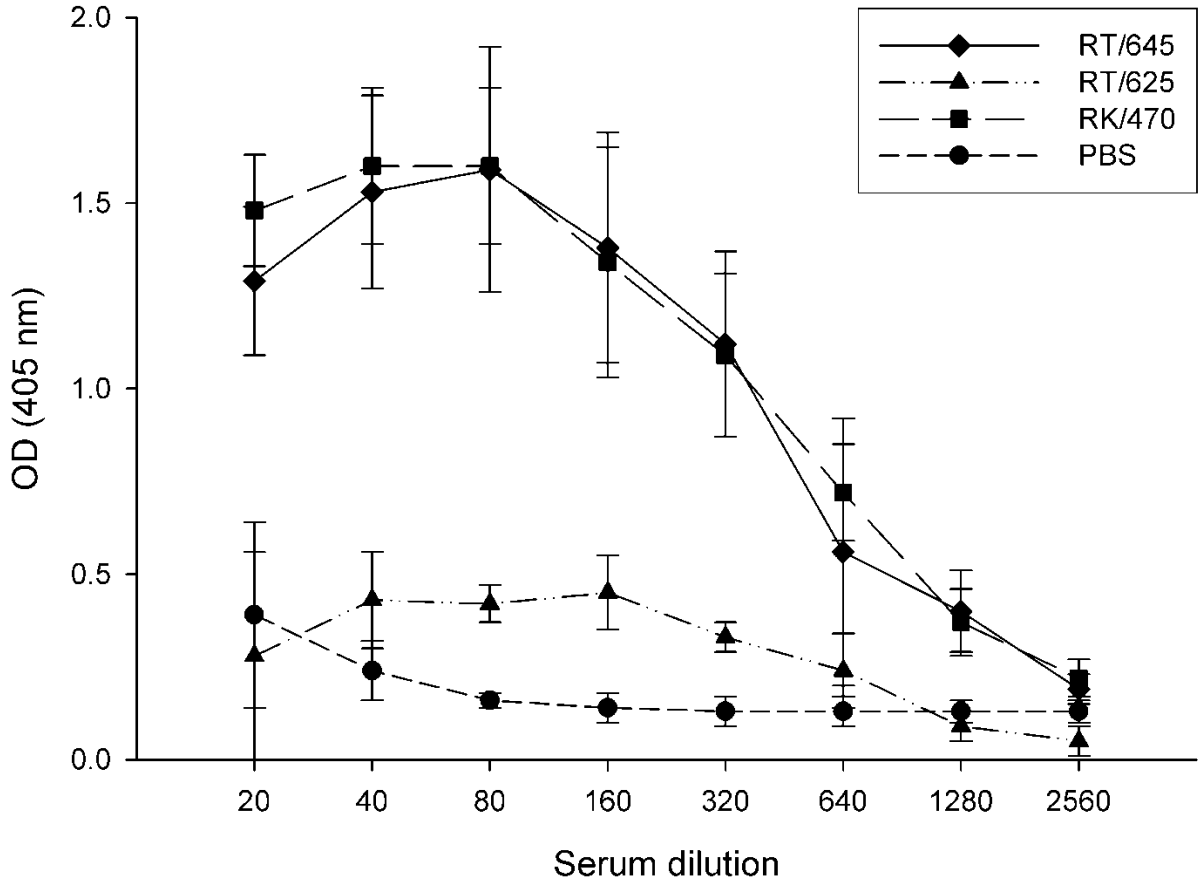


Figure 3.6. Serum antibody response in mice after wild bird AIV infection. Groups of 5 mice were inoculated with 20MID_{50} of RT/625, RT/645, or RK/470 and sera were collected day 23 postinoculation. Sera from individual mice were tested against whole virus and allantoic fluid, and the difference was averaged and plotted. Sera from PBS inoculated mice were tested against allantoic fluid.



Corrigendum to "Avian influenza isolates from wild birds replicate and cause disease in a mouse model of infection"[1]

Recent PCR subtyping in a collaborative laboratory after publication of this article has indicated reclassification of the subtypes of several isolates in this study. Previous subtyping of these isolates that was reported in this paper (Table 3.1) was done by hemagglutinin inhibition and neuraminindase inhibition assays. The changes of the subtypes for the isolates reported in Table 3.1 are as follows:

Virus	Name in study	Subtype in Table 1	New subtype determined by PCR
A/RuddyTurnstone/NJ/650624/02	RT/624	H2N4	H1N1
A/RuddyTurnstone/DE/650621/02	RT/621	H2N9	H1N9
A/RuddyTurnstone/NJ/650638/02	RT/638	H2N9	H1N9
A/RuddyTurnstone/DE/650645/02	RT/645	H2N9	H1N9
A/RuddyTurnstone/DE/650580/02	RT/580	H2N9	H1N9

Additionally, the isolate A/RuddyTurnstone/DE/650645/02 (RT/645) was used for pathogenesis studies, therefore, figures captions for Figures 2-6 are affected by the subtype change of this virus and instead of reading RT/645 (H2N9), should read RT/645 (H1N9). This subtype change for these isolates does not affect any of the results or conclusions presented. We would like to thank Becky Poulson for the PCR work performed that reclassified the isolate subtypes.

---

[1]Driskell, E.A., C.A. Jones, D.E. Stallknecht, E.W. Howerth, and S.M. Tompkins. 2011. *Virology* 413: 321. Reprinted here with permission of publisher.

CHAPTER 4

LOW PATHOGENIC AVIAN INFLUENZA ISOLATES FROM WILD BIRDS REPLICATE AND TRANSMIT IN FERRETS WITHOUT PRIOR ADAPTATION[1]

---

[1]Driskell, E.A., J.A. Pickens, J. Humberd-Smith, J.T. Gordy, K.C. Bradley, D.A. Steinhauer,, R.D. Berghaus, D.E. Stallknecht, E.W. Howerth, and S.M. Tompkins. To be submitted to *PLoS Pathogens*.

ABSTRACT

Direct transmission of avian influenza viruses to mammals has become an increasingly investigated topic during the past decade; however, isolates that have been primarily investigated are typically ones originating from human or poultry outbreaks. Currently there is minimal comparative information on the behavior of the innumerable isolates that exist in the natural wild bird host. We have previously demonstrated the capacity of numerous current circulating North American wild bird isolates to infect and induce lesions in the respiratory tract of mice. In this study, two isolates from shorebirds that were previously examined in mice (H1N9 and H6N4 subtypes) are further examined through experimental inoculations in the ferret with analysis of viral shedding, histopathology, and antigen localization via immunohistochemistry to elucidate pathogenicity and transmission of these viruses. Using sequence analysis and glycan binding analysis, we show that these avian isolates have the typical avian influenza binding pattern, with affinity for cell glycoproteins/glycolipids having terminal SA residues with α 2,3 linkage [Neu5Ac(α2,3)Gal]. Despite the lack of α2,6 linked SA binding, these AIVs productively infected both the upper and lower respiratory tract of ferrets, resulting in nasal viral shedding and pulmonary lesions with minimal morbidity. Moreover, we show that one of the isolates is able to transmit to ferrets via direct contact, despite its binding affinity for α 2,3 linked SA residues. These results demonstrate that avian influenza viruses, which are endemic in aquatic birds, can potentially infect humans and other mammals without adaptation. Finally this work highlights the need for additional study of the wild bird subset of influenza viruses in regard to surveillance, transmission, and potential for reassortment, as they have zoonotic potential.

AUTHOR SUMMARY

The emergence of the 2009 H1N1 influenza virus as the first influenza pandemic of the 21st century highlights the potential of pandemic influenza originating from avian reservoirs as it has a tripartite genetic composition that includes swine, human, and avian components. While highly pathogenic avian influenza viruses (AIVs), particularly of the H5 subtype, have been extensively studied in mammals, much less is known about the AIVs that widely circulate in the reservoir species, wild aquatic birds. Here we show that North American AIVs from shorebirds readily infect mammals using a ferret model. Furthermore, although these viruses demonstrate a classic avian influenza binding pattern, which is thought to be a barrier for effective mammalian infection with avian influenza viruses, both isolates exhibited robust nasal shedding and one of the isolates transmitted to naïve ferrets. While these viruses cause limited clinical disease, they replicate to high titer and cause pathology similar to other human influenza viruses. Our study demonstrates the ability of AIVs present in shorebirds to infect mammals without any adaptation, providing an avenue for introduction of avian viruses and the opportunity to reassort with mammalian viruses generating novel, potentially pandemic influenza viruses.

INTRODUCTION

The host and virulence range for avian influenza viruses (AIV) continues to surprise, with numerous cases of direct transmission from birds to mammals that result in a range of disease including pneumonia, conjunctivitis, and occasionally systemic disease [1,2,3]. Although transmission of AIV to humans resulting in disease has been limited to poultry adapted viruses, there is evidence of both direct transmission of AIV to other mammalian species [3,4,5] and experimental evidence that numerous AIV hemagglutinin (HA) subtypes can infect mammals [5,6,7,8,9,10]. However, there remains a great void of knowledge regarding the capacity of AIVs to infect mammals, especially related to AIVs from the wild bird reservoir. Human AIV infections have been limited to the H5, H7, and H9 subtypes [2,11,12,13,14] and these viruses are of concern because they have a pandemic potential if they become highly transmissible in the human population. Recent studies have demonstrated the high compatibility of avian and human influenza reassortants *in vitro* and *in vivo* and generation of viable reassortants *in vivo* in ferrets, further raising the concern of the natural generation of a pandemic strain [15,16,17,18]. Examining the capacity of a spectrum of wild bird AIVs to infect mammals is necessary to complete our understanding of AIV host range restrictions and to better define potential risks of mammalian infection and viral reassortment.

We have previously screened wild bird AIVs in a mouse model and demonstrated their varying capacity to replicate in the lung of mice, with some isolates exhibiting robust pulmonary replication regardless of HA subtype and causing mild clinical disease [19]. Ferrets are a better model for influenza infection and transmission in humans as they are naturally susceptible to the virus and have a similar distribution of sialic acid glycans in the respiratory tract; they have also been used for numerous studies of AIV isolates that have resulted in human disease [20,21,22].

In this study, two wild bird AIV isolates (H1N9 and H6N4 subtypes) that exhibited robust pulmonary replication in mice were further studied in a ferret model to better assess pathogenesis and transmission capacity in mammals.

Viral contributors to host range restriction and virulence of AIVs in mammals have been demonstrated to be multifactorial. The interaction between the major viral glycoprotein, the hemagluttinin (HA) and the host cell sialic acid receptors is considered critical for establishing an influenza infection, and species specific binding restrictions have been identified. Influenza viruses of avian origin preferentially bind terminal sialic acids with a α2,3 linkage located in cells in the gastrointestinal tract of birds and on the ciliated cells and type II pneumocyte in the human respiratory tract [23,24,25,26,27,28]. Conversely, human influenza viruses exhibit preferential binding to terminal sialic acids with a α2,6 SA linkage located most prominently on non-ciliated cells of the human upper respiratory tract (nasopharynx and trachea) [23,27,29,30,31,32]. It is thought that the receptor specificity of influenza viruses is a large component of host restriction; where in some AIV cases (H5, H7, and H9 subtypes), the viruses are able to infect and cause disease in humans yet exhibit poor human to human transmission [14,33,34,35].

The amino acid residues contributing to α2,3 versus α2,6 SA binding specificity have been described for some viruses and mutation analysis has shown some of these residues to be directly involved with altering viral receptor specificity. In human H3 strains, amino acids Leu226 and Ser228 (H3 numbering) result in α2,6 SA binding, where avian strains that preferentially bind α2,3 SA receptors exhibit a Gln226 and Gly228 amino acid sequence [26,27,32,36]. Amino acid residues 138, 190, 194, and 225 (H3 numbering) have also been shown to be differentially conserved in avian and human influenza viruses [32].

Here we examine the potential for infection and transmission of the H1N9 (RT/645) and H6N4 (RT/625) AIV isolates. Using sequence, *in vitro* binding, and glycan microarray analysis we determined the receptor specificity of these viruses. Using the ferret model, the most representative animal model of human influenza virus infection, we performed *in vivo* assessment of the potential for infection, replication, and transmission of these viruses in mammals. Despite a dominant α2,3 (avian) binding specificity we demonstrate that both of these viruses replicate in both the upper and lower respiratory tract of ferrets, inducing pulmonary lesions, but resulting in little morbidity. Moreover, we demonstrate that one of these viruses (RT/645, H1N9) is able to transmit via direct contact, despite its dominant avian α2,3 SA binding preference. These findings suggest that transmission of wild bird AIVs to mammals is not restricted to specific subtypes and further investigation is warranted to better understand their zoonotic potential.

