DETERMINANTS OF NORTH AMERICAN LOW PATHOGENIC AVIAN INFLUENZA VIRUS TROPISM

by

JENNIFER ANN PICKENS

(Under the Direction of S. Mark Tompkins)

ABSTRACT

Avian influenza viruses are endemic in wild aquatic birds and were generally thought to be apathogenic in humans until the 1997 outbreak of an extremely virulent highly pathogenic avian influenza (HPAI) H5N1 virus that resulted from direct transmission of a solely avian virus to humans. Since then, much of the research has concentrated on a limited number of HPAI subtypes and little is known about the pandemic potential of low pathogenic avian influenza (LPAI) strains from the natural reservoir. Most LPAI require exogenous trypsin to replicate in cell culture and is often an indicator of potential pathogenicity. Here a collection of 419 North America LPAI isolates was examined for their ability to replicate independent of exogenous trypsin in cell culture. Approximately 10% of the isolates replicated to high viral titers in various mammalian cell lines independent of trypsin. Two LPAI isolates, RT/645 (H1N9) and RT/625 (H6N1) replicated and induced pulmonary lesions in the ferret and mouse model systems without prior adaptation. The objective of this research was to examine the genetic features that mediate tropism and replication capacity of selected LPAI isolates, as well as host features that contribute to trypsin independent phenotype and the

transmission observed in mammals. A subset of twenty-three LPAI isolates that exhibited the trypsin independent phenotype were examined and all maintained the classical avian monobasic HA cleavage site and α2,3-linked sialic acid binding affinity. The trypsin independent phenotype did not appear to be mediated by the viral hemagglutinin or neuraminidase glycoproteins alone but was instead facilitated by virus infection. Using a protease inhibitor screen, the RT/645 and RT/625 isolates were shown to induce alternative proteases to mediate HA cleavage independent of serine proteases and exogenous trypsin. It is not clear why some LPAI isolates induce proteases while other viruses do not. It is certain that not all LPAI viruses adhere to the protease requirements described in the literature for proteolytic HA activation. These studies help to provide a better understanding of the genotypes and/or phenotypes associated with avian influenza viruses that infect, replicate, and transmit in mammalian cell culture, human airway cell cultures, and ferrets.

INDEX WORDS: Avian influenza, LPAI, trypsin, HA cleavage, proteases

DETERMINANTS OF NORTH AMERICAN LOW PATHOGENIC AVIAN INFLUENZA VIRUS TROPISM

by

JENNIFER ANN PICKENS

BS, Georgia State University, 2003

MS, Georgia 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

DETERMINANTS OF NORTH AMERICAN LOW PATHOGENIC AVIAN INFLUENZA VIRUS TROPISM

by

JENNIFER ANN PICKENS

Major Professor: S. Mark Tompkins

Committee: Mark Jackwood
David Stallknecht
David Steinhauer
David Suarez

Electronic Version Approved:

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

DEDICATION

I dedicate this to my Grandfather for always encouraging me to reach for my dreams no matter what obstacles I faced and instilling in me that anything is possible with hard work, dedication, and love. With love, I found not only myself but the man of my dreams… I dedicate this to Kevin for always believing in and loving me through all of life's craziness.

ACKNOWLEDGEMENTS

When you start a graduate school, you have no idea what it takes to survive the experience and how the people around you help make it truly great. First, I have to thank Dr. Tompkins for always having an open door and taking time to talk through experiments, discuss results, and broaden my scientific horizons. Even though I may not have been aware at the time, his nudging or in some cases pushing me out of my comfort zone showed me that there are multiple ways to approach research and the confidence to "own my data"!! Thank you, Dr Tompkins for believing in me… I have to also thank my committee for taking the time to meet and discuss my project and results. Thank you, Drs. Steinhauer and Suarez for taking the time and lab space to allow me to explore new methods and approaches to answer the constantly evolving questions associated with my research. Thank you to Dr. Stallknecht for providing the over 400 avian influenza viruses examined in my project… without the hard work of your team to collect these viruses, I would not have had a project. I appreciate Dr. Jackwood for providing thoughtful insight on the scientific approach and molecular techniques that helped me throughout my project.

I have so much appreciation for all my friends and co-workers at the Animal Health Research Center. I have to thank Cheryl Jones and Frank Michel for all their help and support in accomplishing daily tasks that at times seemed impossible. I could not have made it without the help of Jackelyn, thank you for always being a positive and supportive force in the lab and as a friend.

My friends at UGA and Emory have been a major part of what I enjoyed the most of graduate school. From the beginning, one friend and confidant has been there for me and I dread the day in the near future when I do not get to see him everyday… Jon, thank you for being such a wonderful friend and taking the time to listen whether it was about science or just to talk things out. I am going to miss you. In you, I have found a life long friend and those are a rare find. To one of my closest friends, Victoria, thank you for making some of the most difficult days in the lab and graduate school bearable. I have had some of my most memorable times in your company and you have always been game for activities. Thank you for always being there for me whether it was an impromptu wine night, running a ½ marathon, taking one for the team, therapeutic shopping, or just hanging out. I could not have survived the graduate school experience without you and I am so grateful to have such a great friend. The entire AHRC gang has been a huge part of daily life and I have so many memories of our time together, such as kayaking the Broad River, whitewater rafting, the bouncy house party, getting lost on our way to conferences… Thank you, Julie, for always having an opinion and not being afraid to express it. I am always amazed by your dedication to your friends, family, and your work. Danny D, we hit it off from the start and I appreciate your drive and ambition to being the best and that dedication overflows into your friendships. Thank you for being a friend… Valerie, we have been in this from day one and you have always been someone I could confide in about almost anything. Thank you for taking the time to listen… Tiffany, who can forget you, with your infectious and contagious laugh, you have always been game for anything adventurous. Thank you. Finally, Lauren, Alaina, Caleb, and Anthony… you guys were so much fun and I have so many wonderful memories of our

time together at conferences, mixers, and nights out on the town. There are several friends that are among the AHRC alumni and that I have met along the way that I want to thank… Christine, thank you for being such a positive influence and confidant through some of the most difficult and challenging times. You have helped me in so many ways and I am forever grateful to have you as a friend. There are people that cross your path and that you know from your first meeting that you have just found a true friend. Summer, you are one of those type of friends. I cannot tell you how much I appreciated your help, guidance, and kindness. It has meant so much to me.