MATERIALS AND METHODS

Viruses

Avian influenza viruses used were cloacal swab isolates from wild birds in the United States acquired from Southeastern Cooperative Wildlife Disease Study (Athens, GA). Viruses were isolated from cloacal swabs in 9 day old embryonated chicken eggs (ECE) at 37°C for 72 hours and then minimally passaged (3 or fewer passages) in ECEs. Select isolates were screened in a previous study in Balb/c mice[19]. Two isolates that exhibited efficient pulmonary replication and induced pulmonary lesions in Balb/c mice were selected for *in vivo* studies in ferrets. Isolates included A/Ruddy Turnstone/650625/02 (abbreviated RT/625, H6N4 subtype) and A/Ruddy Turnstone/650645/02 (abbreviated RT/645, H1N9 subtype). The original low passage isolates, once selected by screening methods, were grown once more in 9 to 10 day old

ECE to generate a stock of the virus. Stock viruses were aliquoted and stored at -80°C until use. Stock virus titers were determined by plaque assay on MDCK cells.

Sequencing

Total viral RNA was extracted from AIV infected allantoic fluid using the RNeasy kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's protocol. One-step RT-PCR was performed on viral RNA using a universal primer set (Uni12/Inf-1 [5′-GGGGGGAGCAAAAGCAGG-3′ and Uni13/Inf-1 [5′-CGGGTTATTAGTAGAAACAAGG-3′) as previously described [58]. All 8 segments were generated, the HA segment was excised and gel purified using the QIAquick Gel Extraction kit (QIAGEN, Inc., Valencia, CA). The HA was sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with subtype specific primers (primer sequences available upon request).

Erythrocyte Binding Assays

Fresh turkey, guinea pig, and equine erythrocytes were thoroughly washed with 1X PBS and resuspended to 1% v/v in 1X PBS/.5%BSA. A standard hemagglutination assay was performed for each AIV isolate against all types of erythrocytes, and appearance of agglutination was scored after a 60-min incubation period.

Glycan Array Binding

Viruses were cultured on MDCKs for 72 hours in 1X Minimal Essential Medium supplemented with 1μg/ml TPCK [L-(tosylamido-2-pheyl) ethyl chloromethyl ketone]-treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ). The viral supernatant was collected and centrifuged at 5,000 RPM for 5 minutes to remove cell debris before viral purification. Each AIV isolate was purified on a 25% sucrose cushion and resuspended in 1X PBS with 1mM EDTA. Briefly, each virus was purified through a 25% sucrose gradient by high

speed centrifugation at 28,000 rpm at 4°C for 3 hours. The purified viruses were resuspended in1XPBS with 1mM EDTA on ice for 4 hours and stored at -80°C. Viral titers were determined by standard plaque assay on MDCK cells. All purified strains were labeled with 25 µg of Alexa488 dye in 1M $NaHCO_3$ (pH 9) for 1 hour. To remove residual dye, each sample was dialyzed in a 7000 MWCO Slide-A-Lyzer MINI dialysis cassette (Thermo Scientific) against PBS with 1mM EDTA overnight. The labeled viruses were analyzed via glycan microarray by the Core H of the Consortium of Functional Glycomics (www.functionalglycomics.org), where 70 µl of labeled virus was added to glycan microarray slide and incubated at 4°C for 1 hour. Each microarray was scanned by Perkin-Elmer ProScanAray that detected SA binding peaks designated as relative fluorescent units (RFUs).

Ferrets

Castrated male Fitch ferrets (Triple F Farms, Sayre, PA), 3 months old and seronegative to circulating human H1N1 and H3N2 influenza viruses, were used for the study. Ferrets were housed in a BSL2 facility in HEPA filtered isolator caging (Allentown, Allentown, NJ). A subcutaneous temperature transponder (BMDS, Seaford, DE) was implanted in each ferret for identification and temperature measurement. Studies were conducted under guidelines approved by the Institutional Animal Care and Use Committee of the University of Georgia and the Centers for Disease Control and Prevention.

Infection and direct transmission study

Seven ferrets were inoculated per virus (four for tissue examination and three for transmission study) and three additional naive ferrets were used to assay direct contact transmission. In the contact trials, one direct contact ferret was housed with one inoculated ferret as paired cage mates. An additional three ferrets were mock infected with allantoic fluid in PBS

for negative controls for nasal washes, serology, and complete blood counts (CBC). Inoculated ferrets were lightly anesthetized with isoflurane and intranasally inoculated with 5 X 10^5 PFU in 500 µL of sterile PBS (250 µL of per nostril) of either RT/625 or RT/645. Direct contact ferrets were placed with inoculated ferrets twenty-four hours post inoculation. An additional study was performed with RT/645 as previously described to confirm direct contact transmission, using three inoculated and three direct contact ferrets. Temperatures were monitored for four days to establish baseline then temperature, weights, and complete blood counts were monitored in inoculated ferrets on days 1, 3, 5, 7, 10, 13, 18, and 21 pi and in direct contact ferrets on days 2, 4, 6, 9, 12, 17, and 20 post contact (pc). Nasal washes were sampled from ferrets on days 1, 3, 5, 7, 10 pi or days 2, 4, 6, 9, 12, and 17 pc to monitor for viral infection. For nasal washes, ferrets were anesthetized with 4 mg ketamine via intramuscular injection and 1 mL of sterile PBS with penicillin (4000 U/ml) (Calbiochem, Gibbstown, NJ), streptomycin (800 µg/ml) (Sigma, St. Louis, MO), polymyxin B (400 U/ml) (MP Biochmemicals, LLC, Solon, OH), and gentamicin (100 µg/ml) (Gibco, Carlsbad, CA) was introduced into the nostrils to induce sneezing and collected in specimen cups. For repeat study ferrets, temperature, weights, and nasal washes were performed for inoculated ferrets and transmission ferrets on days 1, 3, 5, 7, 9, 11, 13 and 15 pi and on days 2, 4, 6, 8, 10, 12, and 14 pc. Repeat study ferrets were humanely euthanized at day 21 pi (day 20 pc) and samples of lung from all ferrets were fixed in neutral buffered formalin for histopathology.

Determination of Viral Titers

Nasal washes were immediately tested via real time RT-PCR to aid in determining days for sample collection. Briefly, viral RNA was extracted from nasal wash by using RNeasy mini kit (QIAGEN, Inc., Valencia, CA) and the Qiagen one-step RT-PCR kit was used for RRT-PCR

with a Stratagene MX300P/3005P thermocyler and Mx Pro QPCR software (La Jolla, CA). Reaction mixture and PCR cycling protocol is available upon request. An influenza virus matrix gene specific primer and probe set were used as follows: primer M+25, sequence AGA TGA GTC TTC TAA CCG AGG TCG; primer M-124, sequence TGC AAA AAC ATC TTC AAG TCT CTG; and probe M+64, sequence FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA (Biosearch Technologies, Novato, CA) [59].

Four ferrets per virus were humanely euthanized (two on day 3 pi and two on day 7 pi per virus) and lung, nasal turbinate, liver, spleen, and olfactory bulb were sampled under sterile conditions and frozen at -80°C for virus isolation. Based on histopathology and viral titers from the lung, we suspected early pulmonary infection with rapid clearance, therefore an additional two ferrets were intranasally inoculated as previously described with RT/645 and humanely euthanized at day 2 pi with fresh lung collected to examine the earlier time point. Tissues were later homogenized in 1 mL PBS with antibiotics, clarified by centrifugation, and 100 µL of clarified homogenate was inoculated into 9 to 10 day old ECEs for virus isolation (4 eggs per sample, 72 hour incubation). Nasal washes and clarified lung homogenate were titrated in MDCK cells with serial 1:10 or 1:3 dilutions with a limit of detection of 1.5 $\log_{10}TCID_{50}$/mL (nasal wash) or 1.3 to 1.0 $\log_{10}TCID_{50}$/gram (lung).

Serology and Hematology

At day 21 pi (day 20 pc), blood was collected and seroconversion was determined via hemagluttination inhibition (HI) and microneutralization (MN) assays. All blood samples were analyzed the same day as sample collection. Complete blood counts were performed using VetScan analyzer (Abaxis, Union City, CA). Leukocyte counts were log transformed and statistically analyzed using repeated measures ANOVA (Stata version 11.0) to examine

differences between viral groups and days pi. Degrees of freedom for F-tests of repeated measures factors were adjusted using the Greenhouse-Geisser estimate of epsilon to correct for any departures from the sphericity assumption. All testing assumed a two-sided alternative hypothesis, and P-values <0.05 were considered significant.

Histopathology and Immunohistochemistry

Lung (cranial and caudal lobes), trachea, tracheobronchial lymph node, esophagus, heart, spleen, liver, stomach, small intestine, large intestine, pancreas, mesenteric lymph node, kidneys, adrenal gland, bladder, brain, and nasal turbinates were collected on days 3 and 7 pi from inoculated ferrets (two ferrets per virus per day, the same ferrets as described for virus isolation in fresh tissues). The additional two ferrets that were intranasally inoculated with RT/645 and humanely euthanized at day 2 pi had the same set of tissues collected for histopathology and immunohistochemistry. All tissues were preserved in 10% neutral buffered formalin. Tissues were routinely processed, embedded and stained with hematoxylin and eosin. Immunohistochemical staining was performed on lung, trachea, and nasal turbinates for all ferrets. Immunohistochemistry was performed using a commercially available goat polyclonal antibody to the nucleoprotein of influenza A virus at a 1:10,000 dilution (Biodesign International, Sako, Maine). Tissues were deparaffinized and blocked with a commercial protein blocking agent (Dako Cytomation, Carpinteria, CA) and a linked strepavidin-biotin immunoperoxidase system was used for immunolabeling. The reaction was visualized with 3, 3'-diaminobenzidine substrate (Dako Cytomation, Carpinteria, CA).

RESULTS

Wild bird avian influenza viruses replicated in ferrets but exhibited low virulence

Infection in RT/625 and RT/645 inoculated ferrets and in RT/645 direct contact ferrets was demonstrated with presence of virus in nasal washes and seroconversion despite minimal clinical signs (Table 4.1). Three ferrets infected with RT/625 (H6N4) had transient, mild weight loss that was most prominent day 1 pi (Table 4.1), but two of these ferrets took an additional two to six days to return to pre-infection weight. In contrast, six out of seven ferrets infected with RT/645 (H2N9) had mild weight loss most prominent at day 1 post inoculation (pi) (Table 4.1) in which it took up to four days to return to pre-infection weight. The average temperature of RT/625 and RT/645 inoculated ferrets was most elevated on day 1 pi (Table 4.1). Direct contact RT/645 ferrets had elevated temperatures that did correlate with shedding of virus in nasal washes, while direct contact RT/625 ferrets (did not become infected) had rare, inconsistent temperature elevations (data not shown). Sneezing was not observed in any group of ferrets and all ferrets remained bright and alert through the duration of the study. There were no statistically significant differences in the total or differential leukocyte parameters for group by day interactions. However, mean lymphocytes (mean +/- standard error) decreased in both RT/625 (3.50 +/- 0.06 pre inoculation to 3.26 +/- 0.06 day 1 pi) and RT/645 (3.76 +/- 0.06 pre inoculation to 3.61 +/- 0.09 day 1 pi) inoculated groups compared to the allantoic inoculated group (3.46 +/- 0.05 pre inoculation to 3.45 +/- 0.06 day 1 pi) for day 1 pi. Mean lymphocytes then increased for the RT/645 (4.06 +/- 0.16 day 7 pi) inoculated group compared to the allantoic inoculated group (3.67 +/- 0.11 day 7 pi) and remained elevated through day 18 pi (Supplemental Table 4.1).

Both H6N4 and H2N9 wild bird avian influenza viruses replicated in the upper respiratory tract of ferrets

Both RT625 and RT645 demonstrated replication in the upper respiratory tract of ferrets, evident by nasal washes and immunohistochemistry (IHC). Although both RT/625 and RT/645 replicated in the nasal cavity, RT645 replication was more robust, reaching consistently higher titers than RT625 for days 3 and 5 pi with shedding occurring consistently longer (Figure 4.1 and Table 4.1). Lesions of influenza infection were present in the nasal turbinates of ferrets infected with RT/625 and RT/645 (Figure 4.2A), however, only a single ferret in the RT/625 group (day 3 pi) had lesions while nasal turbinates of all four ferrets infected with RT/645 had lesions (both days 3 and 7 pi). This correlates with the viral titers obtained from nasal washes of these ferrets, as viral shedding quickly declined over a short period of time in this group with some ferrets shedding virus only up to day 3 pi (Figure 4.1). Lesions in the nasal turbinates included infiltration of the submucosa with mild to moderate numbers of lymphocytes with fewer plasma cells and occasional submucosal edema on both days 3 and 7 pi, with an increased amount of inflammation on day 7 pi for RT/645 (Figure 4.2A). The presence of influenza viral antigen was confirmed by abundant strong intranuclear and frequent intracytoplasmic staining on IHC for the nucleoprotein (NP) of influenza A in the nasal turbinates epithelium (Figure 4.2B). Positive staining occurred on day 3 pi in the nasal turbinates of ferrets that had lesions and was not present by day 7 pi for both viruses.

Both H6N4 and H2N9 wild bird avian influenza viruses replicated in the lower respiratory tract of ferrets

There was evidence of replication of both RT/625 and RT/645 in the lung of inoculated ferrets, with similar histopathologic progression of lesions for both viruses. Influenza was

detected in the lung for both viruses for all ferrets day 3 pi using virus isolation in ECEs, but was not isolated for day 7 pi from the lung for either group of ferrets (Table 4.2). For both viruses, histopathologic change in the lung on day 3 pi was characterized by a small amount of mucus admixed with neutrophils and macrophages within the lumens of bronchi and large bronchioles with peribronchial inflammation, primarily lymphocytes, that surrounded and occasionally infiltrated peribronchial glands (Figure 4.2G). There was positive intranuclear immunoreactivity against the NP of influenza A in small numbers of bronchiolar epithelial cells in the lungs of ferrets infected with RT/625 and RT/645 on day 3 pi (Figure 4.2F). No influenza antigen was present via IHC in the lung of ferrets infected with either of the two viruses on day 7 pi, supporting that virus was cleared from the lung by that time point. Rare peribronchial glands contained cellular debris and necrosis of the glandular epithelium, with presence of influenza antigen in the epithelial cells demonstrated via IHC on day 3 pi (Figure 4.2H). Smaller bronchioles were frequently filled with neutrophils, macrophages, and cellular debris and lined by a mixture of ectatic and very plump epithelial cells, indicative of early repair after previous epithelial damage (Figure 4.2D, E). Smaller bronchioles still had intraluminal inflammatory exudate and epithelial regeneration on day 7 pi. Peribronchiolar alveoli of affected bronchioles were occasionally filled by macrophages, but notably, there were minimal alveolar changes. Some pulmonary vessels had small perivascular cuffs composed primarily of lymphocytes. On both day 3 and day 7 pi for both viruses, the caudal lung lobes were more affected in severity and extent than the cranial lung lobes. Importantly, only segmental areas of the lung were affected in ferrets inoculated with either virus, with some lung lobes in individual ferrets having no histopathologic lesions at all. There was evidence of viral replication in the trachea, with rare neutrophils present in the epithelium for both RT/625 and RT/645 and rare positive intranuclear

epithelial immunoreactivity for the NP of influenza for one RT/645 inoculated ferret. Lung in the three inoculated ferrets from repeat transmission studies was examined microscopically at day 21 pi; these tissues had no significant lesions, indicating that complete resolution of pulmonary damage and inflammation had occurred by this time.

As there was evidence of pulmonary damage with regeneration, we suspected that viral replication in the lung had already peaked by day 3 pi. A titer could not be obtained for the lung via $TCID_{50}$ assay for day 3 pi for either virus (limit of detection=1.3 to 1.0 $\log_{10}TCID_{50}/g$) despite positive virus isolation. Therefore, two additional ferrets were inoculated with RT/645 and lung was examined at day 2 pi. In the day 2 pi ferret lung, a virus titer was obtained using a 1:3 dilution scheme (Table 4.1). Histopathologic changes in the lung on day 2 pi also confirmed suspicion of early viral replication, damage, and clearance, as the affected bronchiolar epithelium had necrosis and sloughing without indication of the regeneration that was observed on day 3 pi (Figure 4.2C). Positive intranuclear immunoreactivity against the NP of influenza A was also observed in small numbers of bronchiolar cells in the lungs for day 2 pi.

Extra-respiratory detection of wild bird influenza viruses in ferrets

Few changes in other organs were observed on histopathology. There was mild to moderate gross enlargement of tracheobronchial lymph nodes with microscopic lesions of moderate follicular hyperplasia as a result of the antigenic stimulation in all ferrets (both RT/625 and RT/645, days 3 and 7 pi). Perivascular cuffs of lymphocytes were present in the olfactory nerves and olfactory bulbs of the brain for one of the two ferrets infected with RT/625 on day 7 pi and one of the two ferrets infected with RT/645 on day 7 pi, although no influenza antigen was detected via immunohistochemistry on day 3 pi in the olfactory bulb for any of the ferrets. However, these lesions in combination with positive virus isolation on olfactory bulb in one of

the ferrets infected with RT/625 on day 3 pi, are supportive of probable direct extension of virus from the infected nasal epithelium, as has been shown in numerous experimental intranasal influenza inoculations (Table 4.2) [6,21,37]. Virus isolation was also performed on several other organs with sporadic positive results for rectal swabs, liver, and spleen, despite the absence of histopathologic lesions in these organs (Table 4.2).

Wild bird H1N9 avian influenza viruses transmitted between ferrets

Interestingly, RT/645 exhibited contact transmission between ferrets consistently in all three ferret pairs, but RT/625 did not directly transmit (Figure 4.1). All three direct contact ferrets that became infected with RT/645 had similar peak viral titers (average peak of 5.2 $\log_{10}$ $TCID_{50}$ for transmission ferrets and average peak of 5.4 $\log_{10}$ $TCID_{50}$ for inoculated ferrets) with similar length of shedding time (5 days for both groups) in the nasal wash compared to inoculated ferrets, although the time point of transmission varied greatly between pairs. Transmission variability may be somewhat explained by the varied time point of peak virus in the inoculated ferrets, which matched the pattern of transmission to the paired direct contact ferret (e.g. the later the peak virus in the inoculated ferret, the later the paired contact ferret had indication of transmission). Again, in ferrets that were infected via direct contact transmission, ferret health including clinical signs and morbidity parameters (temperature/weight loss) were minimally affected (Table 4.1). Direct contact transmission of RT/645 was repeated with a second study, and subsequently confirmed by two out of three direct contact ferrets becoming infected (data not shown).

Wild bird avian influenza viruses exhibited typical AIV receptor specificity

Effective transmission by RT/645 raised questions regarding potential mechanisms for transmission. Segment four, encoding the HA gene was sequenced for each virus to examine

receptor specificity as compared to HA sequences defined in the literature. Both RT/625 and RT/645 isolates contained glutamic acid (E) and glycine (G) at positions 190 and 225 of the HA, in contrast to human influenza strains, A/North Carolina/1/1918 and A/Pennsylvania/08/2008, that contain aspartic acid (D) at both 190 and 225 position (Table 4.3) that is associated with α2,6 linked sialic acid receptor specificity [38,39]. The glutamine (Q) at position 226 and glycine (G) at position 228 of the HA are characteristic of avian strains but have been also been identified in human strains and have been shown to influence α2,3 linked sialic acid receptor specificity. The RT/625 and RT/645 isolates contained the HA amino acid residues most commonly present in avian influenza strains that exhibit α2,3 linked sialic acid specificity.

To functionally assess the sialic acid receptor specificity of RT/645 and RT/625, we examined the erythrocyte binding of the avian influenza strains. Both the RT/645 and RT/625 isolates were able to agglutinate equine erythrocytes to a 512 HAU/ml titer (Table 4.4), while the human influenza strains (A/New Caledonia/20/1999 and A/California/04/2009) generated no detectable titer. Red blood cells (RBCs) from most species express both α2,3 and α2,6 linked sialic acids, but equine erythoctyes are unique in that they exhibit predominantly α2,3 linked SA receptors. Both RT/625 and RT/645 agglutinated guinea pig and turkey RBCs to similar levels as compared to a human influenza. Turkey eythrocytes contain a mixture of α2,3 and α2,6 linked sialic acid linked receptors and guinea pig RBCs express largely α2,6 linked sialic acids with lower levels of α2,3 linked sialic acid receptors [40]. While the mixed α2,3 and α2,6 linkages on these RBCs precludes determination of definitive α2,6 linked sialic acid binding, the lack of clear changes in binding to erythrocytes expressing predominantly α2,6 linked sialic acids suggests limited binding to these glycans.

To further define the receptor specificity of the viruses, glycan microarrays were utilized to determine the precise sialyl-oligosaccharide binding profile for RT/645 and RT/625 as compared to a previously defined seasonal H1N1 human influenza virus, A/ Pennsylvania/08/2008 [39]. Purified and fluorescently labeled viruses were submitted to Core H of the Consortium for Functional Glycomics and binding was assessed against 511 glycans (Supplemental data). The binding motif for all three viruses shows that the RT/645 and RT/625 predominantly bind oligosaccharides that contain N-acetylneuraminic acid α2,3 moieties with little binding observed for the α2,6 and α2,8 linked sialic acid containing glycans (Figure 4.3A,B). There was limited N-glyconeuraminic acid (NeuGc) recognition for the RT/645 and RT/625 with only the α2,3 NeuGc glycans demonstrating binding. The NeuGc moieties are the predominant sialic acid species on equine erythrocytes, where the RT/645 and RT/625 α2,3 NeuGc SA binding preference corroborates the horse erythrocyte agglutination data (Table 4.4) [41]. In contrast, the human control strain (A/Pennsylvania/08/2008) demonstrated the predicted α2,6 linked sialic acid receptor binding properties (Figure 4.3C) with minor α2,3 linked sialic acid binding, which is common among human influenza viruses. This uncharacteristic α2,3 linked sialic acid receptor specificity is often the result of fucosylated and sulfated modifications to the oligosaccahrides enhancing suboptimal sialic acid receptor recognition, which, as previously shown, appears to be the case for the human control strain (A/Pennsylvania/08/2008) [39].

DISCUSSION

It has been demonstrated that some low pathogenic H6, H7, and H9 AIV subtypes have the ability to replicate in ferrets; however studies that have demonstrated this have primarily examined poultry adapted isolates and have limited scope in examining the full pathogenesis of

these infections [6,7,10]. We have shown that current circulating North American wild bird LPAIVs do have a capacity to infect and replicate in mammals and we have expanded that knowledge by demonstrating that subtypes of "lesser concern" can also directly infect and replicate in mammals, even with the potential for direct contact mammal to mammal transmission (Table 4.1, Figure 4.1). Our isolates were passaged in embryonated chicken eggs, which may lend to some adaptive mutations; however, we kept serial passages low to minimize alteration of the original viruses. Interestingly, these viruses replicated to relatively high titers in the upper respiratory tract of the ferret and induced lesions in both the upper and lower respiratory tracts, but minimal disease was observed clinically with complete resolution of pulmonary lesions (Table 4.1, Figures 4.1-4.2). Importantly, lesions and antigen localization in the lung indicate there was no alveolar replication of the virus. Alveolar localization of influenza with alveolar lesions has been associated with increased virulence [42]. Additionally, the results of virus titration and histopathology comparing days 2 and 3 pi is supportive that infection was rapid, minimal, and had rapid clearance in the lower respiratory tract. These observations may have played a role in the minimal induction of disease for these two viruses in the ferret. A study that examined a variety of AIVs of the H6 subtype not only demonstrated replication with variable morbidity in ferrets, but also showed no correlation between the ability to infect the ferrets and the source of the virus (e.g. wild bird vs. poultry) [7]. This study provides additional indication that the source of virus may not be as important of a factor in transmission to mammals, regardless of subtype, although many other factors would play a role in natural transmission including host interactions and amount of virus shed.