I want to thank my family for all of their support through all of my academic pursuits. Yes, Mom, I am finally done with school after being a perpetual student. Thank you, Mom, for always being a driving force and showing me the will and strength it takes to pursue my dreams. To my Dad… Thank you for being such a solid support system during the highs and lows of graduate school and at times when I doubted myself, you helped me find the confidence to move pass the obstacles and stay true to myself. My dear grandmother, you have always been there for me and have provided the example by which I live my life. You are a true inspiration to me. Thank you, Jonathan… You are one of the true grand surprises of my life and I am so proud to have you as my brother. We are in this together and your support has meant so much to me. Finally, Carolyn, you are like a sister to me and you have been there for me for as long as I can remember and I am so lucky to have you in my life.

My old school friends have held a special place in my heart and their support has helped me through some of the most trying times of my life. I need to thank my oldest and dearest friend, Kim who has been a true friend throughout most of my life and we

have been through so much together. I can always count on you and there are not enough words in the universe to describe how grateful I am to have such a wonderful friend. Jamie, you and I have been so close no matter the miles that separate us and your support and encouragement have helped me make it this far. Thank you so much Kim and Jamie!!

Last but not least… I am so lucky to have found such an amazing friend, partner, and supporter… Kevin, you have changed my life and I am so thankful to have you in it. Thank you for taking the time to help and encourage me; even in times when I was full of doubt, you always knew the right thing to say. Thank you for taking the time to read my chapters, talk through my results, and to just listen. I am so grateful. You are an inspiration to me and your encouragement has meant the world to me. Thank you for being you…

Thank you all so much.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Influenza A viruses have caused millions of deaths worldwide over the last century. Despite the advances in modern medicine and influenza research, it still poses a major health threat. Annual influenza epidemics result in an average of 41,000 deaths and 200,000 hospitalizations in the United States (12, 14, 54). Influenza A viruses are of the most diverse RNA viruses, which can be attributed to its segmented, negative sense genetic configuration. The constant evolution of the virus requires that the seasonal influenza vaccine be evaluated annually to determine its efficacy against circulating strains. The segmented nature of influenza A virus genome also allows for major changes in the virus, which can result in pandemics and within the last century, there have been 4 major influenza pandemics, resulting in millions deaths worldwide (27, 34, 53). For this reason, a primary goal of influenza research is identification of potential sources of the next pandemic influenza virus. All influenza pandemics were the result of reassortment events between avian, swine, and/or human viruses (18, 29, 31, 35, 52). Due to the segmented nature of influenza, during co-infection, it is possible for the virus to exchange gene segments, generating novel influenza subtypes that can potentially infect and be transmitted among an immunologically naive population, causing substantial morbidity and mortality. For example, phylogenetic analysis of the 1918, 1957, and 1968 pandemic strains revealed that each acquired avian derived HA glycoproteins (35, 38, 39,

52, 57). In 2009, a novel H1N1 influenza A virus that was a reassortment of avian, swine, and human influenza gene segments emerged in North America and spread across the world in weeks, causing the first pandemic of the 21st century (1, 18). The 2009 H1N1 pandemic strain was able to infect the human population and was readily transmitted without adaptation. Fortunately, the 2009 H1N1 pandemic was mild as compared to other pandemics because of pre-existing immunity due to priming by circulating influenza strains (19, 26). It is not clearly defined what viral mechanisms are in place to facilitate reassortment events and what role the host plays in permitting such events.

All 16 HA and 9 NA subtypes are maintained in birds of the orders *Anseriformes* (waterfowl) and *Charadriiformes* (shorebirds and gulls) (28, 45, 56). There is phylogenic evidence that all human influenza strains originated from the large virus reservoir indigenous to wild aquatic birds and so there is ongoing global surveillance of influenza in aquatic bird population to characterize the pandemic potential of avian influenza viruses (AIV) (16, 48, 58). It was generally thought that avian influenza viruses were apathogenic in humans, but that notion changed with the 1997 H5N1 and 2003 H7N7 outbreaks of highly pathogenic avian influenza (HPAI) strains that were exceptionally virulent in humans even though they exhibited poor human-to-human transmission (10, 49, 50, 60). Since the 1997 H5 HPAI occurrence, there have been sporadic outbreaks throughout Asia, which have resulted in greater than 330 human deaths and the mass culling of birds (49, 50, 59, 60). It has been hypothesized that HPAI strains are the result of H5 and H7 low pathogenic avian influenza (LPAI) strains that are perpetuated for prolonged periods in poultry, where the accumulation of basic amino acids within the

LPAI HA cleavage site results in HPAI isolates that are susceptible to intracellular cleavage by ubiquitously expressed cellular proteases (9, 20, 23, 24, 46, 47, 55). The polybasic HA cleavage site is a primary determinant for the HPAI phenotype and indicative of HPAI systemic dissemination. Conversely, LPAI strains undergo extracellular cleavage by localized trypsin-like proteases of the gastrointestinal or respiratory tract of birds, restricting their tissue tropism (25, 30). The presence of a polybasic cleavage site has emerged as a primary determinant of AIV virulence (22, 28, 40, 51). Virus receptor specificity and polymerase activity have also emerged as determinants of potential for infection in mammals and humans. All influenza viruses mediate infection through sialic acid receptors on the surface of cells. Avian viruses bind preferentially to α2,3-linked sialic acids of the gastrointestinal and respiratory tract in birds, while human viruses prefer sialic acids with α2,6 linkages located most prominently on non-ciliated cells of the human upper respiratory tract (11, 33, 36, 37, 43). Thus it is thought that the limited expression of appropriate receptors in the upper respiratory tract limits the infection of humans with avian influenza viruses. Similarly, avian viruses have higher optimum temperatures of replication than human viruses, and while the core body temperature of humans is approximately 37°C, the temperature in the upper respiratory tract is cooler, approximately 33°C (32). Permissiveness for replication at lower temperatures has been associated with viral polymerase activity and mutations in the polymerase can influence transmission in mammals (17, 21, 32, 42). While these various features have been associated with infection, transmission, and virulence of AIVs in poultry and mammals, much of this work has been limited to a handful of HPAI and pandemic viruses, and in contrast, little is known about the LPAI viruses circulating the

wild birds. It is essential to understand the potential hazards and risks linked with AIV transmission between the avian reservoir and mammals.