Pulmonary replication of these two AIVs was rapid in onset and rapid to resolve regardless of magnitude of virus and pulmonary lesions. This rapid transient infection may be

related to inoculation methods, which place a large dose of virus in an anesthetized ferret where it becomes inhaled deep into the respiratory tract. This confounds risk assessment, as natural exposure would be through a fomite or aerosol droplet from another infected individual. Early pulmonary viral titers and histopathology have not been examined in transmission ferrets in other transmission experiments or in this study. This would be an interesting component to evaluate to aid in determining the pulmonary replication capacity of these AIVs in a more realistic transmission setting.

Few studies have examined hematologic parameters in influenza infected ferrets, but investigation into this is worthy as it may be a good measure of clinical disease [43]. Examining clinical pathologic findings of such a small sample size can be difficult, given the marked variation between individuals. Nonetheless, there was a trend in both groups RT/625 and RT/645 compared to the allantoic inoculated group of a decrease in lymphocytes on day 1 pi that resolved by day 3 pi for individual animals. Lymphopenia is well established to occur in the very early stages of viral infections. Indeed, it has been demonstrated that experimental ferret infections with HPAI H5N1 isolates result in a profound lymphopenia days 3 and 5 pi [44]. Also, ferrets infected with a variety of H1N1 influenza viruses had a decrease in lymphocytes days 3 and 7 pi [43]. For RT/645 inoculated animals, lymphocytes were increased between days 7 pi through 18 pi for many individuals. Lymphocytosis has not been described in ferrets experimentally infected with influenza, although this finding seems logical, given that chronic antigenic stimulation can induce increases in lymphocytes resulting in peripheral lymphocytosis.

Infectivity of AIVs in mammals and humans is thought to be reliant on the viral hemagglutinin binding sialic acid (SA) residues on host cells, and differences in binding between mammalian versus avian influenza viruses are suggested to be partially responsible for host

specificity and localization of infection [24,45]. It is thought that the avian influenza preference for binding α2,3 linked SA receptors compared to the human influenza preference for binding α2,6 linked SA receptors provides somewhat of a barrier to transmission in ferrets and humans due to the paucity of α2,3 linked SA in the upper respiratory tract. However, there is a presence of α2,3 linked SA in the lower respiratory tract in both of these species, and it has been proposed that if enough influenza can be deposited in the lower respiratory tract, pulmonary infection will predominate [42]. We did observe infection and replication in the lung of these ferrets for both RT/625 and RT/645 that supports viral attachment in the lung (bronchioles, but not alveoli), however, we also observed robust upper respiratory tract infections that were more productive with higher viral titers present than compared to the lung despite the alpha α2,3 SA binding preference (Table 4.1, Figures 4.1-4.2).We and others typically observe that ferrets infected with human influenza viruses have viral replication restricted to the upper respiratory tract [46,47]. The absence of pulmonary infection is confirmed by negative virus isolation, absence of lesions on histopathology, and absence of viral antigen on IHC. Very mild pulmonary infections have been demonstrated in other laboratories with human influenza viruses in experimental ferret infections, however severe pneumonia is only associated with highly virulent human influenza viruses or highly pathogenic avian influenza viruses [37,42,48]. In this study, neither RT/625 nor RT/645 virus appeared to infect alveolar epithelial cells (Figure 4.2) yet, both viruses did infect mouse and feline alveolar epithelial cells in other *in vivo* experimental trials in our laboratory. Other experiments have demonstrated replication of AIVs in the upper and lower respiratory tract of ferrets with some isolates having higher replication in the nasal turbinates and others with higher replication in the lung [6,7,21]. Clearly, cellular tropism in an influenza infected host

is complex, and while cellular SA ligands for HA binding is certainly an important component, additional mechanisms are likely at work.

In our study, the H1N9 subtype AIV (RT/645) exhibited direct contact transmission between ferrets. Aerosol transmission was not explored in this study due to lack of a validated aerosol transmission model in our facilities, however, it would be an interesting next step. Many experiments have established the importance of receptor binding in influenza transmission, demonstrating no contact or aerosol transmission in viruses that have a binding preference for α2,3 linked SA over α2,6 linked SA [10,49,50]. Both the RT/645 and RT/625 isolates have avian specific α 2,3 linked SA receptor binding as shown in the erythrocyte binding assays. There are no apparent HA amino acid residues that would suggest an altered receptor specificity and this assumption is supported by the glycan microarray analysis, where RT/645 dominantly bound glycans having α2,3 SA linkages. However, there may be other unidentified amino acids in the HA or other viral gene segments that mediated the direct transmission of the RT/645 in ferrets. Direct contact RT/625 ferrets did not become infected and did not seroconvert (Figure 4.1). Perhaps this is due to lower levels of viral shedding for a shorter period of time in RT/625 infected ferrets as compared to RT/645 infected ferrets.

The viral polymerase has also been suggested to potentially have a role in efficient avian to mammalian transmission, replication, and localization of viral infection based upon differences in temperature for optimal replication, tissue/species tropism for replication, rate of replication, and effect on efficiency of viral nuclear transport [51,52,53,54,55,56,57]. Sequence analysis of RT/645 PB2 found avian specific Glu627 and Asp701 residues (data not shown), suggesting that there may be other genetic features contributing to the robust upper respiratory replication and transmission of this H1N9 virus in ferrets.

Our study, in combination with additional studies of AIV infections in ferrets, indicates that there is a capacity for wild bird AIVs, subtype notwithstanding, to directly infect mammals with minimal clinical signs. The results support the potential for direct interspecies transmission or formation of a viable AIV reassortant. Although we have demonstrated the low virulence and rapid clearance of these AIVs, possibilities for reassortment in susceptible wild and domestic mammalian species make these species of particular interest and worth further investigation. Furthermore, the variable magnitude of seroconversion despite productive influenza infection could make surveillance and monitoring for mammalian infection with AIVs difficult. Together, these studies support the need for expanded analysis of influenza viruses from their reservoir species as understanding of the mechanisms of infection and transmission is incomplete and subsequent risk assessment imperfect.

ACKNOWLEGMENTS

The authors wish to thank the Animal Resources personnel at the College of Veterinary Medicine, University of Georgia for excellent animal husbandry. We would also like to thank histotechnicians in the Veterinary Pathology Department at University of Georgia for their assistance, especially Abbie Butler for her outstanding immunohistochemistry support. Additional thanks go to Taiana Costa, Deb Carter and Jon Gabbard for superior assistance with animal work and Cheryl Jones for excellent technical support at the College of Veterinary Medicine, University of Georgia.

REFERENCES

1. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, et al. (2005) Avian influenza A (H5N1) infection in humans. The New England Journal Of Medicine 353: 1374-1385.

2. Belser JA, Bridges CB, Katz JM, Tumpey TM (2009) Past, present, and possible future human infection with influenza virus A subtype H7. Emerging Infectious Diseases 15: 859-865.

3. Klopfleisch R, Wolf PU, Uhl W, Gerst S, Harder T, et al. (2007) Distribution of lesions and antigen of highly pathogenic avian influenza virus A/Swan/Germany/R65/06 (H5N1) in domestic cats after presumptive infection by wild birds. Veterinary Pathology 44: 261-268.

4. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, et al. (2004) Avian influenza H5N1 in tigers and leopards. Emerging Infectious Diseases 10: 2189-2191.

5. Song D, Kang B, Lee C, Jung K, Ha G, et al. (2008) Transmission of avian influenza virus (H3N2) to dogs. Emerging Infectious Diseases 14: 741-746.

6. Belser JA, Lu X, Maines TR, Smith C, Li Y, et al. (2007) Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. Journal of Virology 81: 11139-11147.

7. Gillim-Ross L, Santos C, Chen Z, Aspelund A, Yang CF, et al. (2008) Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. Journal of Virology 82: 10854-10863.

8. Hinshaw VS, Webster RG, Easterday BC, Bean WJ, Jr. (1981) Replication of avian influenza A viruses in mammals. Infection and Immunity 34: 354-361.

9. Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, et al. (2007) Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. Journal of Virology 81: 10558-10566.

10. Wan H, Sorrell EM, Song H, Hossain MJ, Ramirez-Nieto G, et al. (2008) Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One 3: e2923.

11. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, et al. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. Journal of Clinical Microbiology 43: 5760-5767.

12. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, et al. (2004) Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proceedings of the National Academy of Sciences of the United States of America 101: 1356-1361.

13. Guo Y, Li J, Cheng X (1999) Discovery of men infected by avian influenza A (H9N2) virus. Chinese Journal of Experimental and Clinical Virology 13: 105-108.

14. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, et al. (2004) Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 363: 587-593.

15. Octaviani CP, Ozawa M, Yamada S, Goto H, Kawaoka Y (2010) High level of genetic compatibility between swine-origin H1N1 and highly pathogenic avian H5N1 influenza viruses. Journal of Virology 84: 10918-10922.

16. Sun Y, Qin K, Wang J, Pu J, Tang Q, et al. (2011) High genetic compatibility and increased pathogenicity of reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza viruses. Proceedings of the National Academy of Sciences of the United States of America 108: 4164-4169.

17. Chen LM, Davis CT, Zhou H, Cox NJ, Donis RO (2008) Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. PLoS Pathogens 4: e1000072.

18. Jackson S, Van Hoeven N, Chen LM, Maines TR, Cox NJ, et al. (2009) Reassortment between avian H5N1 and human H3N2 influenza viruses in ferrets: A public health risk assessment. Journal of Virology 83: 8131-8140.

19. Driskell EA, Jones CA, Stallknecht DE, Howerth EW, Tompkins SM (2010) Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. Virology 399: 280-289.

20. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, et al. (2006) H5N1 virus attachment to lower respiratory tract. Science (New York, N Y) 312: 399.

21. Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, et al. (2005) Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. Journal of Virology 79: 11788-11800.

22. Yen HL, Lipatov AS, Ilyushina NA, Govorkova EA, Franks J, et al. (2007) Inefficient transmission of H5N1 influenza viruses in a ferret contact model. Journal of Virology 81: 6890-6898.

23. Thompson CI, Barclay WS, Zambon MC, Pickles RJ (2006) Infection of human airway epithelium by human and avian strains of influenza a virus. Journal of Virology 80: 8060-8068.

24. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, et al. (2006) Avian flu: influenza virus receptors in the human airway. Nature 440: 435-436.

25. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proceedings of the National Academy of Sciences of The United States of America 101: 4620-4624.

26. Connor RJ, Kawaoka Y, Webster RG, Paulson JC (1994) Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205: 17-23.

27. Rogers GN, Paulson JC (1983) Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127: 361-373.

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

29. Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, et al. (2007) Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nature Medicine 13: 147-149.

30. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JS (2007) Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respiratory Research 8: 73.

31. Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Review of Biochemistry 69: 531-569.

32. Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, et al. (1997) Avian influenza A viruses differ from human viruses by recognition of

sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology 233: 224-234.

33. Kemink SA, Fouchier RA, Rozendaal FW, Broekman JM, Koopmans M, et al. (2004) [A fatal infection due to avian influenza-A (H7N7) virus and adjustment of the preventive measures]. Nederlands Tijdschrift voor Geneeskunde 148: 2190-2194.

34. Uyeki TM, Chong YH, Katz JM, Lim W, Ho YY, et al. (2002) Lack of evidence for human-to-human transmission of avian influenza A (H9N2) viruses in Hong Kong, China 1999. Emerging Infectious Diseases 8: 154-159.

35. Claas EC, de Jong JC, van Beek R, Rimmelzwaan GF, Osterhaus AD (1998) Human influenza virus A/HongKong/156/97 (H5N1) infection. Vaccine 16: 977-978.

36. Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, et al. (1998) The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. Journal of Virology 72: 7626-7631.

37. Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, et al. (2005) Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. Journal of Virology 79: 2191-2198.

38. Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, et al. (2005) A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. Journal of Virology 79: 11533-11536.

39. Bradley KC, Jones CA, Tompkins SM, Tripp RA, Russell RJ, et al. (2011) Comparison of the receptor binding properties of contemporary swine isolates and early human pandemic H1N1 isolates (Novel 2009 H1N1). Virology 413: 169-182.

40. Medeiros R, Escriou N, Naffakh N, Manuguerra JC, van der Werf S (2001) Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology 289: 74-85.