LPAI viruses are prevalent in wild aquatic birds found throughout North America and little is known about the pandemic threat they pose (4, 6, 7, 15, 41, 44, 45). The absence of the polybasic cleavage site in the HA protein generally requires the addition of exogenous trypsin to *in vitro* mammalian cell cultures to serve as a surrogate protease for activation of the HA and enable successful virus replication (30). As such, the replication of influenza viruses in cell culture in the presence and absence of trypsin could serve as a potential indicator of virulence, as well as replicative capacity in mammals (2, 3, 5, 8). A repository of North America LPAI isolates, which included 11 of the 16 HA subtypes collected between 1998 through 2006, provided by the Southeastern Cooperative Wildlife Disease Study, were examined for their ability to replicate independent of trypsin *in vitro*. This is a unique phenotype for LPAI strains, which typically require exogenous trypsin to replicate in cell culture (30). Our lab has demonstrated that several AIV isolates of various subtypes were able to replicate to high viral titers and induce lesions in mammals (i.e. mice) without prior adaptation (13). Moreover, we have shown that some of these LPAI strains infect and in one case transmit in the ferret model, which is considered the best representative model to study human infection with influenza virus. The initial screen of the LPAI strains established that understanding of determinants that influence species and tissue tropism remains largely undefined. The objective of this research is to examine the genetic features that mediate tropism and replication capacity, as well as the host features that may contribute to trypsin independent phenotype and the transmission observed in mammals.

The overall hypothesis of the project is that the hemagglutinin of influenza A virus contains features that affect tissue tropism, species tropism, and transmission. The specific aims of this project are to examine the pandemic potential of North American LPAI isolates that exhibit a trypsin independent phenotype with a complete characterization of viral and host factors that influence receptor specificity, proteolytic activation of HA and fusion.

Specific Aim 1: Confirmation of AIV replication in various cell lines (MDCK, MDBK, DF-1, and Vero cells) in the absence of exogenous trypsin. . The *working hypothesis* is that these LPAI viruses are able to replicate in a variety of cell types in the absence of trypsin, where the combination of general permissiveness of the cells and viral mechanisms influence viral titers.

Specific Aim 2: Determine the receptor specificity of the AIV isolates shown to infect human airway cells, as well as wild bird AIV strains shown to infect and transmit in ferrets. The *working hypothesis* is t*he* LPAI isolates with α2, 3 receptor specificity can infect cells despite a paucity of α2,3-linked sialic acids, suggesting other features contribute to tropism.

Specific Aim 3: Characterize the protease requirements for AIV HA cleavage and fusion. The *working hypothesis* is the presence of neuraminidase enhances HA cleavage in the AIV isolates allowing them to infect a wide array of cell types, thus increasing their

pandemic potential and ability to cause disease in mammals. The alternative hypothesis is viral infection induces expression of cellular protease(s) that cleave viral HA.

Completion of these three aims will provide a better understanding of the genotypes and/or phenotypes associated with avian influenza viruses that infect, replication and transmit in mammalian cell culture, human airway cell cultures, and ferrets, which all serve as models for human infection with influenza viruses.

References:

1. 2009. Update: novel influenza A (H1N1) virus infections - worldwide, May 6, 2009. MMWR Morb Mortal Wkly Rep **58:**453-8.
2. **Bertram, S., I. Glowacka, P. Blazejewska, E. Soilleux, P. Allen, S. Danisch, I. Steffen, S. Y. Choi, Y. Park, H. Schneider, K. Schughart, and S. Pohlmann.** 2010. TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J Virol **84:**10016-25.
3. **Bertram, S., I. Glowacka, I. Steffen, A. Kuhl, and S. Pohlmann.** 2010. Novel insights into proteolytic cleavage of influenza virus hemagglutinin. Rev Med Virol **20:**298-310.
4. **Bokma, B. H., C. Hall, L. M. Siegfried, and J. T. Weaver.** 2006. Surveillance for avian influenza in the United States. Ann N Y Acad Sci **1081:**163-8.
5. **Bottcher, E., C. Freuer, T. Steinmetzer, H. D. Klenk, and W. Garten.** 2009. MDCK cells that express proteases TMPRSS2 and HAT provide a cell system to propagate influenza viruses in the absence of trypsin and to study cleavage of HA and its inhibition. Vaccine **27:**6324-9.
6. **Breban, R., J. M. Drake, D. E. Stallknecht, and P. Rohani.** 2009. The role of environmental transmission in recurrent avian influenza epidemics. PLoS Comput Biol **5:**e1000346.
7. **Brown, J. D., and D. E. Stallknecht.** 2008. Wild bird surveillance for the avian influenza virus. Methods Mol Biol **436:**85-97.
8. **Chaipan, C., D. Kobasa, S. Bertram, I. Glowacka, I. Steffen, T. S. Tsegaye, M. Takeda, T. H. Bugge, S. Kim, Y. Park, A. Marzi, and S. Pohlmann.** 2009. Proteolytic activation of the 1918 influenza virus hemagglutinin. J Virol **83:**3200-11.
9. **Chen, J., K. H. Lee, D. A. Steinhauer, D. J. Stevens, J. J. Skehel, and D. C. Wiley.** 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell **95:**409-17.
10. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472-7.
11. **Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:**17-23.
12. **Cox, N. J., and K. Subbarao.** 2000. Global epidemiology of influenza: past and present. Annu Rev Med **51:**407-21.
13. **Driskell, E. A., C. A. Jones, D. E. Stallknecht, E. W. Howerth, and S. M. Tompkins.** 2010. Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. Virology **399:**280-9.
14. **Dushoff, J., J. B. Plotkin, C. Viboud, D. J. Earn, and L. Simonsen.** 2006. Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. Am J Epidemiol **163:**181-7.