41. Suzuki Y, Matsunaga M, Matsumoto M (1985) N-Acetylneuraminyllactosylceramide, GM3-NeuAc, a new influenza A virus receptor which mediates the adsorption-fusion process of viral infection. Binding specificity of influenza virus A/Aichi/2/68 (H3N2) to membrane-associated GM3 with different molecular species of sialic acid. J Biol Chem 260: 1362-1365.

42. van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, et al. (2010) Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. The Journal of Infectious Diseases 201: 993-999.

43. Belser JA, Gustin KM, Maines TR, Blau DM, Zaki SR, et al. (2011) Pathogenesis and transmission of triple-reassortant swine H1N1 influenza viruses isolated before the 2009 H1N1 pandemic. Journal of Virology 85: 1563-1572.

44. Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, et al. (2002) Pathogenesis of avian influenza A (H5N1) viruses in ferrets. Journal of Virology 76: 4420-4429.

45. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, et al. (2007) Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. The American Journal of Pathology 171: 1215-1223.

46. Rowe T, Leon AJ, Crevar CJ, Carter DM, Xu L, et al. (2010) Modeling host responses in ferrets during A/California/07/2009 influenza infection. Virology 401: 257-265.

47. Sweet C, Bird RA, Coates DM, Overton HA, Smith H (1985) Recent H1N1 viruses (A/USSR/90/77, A/Fiji/15899/83, A/Firenze/13/83) replicate poorly in ferret bronchial epithelium. Brief report. Archives of Virology 85: 305-311.

48. McBrayer A, Camp JV, Tapp R, Yamshchikov V, Grimes S, et al. (2010) Course of seasonal influenza A/Brisbane/59/07 H1N1 infection in the ferret. Virology Journal 7: 149.

49. Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, et al. (2007) A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science (New York, N Y) 315: 655-659.

50. Belser JA, Blixt O, Chen LM, Pappas C, Maines TR, et al. (2008) Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. Proceedings of the National Academy of Sciences of the United States of America 105: 7558-7563.

51. Van Hoeven N, Belser JA, Szretter KJ, Zeng H, Staeheli P, et al. (2009) Pathogenesis of 1918 pandemic and H5N1 influenza virus infections in a guinea pig model: antiviral potential of exogenous alpha interferon to reduce virus shedding. Journal of Virology 83: 2851-2861.

52. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, et al. (2007) Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathogens 3: 1374-1379.

53. Steel J, Lowen AC, Mubareka S, Palese P (2009) Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathogens 5: e1000252.

54. Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, et al. (2009) Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog 5: e1000709.

55. Shinya K, Hamm S, Hatta M, Ito H, Ito T, et al. (2004) PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 320: 258-266.

56. Subbarao EK, London W, Murphy BR (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. Journal of Virology 67: 1761-1764.

57. Gabriel G, Herwig A, Klenk HD (2008) Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathogens 4: e11.

58. Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, et al. (2009) Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. Journal of Virology 83: 10309-10313.

59. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, et al. (2002) Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256-3260.

Table 4.1. Morbidity, seroconversion, and viral replication in ferrets inoculated with wild bird avian influenza viruses

Inoculated animals

	Clinical parameters			Virus shedding		Seroconversion	
Virus	**Weight loss (max %, average %)**	**Average temperature increase (day p.i.)[a]**	**Sneezing**	**Virus detection in nasal wash (peak TCID50)[b]**	**Average TCID50/g virus in lung (day p.i.)[c]**	**Number with seroconversion (HI titers)**	**Number with seroconversion (MN titers)**
RT/625	3/7 (6.3, 5.8)	1.6 (1)	0/3	7/7 (5.2)	Not detected (3,7)	3/3 (1:160, 1:160, 1:160)	3/3 (1:2560, 1:1280, 1:640)
RT/645	6/7 (12.8, 4.5)	0.9 (1)	0/3	7/7 (5.8)	5.1 (2); Not detected (3,7)	3/3 (1:40, 1:40, 1:40)	3/3 (1:320, 1:640, 1:320)
Allantoic fluid	0/3	0.3 (1)	0/3	0/3	ND[d]	0/3[e]	ND

Direct contact animals

	Clinical parameters			Virus shedding		Seroconversion	
	Weight loss	**Range temperature increase [a]**	**Sneezing**	**Virus detection in nasal wash (peak TCID50) [b]**	**Average TCID50/g virus in lung (day p.i.)[c]**	**Number with seroconversion (HI titer range)**	**Number with seroconversion (MN titers)**
RT/625	1/3	0.4-1.4	0/3	0/3	ND	0/3	0/3
RT/645	2/3	0.8-1.3	0/3	3/3 (6.0)	ND	3/3 (1:20, 1:40, 1:80)	3/3 (1:320, 1:640, 1:640)

[a]Temperature is in degrees Celsius

[b]Limit of detection for nasal wash 1.5 $\log_{10}$ $TCID_{50}$/mL

[c]Limit of detection for lung day 7 pi both viruses and day 3 pi for RT/625 is 1.3 $TCID_{50}$/g; for days 2 and 3 pi for RT/645 is 1.0 $TCID_{50}$/g

[d]Not done

[e]Tested against both RT/625 and RT/645

Table 4.2. Presence of influenza virus in ferret organs by virus isolation in MDCK cells for ferrets infected with wild bird avian influenza viruses RT/625 and RT/645. For each organ listed, the number of animals with a positive isolation of virus out of the number of animals tested is shown.

Virus	Days post inoculation	Lung	Rectal swab	Intestine	Olfactory bulb	Liver	Spleen
RT/625	3	2/2	2/7	0/2	1/2	1/2	0/2
	5	ND	1/5	ND	ND	ND	ND
	7	0/2	0/5	ND	ND	ND	ND
RT/645	3	2/2	1/7	0/2	0/2	2/2	1/2
	5	ND	1/5	ND	ND	ND	ND
	7	0/2	1/5	ND	ND	ND	ND

[a]Not done for this sample

Table 4.3. Comparison of critical amino acids involved in receptor specificity of influenza hemagglutinin.

				Hemagglutinin Amino Acids[a]				
Virus	**Isolate ID**	**HA Subtype**	**Host**	**190**	**225**	**226**	**228**	**Accession No.**
A/Ruddy Turnstone/DE/650645/2002	RT/645	H1	Avian	E	G	Q	G	
A/Ruddy Turnstone/DE/650625/2002	RT/625	H6	Avian	E	G	Q	G	
A/Duck/Alberta/35/1976		H1	Avian	E	G	Q	G	AF091309
A/South Carolina/1/1918		H1	Human	D	D	Q	G	AF117241
A/New Caledonia/20/1999		H1	Human	N	D	Q	G	AB304818
A/Pennsylvania/08/2008		H1	Human	D	D	Q	G	FJ549047
A/California/04/2009		H1	Human	D	D	Q	G	FJ966082

[a] Hemagglutinin residues using H3 numbering

Table 4.4. Hemagglutination of erythrocytes from different animal species by human and avian influenza viruses.

Virus	Isolate ID	HA Subtype	Host	Hemagglutination Titers[a]		
				Turkey	Equine	Guinea Pig
A/Ruddy Turnstone/DE/650645/2002	RT/645	H1	Avian	1024	512	512
A/Ruddy Turnstone/DE/650625/2002	RT/625	H6	Avian	1024	512	256
A/New Caledonia/20/1999		H1	Human	1024	0	256
A/California/04/2009		H1	Human	64	0	16
A/Pennsylvania/08/2008		H1	Human	128	0	32

[a] Hemagglutination titers are provide as the reciprocal of the highest virus dilution generating agglutination.

Figure 4.1. Nasal shedding and direct contact transmission of wild bird influenza viruses in ferrets. Seven ferrets were intranasally inoculated with $5X10^5$ PFUs of either RT/625 (H6N4) or RT/645 (H1N9) and nasal washes were collected and titered on MDCK cells (days post-inoculation portion of graph). Three naïve ferrets were paired with three of the inoculated ferrets 24 hours post inoculation for each virus group (days post-direct contact portion); nasal washes were collected titered on MDCK cells. Both RT/625 and RT/645 demonstrated replication in the upper respiratory tract of the ferrets, however, viral shedding was consistently greater in magnitude and duration for RT/645. RT/645 demonstrated direct contact transmission, but RT/625 did not transmit to direct contact ferrets.

Figure 4.1.

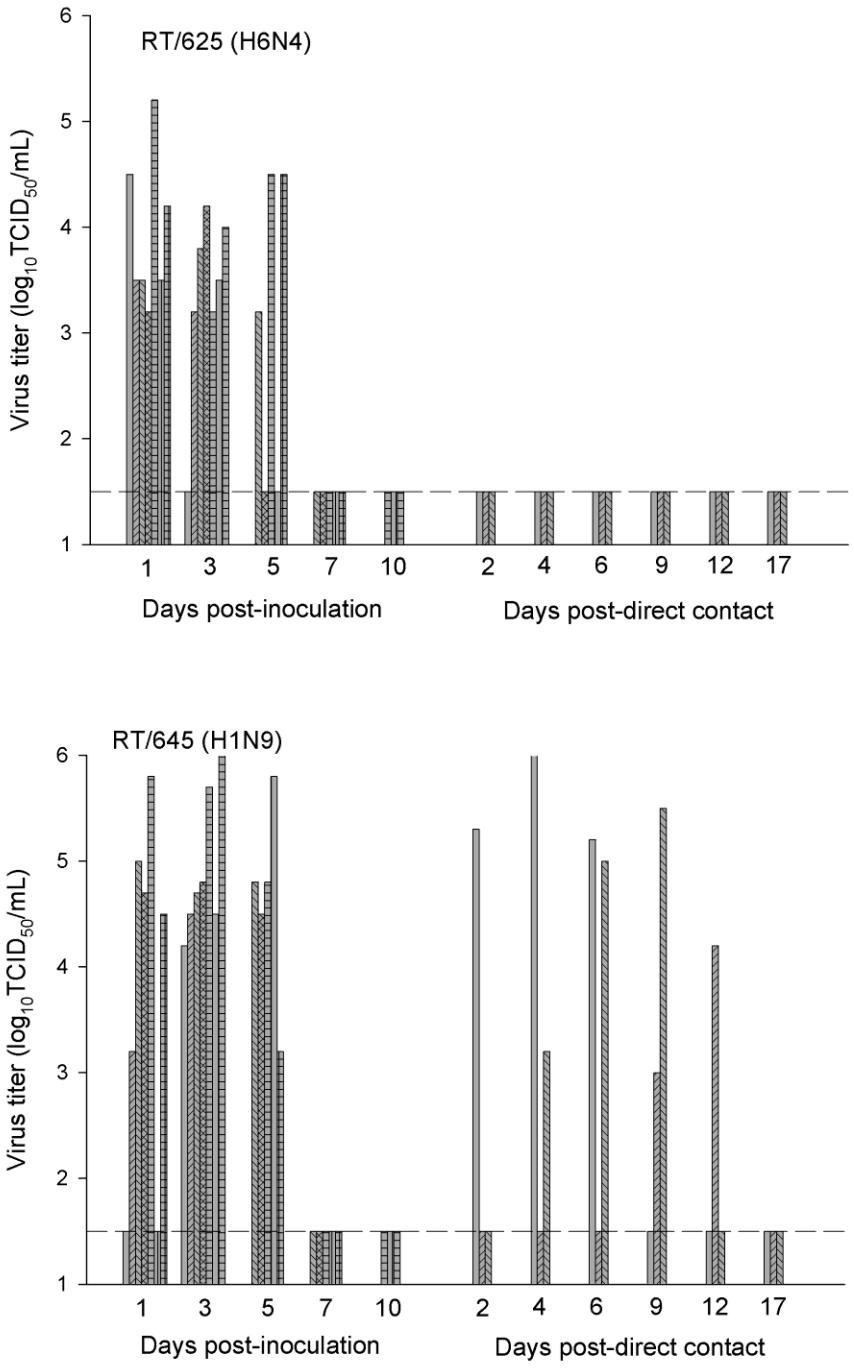

RT/625 (H6N4)
Virus titer ($log_{10}TCID_{50}$/mL)
6
5
4
3
2
1
1 3 5 7 10 2 4 6 9 12 17
Days post-inoculation
Days post-direct contact
RT/645 (H1N9)
Virus titer ($log_{10}TCID_{50}$/mL)
6
5
4
3
2
1
1 3 5 7 10 2 4 6 9 12 17
Days post-inoculation
Days post-direct contact

Figure 4.2. Histopathologic lesions and influenza antigen localization in ferrets inoculated with wild bird influenza viruses. A. Nasal turbinates of ferrets inoculated with RT/625 (H6N4) or RT/645 (H1N9) demonstrated moderate submucosal inflammation (asterisk) (RT/645 d3pi). B. There is widespread strong intranuclear and some intracytoplasmic positive immunoreactivity for the nucleoprotein of influenza A on immunohistochemistry in ferrets inoculated with RT/625 or RT/645 on day 3 pi (RT/645 d3pi). C. Epithelial damage in the lung was early for ferrets inoculated with RT/645, with necrosis in the bronchioles (arrow) and inflammation (asterisks) within and around bronchioles on day 2 pi. D and E. There was evidence of early repair with regeneration of bronchiolar epithelium (arrow) and persistence of inflammation (asterisks) on day 3 pi for ferrets inoculated with RT/625 and RT/645 (RT/645 d3pi). The arrow highlights the stretched and plump bronchiolar epithelial cells, indicating regeneration. F. Presence of influenza antigen was confirmed in ferrets inoculated with RT/625 and RT/645 by strong positive intranuclear staining of bronchiolar epithelial cells with immunohistochemistry on day 3 pi (RT/625 d3pi). G. Inflammation around larger airways in the lung was also present in ferrets inoculated with RT/625 and RT/645, with prominent periglandular bronchial inflammation (asterisk) (RT/625 d7pi). H. There was strong positive intranuclear staining for the nucleoprotein of influenza in the peribronchial glandular epithelial cells on immunohistochemistry on day 3 pi in ferrets inoculated with RT/625 and RT/645 (RT/645 d3pi).

Figure 4.2.

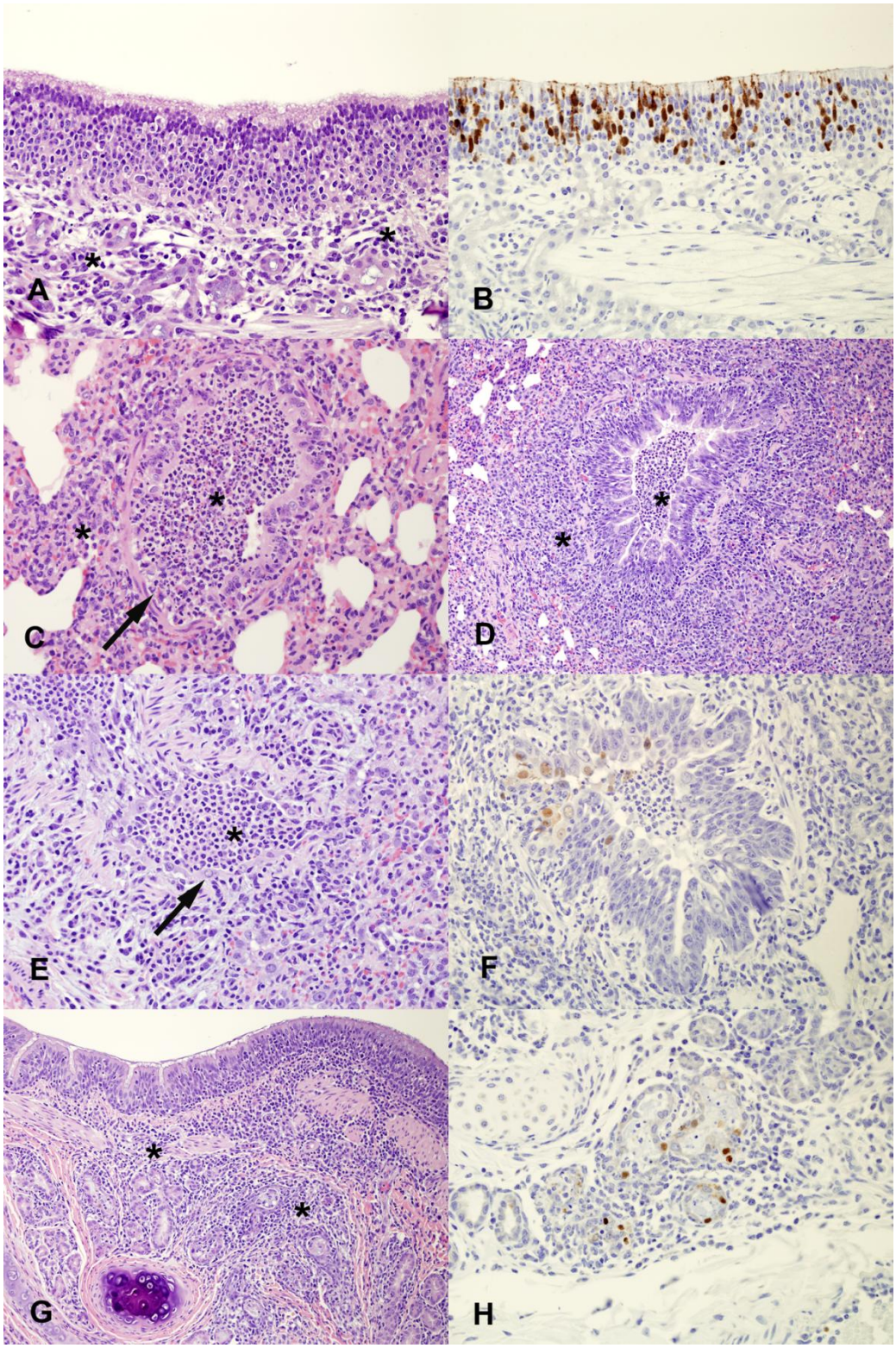
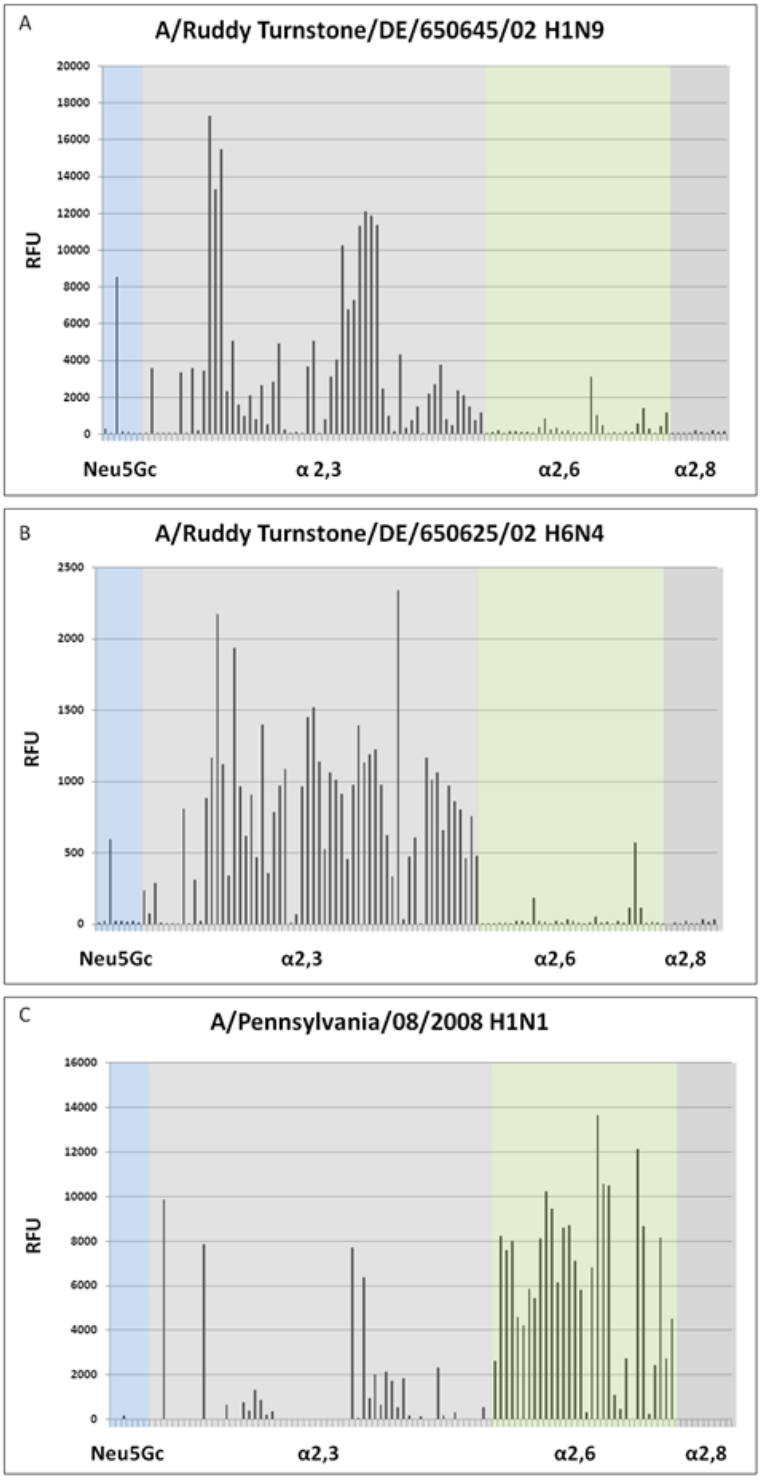


Figure 4.3. Glycan binding analysis of wild bird avian or human influenza viruses. Influenza viruses were propagated in Madin-Darby kidney cells, purified on a 25% sucrose cushion by ultracentrifugation, and labeled with Alexa488 before being applied to the microarray. The data was organized based on Neu5GC, α2,3 SA, α2,6 SA and α2,8 SA glycan structures and represented by different color schemes. Glycan microarray binding analysis was performed by Core H of the Consortium for Functional Glycomics.

A) A/Ruddy Turnstone/DE/650645/02 (H1N9) (**RT/645**), B) A/Ruddy Turnstone/DE/650625/02 (H6N4) (**RT/625**), C) A/Pennsylvania/08/2008 (H1N1)

Figure 4.3.


A
A/Ruddy Turnstone/DE/650645/02 H1N9
RFU
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Neu5Gc
α 2,3
α2,6
α2,8
B
A/Ruddy Turnstone/DE/650625/02 H6N4
RFU
2500
2000
1500
1000
500
0
Neu5Gc
α2,3
α2,6
α2,8
C
A/Pennsylvania/08/2008 H1N1
RFU
16000
14000
12000
10000
8000
6000
4000
2000
0
Neu5Gc
α2,3
α2,6
α2,8

Chapter 4 Supplemental Table. Total and differential leukocyte counts in ferrets inoculated with wild bird avian influenza viruses RT/625 and RT/645. Ferrets infected with RT/645 had significantly higher total leukocyte counts and lymphocyte counts than allantoic inoculated (control) ferrets and ferrets infected with RT/625. Lymphocyte counts peaked at day 7 pi for ferrets infected with RT/645. Although means for individual time points demonstrated statistically significant differences, there were no statistically significant differences between group by day interactions

Chapter 4 Supplemental Table.

Leukocyte	Day pi	Individual ferret leukocyte counts, 3 ferrets per group								
		Allantoic fluid			RT/625			RT/645		
Total leukocytes (cells/µL)	-2	6410	8760	8300	7170	7710	8880	10300	18760	12290
	1	8480	8910	5870	6310	7740	5040	7060	11110	11200
	3	8960	10070	11080	-	6030	11300	13020	17430	13280
	5	12970	7880	10860	8410	10780	6500	12840	13740	18430
	7	10840	10730	10230	9020	7960	9530	35460	19490	13430
	10	8900	10300	12170	6560	7630	8270	15920	20730	13960
	13	13970	6850	11770	12020	10640	6890	10000	18090	20550
	18	22160	12960	13640	21170	10470	16950	25250	19650	12520
	21	13450	11420	6070	8390	8050	12660	9620	5950	13420
Lymphocytes (cells/µL)	-2	2340	3570	8300	2850	2620	4210	4420	7180	6090
	1	2890	3560	2140	1670	2360	1550	2760	5720	4280
	3	3730	3860	2610	-	3200	4650	7130	8210	7500
	5	5410	3270	2470	3760	420	2990	6240	5860	8620
	7	6190	5890	2780	4030	3770	5140	24130	8660	7240
	10	3600	4300	3720	2380	3090	4280	7450	12480	6680
	13	7540	3640	4900	6890	5930	4020	6430	9370	11500
	18	12200	6090	4760	11550	5150	10190	14930	11250	6330
	21	6290	5950	2480	3620	4210	9330	5340	4160	7350
Granulocytes (cells/µL)	-2	3790	5120	5340	3390	5030	4560	5260	10050	6120
	1	4710	5280	3630	4060	4520	3100	3740	3870	5930
	3	4050	6140	7980	-	4530	6570	4540	6870	5680
	5	7470	4540	7460	3640	5130	2890	5130	6020	9680
	7	3800	4750	7370	4920	4140	4330	11120	8260	5650
	10	5240	5280	8370	3660	3780	2970	8360	6560	7180
	13	5890	3170	5930	3530	4640	2820	4140	6800	7290
	18	8250	6560	8120	9470	5180	5890	10170	7920	5270
	21	7070	5400	2930	3650	3680	3250	3400	1610	5990

CHAPTER 5

DOMESTIC CATS ARE SUSCEPTIBLE TO INFECTION WITH SHOREBIRD AVIAN INFLUENZA VIRUSES

[1]Driskell, E.A., C.A. Jones, R.D. Berghaus, D.E. Stallknecht, E.W. Howerth, and S.M. Tompkins. To be submitted to *Veterinary Pathology*.