15. **Fuller, T. L., S. S. Saatchi, E. E. Curd, E. Toffelmier, H. A. Thomassen, W. Buermann, D. F. DeSante, M. P. Nott, J. F. Saracco, C. Ralph, J. D. Alexander, J. P. Pollinger, and T. B. Smith.** 2010. Mapping the risk of avian influenza in wild birds in the US. BMC Infect Dis **10:**187.
16. **Gammelin, M., A. Altmuller, U. Reinhardt, J. Mandler, V. R. Harley, P. J. Hudson, W. M. Fitch, and C. Scholtissek.** 1990. Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol **7:**194-200.
17. **Gao, Y., Y. Zhang, K. Shinya, G. Deng, Y. Jiang, Z. Li, Y. Guan, G. Tian, Y. Li, J. Shi, L. Liu, X. Zeng, Z. Bu, X. Xia, Y. Kawaoka, and H. Chen.** 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.
18. **Garten, R. J., C. T. Davis, C. A. Russell, B. Shu, S. Lindstrom, A. Balish, W. M. Sessions, X. Xu, E. Skepner, V. Deyde, M. Okomo-Adhiambo, L. Gubareva, J. Barnes, C. B. Smith, S. L. Emery, M. J. Hillman, P. Rivailler, J. Smagala, M. de Graaf, D. F. Burke, R. A. Fouchier, C. Pappas, C. M. Alpuche-Aranda, H. Lopez-Gatell, H. Olivera, I. Lopez, C. A. Myers, D. Faix, P. J. Blair, C. Yu, K. M. Keene, P. D. Dotson, Jr., D. Boxrud, A. R. Sambol, S. H. Abid, K. St George, T. Bannerman, A. L. Moore, D. J. Stringer, P. Blevins, G. J. Demmler-Harrison, M. Ginsberg, P. Kriner, S. Waterman, S. Smole, H. F. Guevara, E. A. Belongia, P. A. Clark, S. T. Beatrice, R. Donis, J. Katz, L. Finelli, C. B. Bridges, M. Shaw, D. B. Jernigan, T. M. Uyeki, D. J. Smith, A. I. Klimov, and N. J. Cox.** 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **325:**197-201.
19. **Greenbaum, J. A., M. F. Kotturi, Y. Kim, C. Oseroff, K. Vaughan, N. Salimi, R. Vita, J. Ponomarenko, R. H. Scheuermann, A. Sette, and B. Peters.** 2009. Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. Proc Natl Acad Sci U S A **106:**20365-70.
20. **Guo, X. L., L. Li, D. Q. Wei, Y. S. Zhu, and K. C. Chou.** 2008. Cleavage mechanism of the H5N1 hemagglutinin by trypsin and furin. Amino Acids **35:**375-82.
21. **Hatta, M., Y. Hatta, J. H. Kim, S. Watanabe, K. Shinya, T. Nguyen, P. S. Lien, Q. M. Le, and Y. Kawaoka.** 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog **3:**1374-9.
22. **Horimoto, T., and Y. Kawaoka.** 1995. The hemagglutinin cleavability of a virulent avian influenza virus by subtilisin-like endoproteases is influenced by the amino acid immediately downstream of the cleavage site. Virology **210:**466-70.
23. **Horimoto, T., K. Nakayama, S. P. Smeekens, and Y. Kawaoka.** 1994. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol **68:**6074-8.
24. **Ito, T., H. Goto, E. Yamamoto, H. Tanaka, M. Takeuchi, M. Kuwayama, Y. Kawaoka, and K. Otsuki.** 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol **75:**4439-43.

25. **Ito, T., Y. Suzuki, T. Suzuki, A. Takada, T. Horimoto, K. Wells, H. Kida, K. Otsuki, M. Kiso, H. Ishida, and Y. Kawaoka.** 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. J Virol **74:**9300-5.
26. **Itoh, Y., K. Shinya, M. Kiso, T. Watanabe, Y. Sakoda, M. Hatta, Y. Muramoto, D. Tamura, Y. Sakai-Tagawa, T. Noda, S. Sakabe, M. Imai, Y. Hatta, S. Watanabe, C. Li, S. Yamada, K. Fujii, S. Murakami, H. Imai, S. Kakugawa, M. Ito, R. Takano, K. Iwatsuki-Horimoto, M. Shimojima, T. Horimoto, H. Goto, K. Takahashi, A. Makino, H. Ishigaki, M. Nakayama, M. Okamatsu, D. Warshauer, P. A. Shult, R. Saito, H. Suzuki, Y. Furuta, M. Yamashita, K. Mitamura, K. Nakano, M. Nakamura, R. Brockman-Schneider, H. Mitamura, M. Yamazaki, N. Sugaya, M. Suresh, M. Ozawa, G. Neumann, J. Gern, H. Kida, K. Ogasawara, and Y. Kawaoka.** 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature **460:**1021-5.
27. **Johnson, N. P., and J. Mueller.** 2002. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med **76:**105-15.
28. **Kawaoka, Y., T. M. Chambers, W. L. Sladen, and R. G. Webster.** 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology **163:**247-50.
29. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol **63:**4603-8.
30. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426-39.
31. **Laver, W. G., and R. G. Webster.** 1973. Studies on the origin of pandemic influenza. 3. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology **51:**383-91.
32. **Massin, P., S. van der Werf, and N. Naffakh.** 2001. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol **75:**5398-404.
33. **Matrosovich, M. N., A. S. Gambaryan, S. Teneberg, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, and K. A. Karlsson.** 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:**224-34.
34. **Morens, D. M., and J. K. Taubenberger.** 2009. Understanding influenza backward. JAMA **302:**679-80.
35. **Nelson, M. I., C. Viboud, L. Simonsen, R. T. Bennett, S. B. Griesemer, K. St George, J. Taylor, D. J. Spiro, N. A. Sengamalay, E. Ghedin, J. K. Taubenberger, and E. C. Holmes.** 2008. Multiple reassortment events in the evolutionary history of H1N1 influenza A virus since 1918. PLoS Pathog **4:**e1000012.
36. **Rogers, G. N., and B. L. D'Souza.** 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology **173:**317-22.