ABSTRACT

Cats are susceptible to infection with highly pathogenic avian influenza H5N1 virus, causing concern for zoonotic spread; however, their susceptibility to other AIVs is unknown. Experimental infection of domestic cats with two shorebird AIVs did not result in disease, led to variable pharyngeal viral shedding with one of the viruses, and resulted in limited seroconversion.

INTRODUCTION

Transmission of HPAI H5N1 to domestic and wild felids has been demonstrated naturally and experimentally (*1-7*) resulting in a range of clinical disease; from systemic infection with systemic lesions and death to only seroconversion with minimal disease. Infection with influenza virus in domestic felids is a zoonotic concern given their close relationship with humans; domestic felines have also recently shown susceptibility resulting in severe disease to the pandemic H1N1 (*8-10*). Earlier studies also showed that avian and human origin influenza viruses resulted in shedding in cats in absence of disease (*11*). Cats could not only serve as an intermediate route of transmission of avian influenza viruses to humans, but also may provide a novel opportunity for avian influenza reassortment, potentially resulting in a virus of pandemic concern.

THE STUDY

Avian influenza viruses used in this study originated from ruddy turnstones (*Arenaria interpres*), and were originally isolated from cloacal swabs in 9 day old embryonated chicken eggs (ECE). Viruses were minimally passaged (3 or fewer passages) in ECEs. Select isolates were screened in a previous study in BALB/c mice (*12*). Two isolates that exhibited efficient pulmonary replication and induced pulmonary lesions in BALB/c mice were selected for *in vivo*

studies in cats. Isolates included A/Ruddy Turnstone/ DE/650625/02 (H6N4) (abbreviated RT/625) and A/Ruddy Turnstone/ DE/650645/02 (H1N9) (abbreviated RT/645).

The study included six 5 month old domestic cats (Liberty Research, Inc, Waverly, NY); all were seronegative to circulating H1 and H3 influenza viruses, as determined by hemagglutination inhibition. A subcutaneous temperature transponder (BMDS) was implanted in each cat for identification and temperature measurement. Studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia and CDC. For each of the viruses used, three cats were anesthetized with acepromazine (Boehringer Ingelheim), butorphanol (Fort Dodge), and dexmedetomidine (Pfizer), and intratracheally inoculated with 10^6 PFU of virus. Temperature, weights, complete blood counts, and nasal, pharyngeal, and rectal swabs were sampled on days 0, 1, 3, 5, and 7 post inoculation (pi) under sedation with acepromazine and butorphanol. On day 7 pi, all cats were sedated and humanely euthanized, and lung and trachea were sampled and frozen at -80°C for virus isolation; cranial lung lobes, caudal lung lobes, trachea, tracheobronchial lymph nodes, heart, thymus, spleen, liver, kidney, stomach, small intestine, colon, pancreas, mesenteric lymph nodes, adrenal gland, nasal turbinates, and brain were sampled and fixed in neutral buffered formalin and examined for histopathology.

Immunohistochemistry was performed on an autostainer (DakoCytomation, Carpinteria, CA) with DAB chromagen (Dako Cytomation, Carpinteria, CA) or Vulcan fast red chromagen (Biocare Medical, Concord, CA). Tissues were blocked with Dako Biotin Blocking System (Dako Cytomation, Carpinteria, CA). To detect viral antigen, a goat polyclonal antibody to the nucleoprotein of influenza A virus (Biodesign International, Sako, Maine; diluted 1:10,000) and rabbit biotinylated anti-goat IgG as a secondary antibody (Vector Laboratories, Burlingame, CA)

were used. For double labeling, a mouse monoclonal antibody for cytokeratins AE1/AE3 (Biogenex, San Ramon, CA; diluted 1:200) with Biogenex Multilink kit with HRP label (Biogenex, San Ramon, CA) and a mouse monoclonal antibody to the nucleoprotein of influenza A (Biodesign International, Sako, Maine; diluted 1:1000) with Biogenex Multilink kit with alkaline phosphatase label (Biogenex; San Ramon, CA) was used. Hematoxylin counterstain was used for all immunohistochemistry. Sections of formalin-fixed, paraffin embedded heart from a chicken infected with H5N1 influenza virus incubated with the goat polyclonal or mouse monoclonal primary antibodies for influenza antigen served as a positive control. The same tissue was used as a negative control by replacing the primary antibody with isotype-matched control antibody. For the double labeling immunohistochemistry, lung sections from a domestic cat that died due to trauma treated with the same protocol as double labeled experimental tissues served as negative controls. Virus isolation was performed in 9 day old ECEs for all swabs and fresh tissues.

CBCs were performed using Abaxis. Leukocyte counts were log transformed and statistically analyzed using repeated measures ANOVA (Stata version 11.0) to examine differences between viral groups and days pi. Degrees of freedom for F-tests of repeated measures factors were adjusted using the Greenhouse-Geisser estimate of epsilon to correct for any departures from the sphericity assumption. All testing assumed a two-sided alternative hypothesis, and P-values <0.05 were considered significant.

Cats inoculated with either RT/625 or RT/645 had no clinical evidence of disease; all cats remained alert and playful with minimal weight change. There was no significant group, day, or group by day interaction effect on counts of total leukocytes, lymphocytes, monocytes, or granulocytes. However, there was an overall trend of elevation in total leukocyte counts on day 7

pi for cats inoculated with RT/625 or RT/645 (Table 5.1). Increased leukocyte counts observed pre-inoculation and day 1 pi can likely be attributed to stress response. Despite absence of clinical disease, all cats became infected with RT/625 or RT/645. For RT/645, virus was not isolated from either pharyngeal swabs or tissues (Table 5.2), but all cats seroconverted by day 7 pi as determined by microneutralization assay (Table 5.2). For RT/625, all cats seroconvered; virus was isolated from the lung of one cat on day 7 pi, and two of the cats had evidence of pharyngeal shedding based on positive virus isolation from pharyngeal swabs over several days (Table 5.2). Virus was not isolated from nasal or rectal swabs for RT/645 or RT/625 inoculated cats.

Lesions were primarily restricted to the lung in both RT/625 and RT/645 inoculated cats, and were usually more extensive and severe in the cranial lung lobes, likely a result of the inoculation method (Table 5.2). In the lung, there was a mild to moderate bronchointerstitial pneumonia, characterized by bronchioles that were filled by primarily macrophages and few neutrophils, often with adjacent alveoli filled with macrophages that occasionally affected large areas. Additionally, there was evidence of involvement of bronchiolar and alveolar epithelium, as affected bronchioles were lined by strikingly large, plump epithelial cells and there was prominent type II pneumocyte hyperplasia of alveolar epithelium. Frequently, vessels were surrounded by cuffs of lymphocytes (Figure 5.1). There was a sinus histiocytosis present in the tracheobronchial lymph nodes of all inoculated cats. Both RT/625 and RT/645 inoculated cats had strong intracytoplasmic immunoreactivity in pulmonary and tracheobronchial lymph node macrophages for the nucleoprotein of influenza A (NP) using immunohistochemistry (Figure 5.1). There were also rare bronchiolar epithelial cells and alveolar epithelial cells with strong intranuclear and some intracytoplasmic immunoreactivity for the NP for both RT/625 and

RT/645 inoculated cats (Table 5.2, Figure 5.1). Previous studies examining influenza in cats have shown viral infection and shedding is mostly absent by day 7 pi (*2, 8*).We have examined both of these viruses in ferrets, and virus is cleared from the lung by day 7 pi. Therefore, it is probable that virus was cleared in most of the cats by day 7 pi, as there is evidence of both viruses infecting the lung based on seroconversion and presence of microscopic lesions despite poor recovery of virus from lung and paucity of positive immunoreactivity.

Other studies have demonstrated the capability of influenza to infect bronchiolar epithelium and alveolar epithelium in cats when there is opportunity for exposure deep in the respiratory tract (*3, 5, 7, 8*). We have demonstrated that wild bird influenza viruses have a similar capacity to infect both the bronchiolar and alveolar epithelium in the lung of cats, hemagglutinin subtype notwithstanding, with limited pharyngeal viral shedding. Importantly, this infection occurred with an absence of clinical disease. Domestic cats hunting and eating wild birds may be exposed to AIVs, though productive infection and shedding would likely depend on the isolate and dose of virus upon exposure. Nonetheless, the capacity for cats to be infected with the viruses examined in this experiment further supports the ability of wild bird influenza viruses to infect mammals without adaptation in domestic birds or other mammals; such events could lead to adaptation to domestic cats or reassortment in this species.

ACKNOWLEDGMENTS

The authors wish to thank the Animal Resources personnel at the College of Veterinary Medicine, University of Georgia for excellent animal husbandry. We would also like to thank histotechnicians in the Veterinary Pathology Department at University of Georgia for their assistance, especially Abbie Butler for her outstanding immunohistochemistry support. Additional thanks go to Monique Silva de Franca and Jessica Mobley for superior assistance

with animal work at the College of Veterinary Medicine, University of Georgia. We also thank Xiyan Xu and Alexander Klimov, and the CDC (Atlanta, GA) for providing circulating influenza viruses as antigen sources to confirm seronegativity of study cats.

Support

This work is funded by CDC grant 5U19CI00040102.

REFERENCES

1. Desvaux S, Marx N, Ong S, Gaidet N, Hunt M, Manuguerra JC et al.: Highly pathogenic avian influenza virus (H5N1) outbreak in captive wild birds and cats, Cambodia. *Emerg Infect Dis* 2009, 15(3):475-8.
2. Leschnik M, Weikel J, Möstl K, Revilla-Fernández S, Wodak E, Bagó Z et al.: Subclinical infection with avian influenza A (H5N1) virus in cats. *Emerg Infect Dis* 2007, 13(2):243-7.
3. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S et al.: Avian influenza H5N1 in tigers and leopards. *Emerg Infect Dis* 2004, 10(12):2189-91.
4. Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N et al.: Avian influenza H5N1 in naturally infected domestic cat. *Emerg Infect Dis* 2006, 12(4):681-3.
5. Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R et al.: Avian H5N1 influenza in cats. *Science* 2004, 306(5694):241.
6. Klopfleisch R, Wolf PU, Uhl W, Gerst S, Harder T, Starick E et al.: Distribution of lesions and antigen of highly pathogenic avian influenza virus A/Swan/Germany/R65/06 (H5N1) in domestic cats after presumptive infection by wild birds. *Vet Pathol* 2007, 44(3):261-8.
7. Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA et al.: Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. *Am J Pathol* 2006, 168(1):176-83.

8. van den Brand JM, Stittelaar KJ, van Amerongen G, van de Bildt MW, Leijten LM, Kuiken T et al.: Experimental pandemic (H1N1) 2009 virus infection of cats. *Emerg Infect Dis* 2010, 16(11):1745-7.
9. Sponseller BA, Strait E, Jergens A, Trujillo J, Harmon K, Koster L et al.: Influenza A pandemic (H1N1) 2009 virus infection in domestic cat. *Emerg Infect Dis* 2010, 16(3):534-7.
10. Löhr CV, DeBess EE, Baker RJ, Hiett SL, Hoffman KA, Murdoch VJ et al.: Pathology and viral antigen distribution of lethal pneumonia in domestic cats due to pandemic (H1N1) 2009 influenza A virus. *Vet Pathol* 2010, 47(3):378-86.
11. Hinshaw VS, Webster RG, Easterday BC, Bean WJ, Jr.: Replication of avian influenza A viruses in mammals. *Infect Immun* 1981, 34(2):354-61.
12. Driskell EA, Jones CA, Stallknecht DE, Howerth EW, Tompkins SM: Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. *Virology* 2010, 399(2):280-89.

Table 5.1. Total leukocyte counts in cats inoculated with RT/625 (H6N4) or RT/645 (H1N9) avian influenza viruses.

Cat number Virus RT/625	Total leukocyte counts (cells/µL)				
dpi	-6	1	3	5	7
1	23420	14890	12560	15330	19510
2	9270	-	6920	7720	26880
3	11670	10960	6620	9950	8000
Virus RT/645					
dpi	-6	1	3	5	7
1	17940	-	12980	15230	19370
2	10500	20820	4310	6600	19010
3	13480	22870	21590	12980	25500

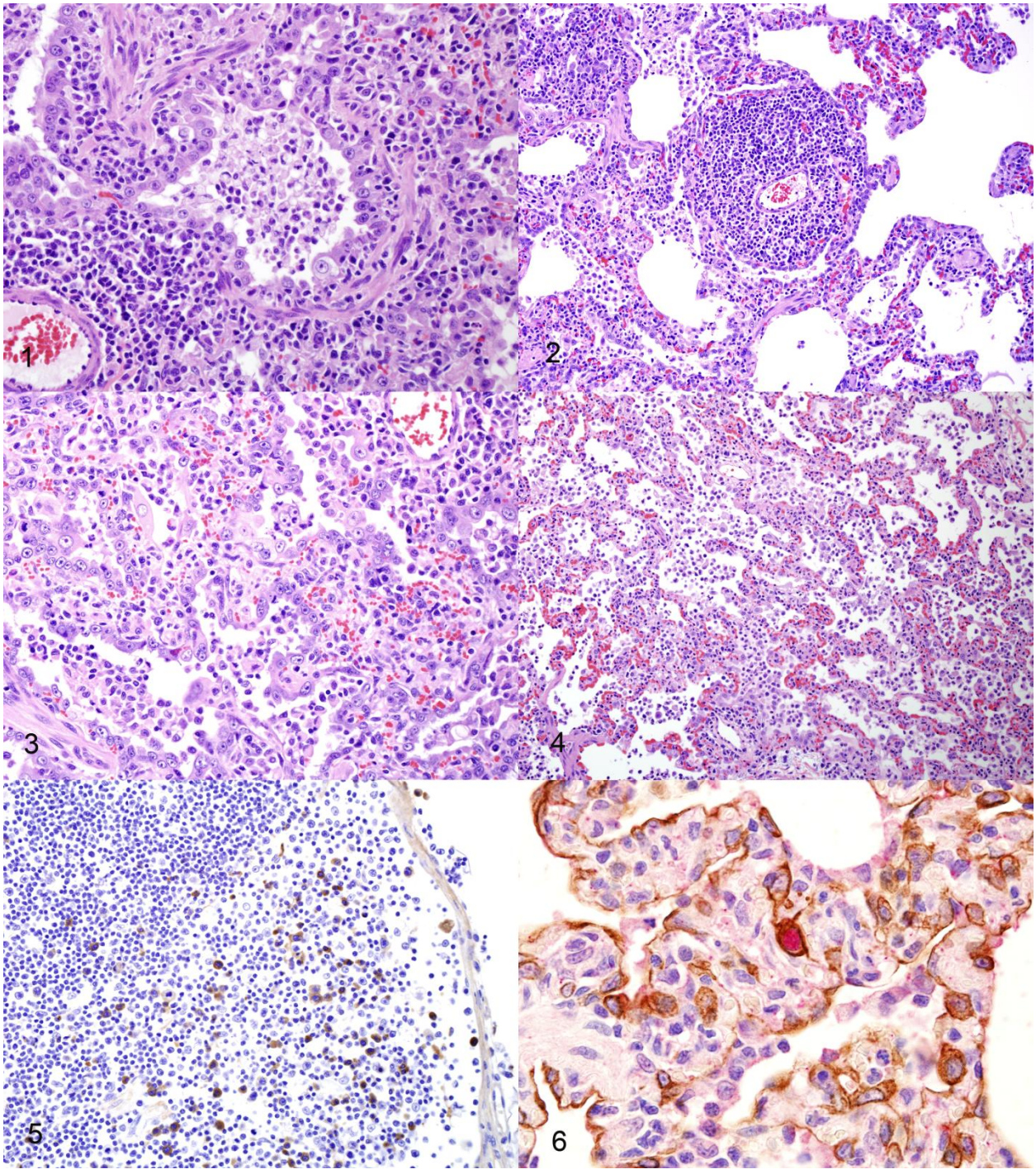
Table 5.2. Viral infection and shedding in cats inoculated with RT/625 (H6N4) or RT/645 (H1N9) avian influenza viruses.

Cat number Virus RT/625	Pharyngeal swab*					Lung*	Trachea*	Pneumonia severity†	Pulmonary IHC‡	Seroconversion (MN titer^{-1})
dpi	0	1	3	5	7	7	7	7	7	7
1	-	-	+	+	+	-	-	up to 10%	-	1:40
2	-	-	+	+	-	+	-	25-50%	+	1:320
3	-	-	-	-	-	-	-	up to 10%	-	1:40
Virus RT/645										
dpi	0	1	3	5	7	7	7	7	7	7
1	-	-	-	-	-	-	-	up to 10%	+	1:20
2	-	-	-	-	-	-	-	25-50%	-	1:80
3	-	-	-	-	-	-	-	10-25%	-	1:80

*Virus detected by virus isolation
†Percent lung affected with inflammation estimated from equivalent microscopic sections between cats for both cranial and caudal lung lobes
‡All cats had positive immunoreactivity in scattered pulmonary macrophages, + indicates positive nuclear immunoreactivity in alveolar epithelial cells

Figure 5.1. Microscopic pulmonary lesions and presence of influenza antigen in cats inoculated with RT/625 (H6N4) or RT/645 (H1N9) avian influenza viruses. (1) Lung; cat; inoculated with avian influenza virus RT/625 (H6N4), day 7 pi. Bronchointerstitial pneumonia with a bronchiolar lumen filled by macrophages and peribronchiolar lymphocytes and plasma cells. Bronchiolar epithelial regeneration is evident with hypertrophied bronchiolar epithelium. HE. (2) Lung; cat; inoculated with avian influenza virus RT/625 (H6N4), day 7 pi. Note thick perivascular cuffs of lymphocytes. HE. (3) Lung; cat; inoculated with avian influenza virus RT/645 (H1N9), day 7 pi. Marked type II pneumocyte hyperplasia with numerous macrophages in alveolar lumens. HE. (4) Lung; cat; inoculated with avian influenza virus RT/645 (H1N9), day 7 pi. Macrophages fill alveolar lumens. HE. (5) Tracheobronchial lymph node; cat; inoculated with avian influenza virus RT/625 (H6N4), day 7 pi. Immunohistochemistry for influenza A viral antigen labels the cytoplasm of macrophages. Streptavidin-biotin immunoperoxidase method with hematoxylin counterstain. (6) Lung; cat; inoculated with avian influenza virus RT/645 (H1N9), day 7 pi. Nuclear immunoreactivity for influenza A viral antigen (red) with cytoplasmic immunoreactivity for cytokeratin AE1/AE3 (brown) in pneumocytes. Streptavidin-biotin immunoperoxidase method (cytokeratin AE1/AE3) and alkaline phosphatase method (NP influenza A) with hematoxylin counterstain.

Figure 5.1



CHAPTER 6

SUMMARY AND CONCLUSIONS

The emergence of avian isolates that can directly infect humans is troubling because changes in host range have the potential to play a large role in the development of influenza pandemics. This fact combined with the severity of disease induced by highly pathogenic H5N1 avian influenza (HPAI H5N1) could be disastrous for the human population. Isolates that potentially transmit to humans, whether or not they directly cause disease, could also contribute to reassortment with other mammalian isolates that could lead to the emergence of a pandemic strain. Studies examining the potential of wild bird AIVs to infect mammals have been limited to only a handful of isolates. Our goal of this work was to systematically examine many of these isolates that have been gathered from extensive surveillance efforts over many years in order to assess these isolates in terms of public health risk.

Our first series of experiments embarked on the large task of screening over 400 North American wild bird AIVs isolates in vitro. From the in vitro screen, 28 isolates were then selected for in vivo screening in mice (Chapter 3). Isolates that replicated without the addition of trypsin on plaquing assays were qualified to be further examined in mouse studies; isolates were then selected to diversify subtype and wild bird species origin for the in vivo screening. The majority of screened AIVs, representing many different subtypes from different wild bird species, were able to replicate in the lung of BALB/c mice without requiring any adaptation. Additionally, eight of these isolates replicated to high titers in the lung. Despite high pulmonary viral titers, these isolates had low pathogenicity for the mice, resulting in minimal clinical

disease. These isolates induced lesions of bronchointerstitial pneumonia with a similar character and influenza antigen was present in the nasal turbinate, tracheal, bronchiolar, and alveolar epithelial cells, localized by immunohistochemistry. Microscopic lesions and immunohistochemical localization of antigen in this study suggests that viral replication was more in alveolar epithelium. Viral kinetics and lesion development in the mouse lung with these wild bird AIVs were similar to other experimental human, mouse adapted, and LPAIV infections in mice. Overall, this study demonstrates that replication of North American wild bird AIVs in the respiratory tract of BALB/c mice is isolate dependent and quite varied, but disease, viral kinetics, and lesions appear similar, regardless of subtype.

A ferret model was used to more accurately examine the public health risk for North American wild bird AIVs to directly infect and transmit in humans (Chapter 4). These experiments characterized the pathogenesis of two shorebird isolates (H1N9 and H6N4 subtypes) in ferrets. Both of these isolates exhibited robust upper respiratory tract replication, demonstrated by viral titers from nasal washes and abundant influenza antigen in immunohistochemical examination of the nasal turbinates, with subclinical to mild clinical disease and minimal upper respiratory tract lesions. The kinetics of viral shedding from the upper respiratory tract is similar to infections with seasonal human influenza viruses. Pulmonary infection was present for both the H1N9 and H6N4 isolates with mild bronchointerstitial pneumonia, though pulmonary infection occurred early post inoculation and was cleared rapidly with marked early bronchiolar epithelial regeneration (day 3 post inoculation). Furthermore, the H1N9 isolate was able to directly transmit despite the retention of α2,3 sialic acid preferential binding (typical avian pattern). There was seroconversion in all ferrets, though seroconversion was not as robust as with human influenza infections in ferrets. Taken together, this study demonstrates there is a capacity

for wild bird AIVs, subtype notwithstanding, to directly infect mammals with minimal clinical signs and also indicates a potential for direct interspecies transmission or formation of viable AIV reassortants, underscoring the potential pandemic risk of AIVs from the reservoir host.

Finally, the domestic cat was investigated as a potential public health risk for human exposure to wild bird AIVs (Chapter 5). The same two shorebird isolates that were examined in the series of ferret experiments were used in cats. Both isolates were able to infect cats, demonstrated by seroconversion, however, only the H6N4 isolate was recovered in tissues of inoculated cats (lung and pharyngeal swabs). Although infections were subclinical, both isolates induced bronchointerstitial pneumonia in inoculated cats. Through this study, we have demonstrated that the domestic cat is susceptible to subclinical infection with wild bird AIVs. This could lead to adaptation or reassortment in this species that may be of public health risk, although parameters for natural infection, such as dose and route of exposure required for infection, are important risk factors that remain to be determined.

The results from these in vivo experimental infections with North American wild bird AIVs demonstrate that the ability for these isolates to infect mammals is quite variable and isolate dependent. For those isolates that can experimentally infect mammals without adaptation, there is minimal clinical disease but typical viral kinetics and pulmonary lesions compared to other influenza A viruses in these species. Notably, there was nasal shedding of these AIVs in ferrets with direct contact transmission. These results implicate that we must be cognizant of the public health risk of circulating wild bird AIVs and continued surveillance of these isolates in wild birds and exposed mammals should be priority.