37. **Rogers, G. N., and J. C. Paulson.** 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:**361-73.
38. **Schafer, J. R., Y. Kawaoka, W. J. Bean, J. Suss, D. Senne, and R. G. Webster.** 1993. Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology **194:**781-8.
39. **Scholtissek, C., W. Rohde, V. Von Hoyningen, and R. Rott.** 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87:**13-20.
40. **Senne, D. A., B. Panigrahy, Y. Kawaoka, J. E. Pearson, J. Suss, M. Lipkind, H. Kida, and R. G. Webster.** 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis **40:**425-37.
41. **Senne, D. A., D. L. Suarez, D. E. Stallnecht, J. C. Pedersen, and B. Panigrahy.** 2006. Ecology and epidemiology of avian influenza in North and South America. Dev Biol (Basel) **124:**37-44.
42. **Shinya, K., S. Hamm, M. Hatta, H. Ito, T. Ito, and Y. Kawaoka.** 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:**258-66.
43. **Shinya, K., and Y. Kawaoka.** 2006. [Influenza virus receptors in the human airway]. Uirusu **56:**85-9.
44. **Stallknecht, D. E., and J. D. Brown.** 2009. Tenacity of avian influenza viruses. Rev Sci Tech **28:**59-67.
45. **Stallknecht, D. E., and S. M. Shane.** 1988. Host range of avian influenza virus in free-living birds. Vet Res Commun **12:**125-41.
46. **Steinhauer, D. A.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.
47. **Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J **11:**2407-14.
48. **Suarez, D. L.** 2000. Evolution of avian influenza viruses. Vet Microbiol **74:**15-27.
49. **Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol **72:**6678-88.
50. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox.** 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science **279:**393-6.
51. **Swayne, D. E.** 2008. Avian influenza, 1st ed. Blackwell Pub., Ames, Iowa.
52. **Taubenberger, J. K.** 2006. The origin and virulence of the 1918 "Spanish" influenza virus. Proc Am Philos Soc **150:**86-112.
53. **Taubenberger, J. K., and D. M. Morens.** 2009. Pandemic influenza--including a risk assessment of H5N1. Rev Sci Tech **28:**187-202.

54. **Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson, and K. Fukuda.** 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA **289:**179-86.
55. **Walker, J. A., S. S. Molloy, G. Thomas, T. Sakaguchi, T. Yoshida, T. M. Chambers, and Y. Kawaoka.** 1994. Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus. J Virol **68:**1213-8.
56. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-79.
57. **Webster, R. G., G. B. Sharp, and E. C. Claas.** 1995. Interspecies transmission of influenza viruses. Am J Respir Crit Care Med **152:**S25-30.
58. **Webster, R. G., K. F. Shortridge, and Y. Kawaoka.** 1997. Influenza: interspecies transmission and emergence of new pandemics. FEMS Immunol Med Microbiol **18:**275-9.
59. **WHO.** 2011. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011.
60. **Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351:**467-71.

CHAPTER 2

LITERATURE REVIEW

Overview of Influenza Viruses

Influenza A Viruses

Influenza viruses belong to the *Orthomyxoviridae* family and consist of three viruses types: influenza A, B and C. Influenza A viruses can infect a wide array of hosts including humans, birds, pigs, horses, dogs, cats, whales, and seals (42, 44, 82, 103, 179, 259). Influenza B viruses have been shown to infect humans and in some cases seals (57, 179, 259). Finally, influenza C viruses primarily infect humans but have been isolated from pigs and dogs (57, 147, 282). Influenza viruses are typically spread by inhalation of virus from coughing, sneezing, or close contact with an infected person or through interactions with fomite contaminated surfaces (5, 10, 13). This review will focus on influenza A viruses, which are comprised of negative-sense, 8 segmented single stranded RNA genomes that encodes up to 11 proteins. The influenza virion is composed of a host derived lipid membrane that houses the structural proteins and the viral genome. The virion morphology varies greatly between clinical and laboratory grown isolates. Viruses propagated in cell culture or eggs exhibit a spherical shape that range from 80 to 120 nm in diameter, where clinical isolates appear filamentous in nature and can be several microns in length (21). The structural surface glycoproteins of the virion are the hemagglutinin (HA), neuraminidase (NA), and matrix ion channel (M2) virus proteins.

The internal structural proteins consist of the matrix protein (M1), nucleoprotein (NP), and the polymerase complex (comprised of the polymerase acid protein (PA), polymerase basic protein 1 (PB1), and the polymerase basic protein 2 (PB2) (57). More recently, the PB1-F2 protein was identified and is encoded from the +1 reading frame of the PB1 protein and is not present in all influenza strains (37). Influenza has two nonstructural proteins: NS1 and NS2/NEP. They are not incorporated into the virion but are abundant in the infected cell (57). Influenza A viruses are subtyped based on their antigenically and genetically diverse hemagglutinin and neuraminidase surface glycoproteins (H1N1), where there are 16 HA and 9 NA subtypes identified in nature and the HA make up 2 distinct phylogenetic groups (group 1 and 2) (57, 248, 270).

Influenza A Replication

The recognition and binding of the viral HA to cellular sialic acid receptor initiates the influenza virus life cycle. This interaction between the virus and host is crucial for influenza pathogenesis, where presence of influenza specific sialic acid receptors at the sites of infection are essential for establishing influenza infections. Sialic acids are 9-carbon sugar moieties that are highly expressed on the surface of most cells. They are penultimate sugars attached through either N- or O-linkages to glycoproteins of host cells and are classified based on α2 linkages to the oligosaccharide backbone. In most cases, influenza viruses recognize N-acetylneuramic acid (Neu5Ac) with α2,3-linkages or α2,6-linakages to galactose, which is commonly referred to as α2,3 Gal–linked and α2,6 Gal–linked sialic acids (172, 247, 262). The α2,3-linked and α2,6-linked N-glycolylneuraminic acid (Neu5Gc) sialic acid moieties are also recognized by influenza but at lesser extent (247). It is the Neu5Ac α2,3 Gal-linked and α2,6 Gal-

linked sialic acids that are one of the primary determinants responsible for the species specific segregation observed between human and avian influenza strains, where avian influenza binds predominantly to α2,3-linked sialic acid receptors found in the gastrointestinal and respiratory tracts of birds, while human influenza strain preferentially recognize α2,6-linked sialic acid receptors of the human upper respiratory tract (9, 40, 45, 87, 105, 153, 193-195).

Once the HA proteins binds a sialic acid receptor, virus entry is achieved through clathrin-mediated, clathrin- and caveolin-independent endocytic pathways (126, 198). Once the virus is endocytosed and the endosome becomes acidified, the M2 ion channel conducts protons across the viral envelope into the interior of the virion, initiating membrane fusion (127, 183, 245). Cleavage of the precursor HA0 polyprotein into the HA1 and HA2 infectious subunits by cellular proteases is required for HA mediated fusion (35, 232). The low endosomal pH results in a conformational change of the HA, which liberates the fusion peptide and coordinates the fusion of the endosomal and viral membranes. Upon fusion, the viral RNA polymerase complex, which includes viral RNA (vRNA) bound to NP and all three polymerase proteins (PB1, PB2 and PA), is released into the cytoplasm and subsequently transported to the nucleus to undergo transcription. All 3 influenza polymerases and the NP contain a nuclear localization signal and undergo active nuclear transport (160, 164, 173). Once in the nucleus, the negative-sense vRNA strand must undergo transcription to generate the two positive-sense RNA species: viral mRNA and complementary RNA (cRNA). Each viral gene segment contains conserved 3' (12 nucleotides) and 5' (13 nucleotides) non-coding regions that are essential for viral mRNA and vRNA synthesis (50, 136, 181, 190, 220).

The 5’ end of the vRNA that is crucial for viral polymerase recognition, while the 3’ end works in combination with the 5’ end in transcription of the viral genome and for some mRNA molecules, it is essential for endonucleolytic processing and establishment of the capped primer (79, 96, 132, 255). The positive-sense viral mRNA acquires a 5’ methylated cap by the “cap snatching” capabilities of the PB2 protein, where the PB2 steals 10 to 12 nucleotides from host cell pre-mRNA and adds it to the 5’ end on the viral mRNA (11, 124, 184). A polyadenylated tail is also required for translation of the viral mRNA and is generated by the stuttering action of viral polymerase complex at the terminal vRNA poly (U) sequence that terminates 15 -17 nucleotides before the end of the vRNA segment (186, 187, 192). In the interim, the other positive-sense RNA, known as complementary RNA (cRNA), is synthesized and is a full length replicate of the viral RNA genome that acts as an intermediate in the generation of negative-sense vRNA that is packaged in progeny virions. The viral mRNA travels from the nucleus to the cytoplasm to undergo translation by host protein synthesis machinery. Newly synthesized NP proteins are transported back into the nucleus to bind vRNA, while the HA, NA and M2 proteins make their way through the endoplasmic reticulum to undergo folding and glycosylation before being trafficked to the plasma membrane via the exocytic pathway (8). Once in the nucleus, the NP undergoes self-oligomerization and encapsulates vRNA to form ribonucleoproteins (RNP), where one NP binds approximately 24 nucleotides of vRNA (117, 177, 200). The newly assembled RNPs utilize CRM1-dependent nuclear export pathways to gain access to the cytoplasm (55, 269). All the synthesized viral proteins and RNP complexes are assembled at the apical plasma membrane, where M1 is thought to be the primary mediator of viral assembly (6,

182). The progeny virions buds off of the host membrane with the assistance of the sialidase activity of the viral NA, which cleaves terminal cell surface sialic acids preventing HA mediated self- binding, allowing virion release (139, 165, 188, 208).

Ecology of Avian Influenza A Viruses

Overview of Avian Influenza Viruses

Even though influenza viruses are able to infect a wide array of species, they are endemic in wild aquatic birds. Avian influenza viruses (AIV) have been isolated from an array of bird species and were first identified in the early 1900's, where it was originally called fowl plaque virus (FPV). It was not until 1950's that FPV was discovered to actually be an influenza virus by Schafer et al. (248). AIV strains have been extensively studied and well characterized. Every human influenza pandemic strain has contained avian derived genetic features. Similar to human influenza classifications, AIV are subtyped based on their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, where all 16 HA and 9 NA subtypes have been identified in circulating wild aquatic bird populations (58, 270).

Avian influenza viruses are further classified as high pathogenic (HPAI) or low pathogenic (LPAI) viruses based on select criteria observed in domestic poultry. The HA polybasic cleavage site is considered a primary pathogenicity determinant, where a monobasic HA cleavage site is observed in LPAI viruses (111, 115, 206). While, a high pathogenic (HPAI) designation is any AIV of H5 or H7 subtype with a polybasic HA cleavage site that exhibits a >75% mortality rate in 6-8 week old chickens injected intravenously with influenza virus (232, 236, 240, 248, 263). If an AIV isolate exhibits

>75% mortality and is not an H5 or H7 subtype, the HA proteolytic cleavage site must be sequenced and compared against other HPAI cleavage sites. The presence of a polybasic HA cleavage site, even in LPAI strains, increases cellular protease recognition. Current theories suggest the high pathogenic H5 and H7 strains originated from low pathogenic H5 and H7 strains that circulated in poultry flocks for prolonged periods of time. There are often mutations or recombination events that result in the accumulation of basic amino acids within the HA cleavage site making it more accessible to a wider array of cellular proteases (46, 99, 102, 113, 141, 207, 240, 241). This simple insertion at the HA cleavage site can enhance the virulence of a virus, potential of transforming a LAPI into a HPAI strain. An additional observation, but not an established requirement for HPAI classification, is that a majority of the HPAI strains exhibit the ability to grow in tissue culture in the absence of exogenous trypsin (15, 20, 120). All viruses that do not fulfill the HPAI criteria are characterized as LPAI viruses (248).

Influenza viruses are perpetuated in aquatic bird populations, where greater than 100 species in 12 avian orders are represented (88, 89, 91, 176, 229, 270). Avian influenza viruses are typically an asymptomatic infection of the gastrointestinal tract that is spread by fecal oral route (90, 230). The two natural reservoirs of AIV are members of the Anseriformes and Charadriiformes orders of birds (4, 22, 248). The Anseriformes include geese, swans, and ducks, while shorebirds and gulls are members of the Charadriiformes (22, 81, 248, 270). Surveillance of wild bird populations in North America reveal distinct HA and NA subtypes isolated from ducks as compared to those isolated from shorebirds and gulls. The H3, H4, and H6 subtypes are most commonly found in ducks, while H5, H7, and H9 subtypes are prevalent in gulls and shorebirds (88,

123). The predominant NA subtypes found in ducks are primarily N2, N6, and N8 subtypes, while the N6 and N9 neuraminidases are the principle subtypes isolated from gulls and shorebirds (270).

Wild aquatic birds are not the only birds susceptible to AIV infections. It is common for domestic birds, such as chickens, turkeys, and even sparrows, to become infected with AIV as a result of direct or indirect contact with infected wild birds (171, 185, 222, 239, 242, 277). Domestic birds can come in contact with infected birds during their migration to or from breeding grounds (90, 123, 229, 230, 270). AIV can also be spread through shared water sources, where the virus can persist for >200 days even at low temperatures (<17^{o}C) (90, 228, 230). The fact that AIV can persist in natural water sources means that as birds migrate, susceptible birds can be infected by AIV contaminated water. Furthermore, it is possible to spread to domestic bird flocks by shared AIV infected drinking water from lakes or rivers (90, 228, 230). It is the constant presence of AIV in wild aquatic birds that perpetuates the seasonal infection of susceptible juvenile birds, where in the spring the infection of newly hatched birds provides a means for maintaining AIV in nature.

Influenza A Viruses Impact on Humans

History of Influenza Pandemics

Within the last century, there have been four major pandemics responsible for >50 million deaths worldwide. These pandemics generally resulted from reassortment events between influenza genomes across different species and subtypes. The first recorded influenza pandemic was the 1918 (H1N1) “Spanish flu” that killed upwards of

40 million people worldwide. Phylogenetic analysis of the 1918 virus revealed that the genetic makeup was predominately of avian origin (189, 254). It remained the primary virus in circulation until the emergence of an H2N2 subtype in 1957. The 1957 (H2N2) "Asia influenza" was responsible for greater than 1 million deaths worldwide (57). The HA, NA, and PB1 genes of the 1957 H2N2 strain were derived from avian strains, with the remaining segments originating from the circulating human strain (112, 202). The next pandemic occurred in 1968, with the emergence of an H3N2 that replaced the circulating H2N2, the "Hong Kong Influenza." The genetic composition of the 1968 H3N2 strain exchanged only 2 segments, containing avian HA and PB1 genes of Eurasian avian lineages (57, 112, 248). In 1977, an H1N1 strain reemerged, the "Russian flu," which was genetically similar to an H1N1 subtype found in circulation from the 1950's. Before the 1970's, one subtype at a time was found in circulation and the emergence of a new subtype displaced the former circulating strain. However, this was not the case with the 1977 H1N1. After its emergence in 1977, it co-circulated with the 1968 H3N2 until a novel H1N1 surfaced in 2009. The novel 2009 H1N1 was the first pandemic of the 21st century and contained a unique combination of genetic segments that had not previously been observed in human or swine influenza viruses. The 2009 H1N1 M and NA gene segments were derived from a Eurasian influenza lineage that emerged in swine in 1979 and appeared to be of avian origin (66). While, the HA, NS, and NP gene segments were derived from the classical swine H1N1 lineage (66). The PB1 was genetically similar to the PB1 of the 1957 and 1968 influenza pandemic strain and appeared to be derived from avian origin (66). Finally, the 2009 H1N1 PA and PB2 gene segments originated from the 1998 swine triple reassortment H1N1 between the

classical H1N1, human H3N2, and America lineage avian strains (14, 66, 211). The first cases appeared in Mexico in early 2009 and were soon found circulating in swineherds. Much of the research done preceding the 2009 H1N1 pandemic concentrated on aquatic birds as the next pandemic source and it was a shock with the emergence of the swine origin H1N1 pandemic strain. It is crucial for scientists to monitor every feasible influenza reservoir as a potential source for the next pandemic strain. If an antigenically novel virus is able traverse species-specific barriers and transmit within an immunologlically naïve human population, the results could be a pandemic of catastrophic proportions.

Avian High Pathogenic Viruses – Brief History of Avian Influenza Viruses

In 1996, there was an outbreak of a highly pathogenic H5N1 in humans in Hong Kong. The virus appeared to be entirely of avian origin and was able to infect humans even though it exhibited poor human-to-human transmission (1, 159, 240, 244, 283). The H5N1 was extremely virulent causing systemic infections, resulting in virus isolated not only from the respiratory tract but also from other various organs (i.e. GI tract and cerebrospinal fluid) (47, 64). The outbreak was linked to humans in close contact with infected birds from live bird markets and as a result Hong Kong officials ordered a mass culling of all birds from contaminated markets (240). Since 2003, there have been a number of severe outbreaks of H5N1 throughout Asian countries, which demonstrated the pandemic threat HPAI strains still pose. According to the WHO 2011 report, there have been a total of 566 cases and 332 deaths linked to H5N1 viruses since 2003 (275).

The H5N1 strains are not the only HPAI subtypes found to infect humans. There have also been sporadic cases of H7N7, H7N3, and H9N2 AIV infections in humans that resulted from close contact with infected birds (29, 59, 93, 162, 205). The H7 subtypes, along with H5 strains, are currently the only subtypes with high pathogenic designation. The H7 HPAI viruses can manifest a range of conjunctivitis related illnesses. In the LPAI H7 strains, mild cases of conjunctivitis have been reported, while the HPAI cases can be fatal (59, 85). There is no definitive number of reported H7 cases because most cases are clinically mild symptoms and are often misdiagnosed.

Determinants of Pathogenicity for Avian Influenza

Antigenic Variations on Avian Influenza A Viruses

It is hypothesized that influenza viruses originated in waterfowl because AIV viruses are so well adapted and are generally asymptomatic in these hosts. The HA and NA segments isolated from wild birds are highly conserved and exhibit low mutation rates when maintained in the avian reservoir. They are optimally adapted to their avian host and undergo minimal evolution, thus they are considered to be at evolutionary stasis (57, 70, 72, 270). Once influenza infects a new host, it undergoes a rapid rate of evolution becoming more adapted to the new host with each round of replication and less likely to replicate in its original host (24, 28, 71, 238, 270). The high mutation rate observed for the HA and NA segments is the result of constant selective immune pressure from the new host, such as neutralizing antibodies that primarily target the HA and to a lesser degree the NA antigenic sites. For example, the human H3 subtype has five antigenic sites on the HA1, around the globular head, that are recognized by host

neutralizing antibodies resulting in inhibition of virus binding to cellular receptors (45, 129, 219). It is these neutralizing antibodies that provide protection in the host and are elicited upon administration of the influenza vaccine (130, 271, 272). The selective immune pressure drives the virus to produce genetically diverse virus populations through point mutations that can result in immunologically unique HA and NA antigenic sites. This phenomenon is referred to as antigenic drift and is the result of point mutations that accumulate over time, most commonly in the HA and NA antigenic sites, allowing the virus to evade the host's immune system (57). It is because of antigenic drift that the influenza vaccine is evaluated yearly to analyze its efficacy against circulating influenza strains.

The mutation rate for HA proteins is approximately 6.7×10^{-3} substitutions per nucleotide per year, while NA proteins exhibit on average 3.2×10^{-3} substitutions per nucleotide per year (180, 223, 224, 278). The genetic diversity observed is generated as a result of the low-fidelity, error-prone RNA dependent RNA polymerase (RdRp) that produces replication errors in $1/10^4$ bases per replication cycle, resulting in a dynamic heterogenous viral population (i.e. quasispecies) (52). The influenza RdRp lacks the proofreading capabilities generating upwards of 10^9 nucleotide errors per round of replication (180, 233). AIV and human strains share the trait of RdRp mediated genetic diversity and is one of the main contributing factors associated with antigenic drift.

In addition to antigenic drift, influenza viruses further facilitate genetic diversity through a process called antigenic shift. The segmented nature of the influenza genome lends itself to antigenic shift. This is achieved when two different subtypes (i.e. H1N1 and H7N9) infect the same cell simultaneously and exchange segments resulting in a

novel subtype strain (e.g. H7N1). There is evidence of antigenic shift in the phylogenetic analysis of each pandemic strain, where each strain contained unique combinations of swine, avian, and/or human viral segments. Swine have been regarded as the "mixing vessels" for influenza viruses, because they express both α2,3-linked and α2,6-linked sialic acid receptors that can be utilized by both avian and human influenza strains to augment the potential of antigenic shift (101, 201, 246). However, more recently, there is evidence that contradicts the swine "mixing vessel" paradigm. Trebbien et al. demonstrated the distribution of α2,6-linked and α2,3-linked sialic acids in the respiratory tract of swine was more similar to that in humans than previously thought, where the α2,6-linked sialic acids were the predominant receptor present throughout the swine respiratory tract, with reduced expression of α2,3-linked moieties in the lower respiratory tract (256). It appears that the potential role swine play in antigenic shift and zoonotic transmission of influenza viruses remains unclear. However, one of the most current examples of swine's potential role in antigenic shift was the emergence of the 2009 H1N1 that exhibited a novel combination of American and Eurasian swine lineages that was readily transmitted to and within the human population without adaptation (66).

Molecular Pathogenicity Determinants of Avian Influenza A Viruses

The HA protein is a primary virulence determinant of influenza viruses because cleavage HA is required for viral entry and thus impacts the overall infectivity of the virus. The hemagglutinin is a homotrimeric type I glycoprotein that is responsible for receptor binding and fusion. There are approximately 400 – 500 HA glycoproteins on the surface of spherical virions (197). The HA protein is translated as a polyprotein (HA0)

and must undergo cleavage by cellular proteases, generating HA1 and HA2 infectious subunits connected by a single disulfide bond (35, 276). Upon cleavage, the HA1 mediates receptor binding, where specific HA1 domains are known to interact with sialic acid receptors. The HA1 190 helix (190 and 194 residues) and 130 loop (residues 135, 136, and 137) are involved in sialic acid recognition, while the 220 loop, residues 226 and 228, help define receptor specificity for various influenza strains and subtypes (40, 45, 150, 163, 195, 276). The fusion peptide is located at the N-terminus of the HA2 subunit and liberated upon cleavage by host proteases within the acidified endosome. It is a class I viral fusion protein made up of 23 residues and is highly conserved among all 16 influenza subtypes (83, 174). Any mutations to the fusion peptide or changes to its length that eliminates its fusogenic function could be detrimental to the virus' survival (43, 83, 128, 218). Proteolytic cleavage alone is not sufficient to facilitate viral fusion but rather acid induced irreversible conformational changes to HA2 subunit is critical for fusion, where an endosomal pH range of pH 5.0 to 6.0 must be achieved in order for the fusion peptide to be liberated in order to mediate fusion between the viral and endosomal membranes (23, 217, 274). Once fusion occurs, the virus releases its genetic payload into the host's cytoplasm, where it is then transported to the nucleus to undergo viral synthesis.

There have been many investigations examining HA structure and function that have helped to define the role of HA in viral infection. Some modifications to the HA receptor binding site have been shown to influence receptor specificity. The HA receptor binding sites of AIV strains contain conserved avian genotype of amino acids (A138, E190, G225, Q226, and G228) (69, 150, 258). Vines et al. showed that mutations L226Q

and S228G within HA from the human H3 subtype influenza strain changed the receptor specificity to recognize avian α2,3-linked sialic acid recognition (264). Furthermore, Tumpey et al. demonstrated that two amino acid changes (D190E and D225G in H3 numbering) in the 1918 (H1N1) HA eliminated viral respiratory droplet transmission in the ferret model and altering receptor specificity from human α2,6-linked to avian α2,3-linked sialic acid receptors (258). Receptor specificity of the virus may also be altered through mutations that modify glycosylation sites near the HA receptor binding site, where elimination of a glycosylation site increases the affinity for a specific receptor. For example, Yen et al. demonstrated that the elimination of a glycosylation site (residue 158) paired with a mutation (S227N) increased the affinity of an H5N1 virus for the human α2,6-linked sialic acid receptor resulting in reduced systemic infections in mice but not in chickens (280). Any mutations that alter the receptor specificity of an AIV could provide a mechanism by which an AIV strain would be better adapted to cross species specific barriers.

The HA cleavage site is an essential factor involved in determining viral pathogenicity. LPAI isolates typically express monobasic cleavage sites, while HPAI strains typically contain polybasic HA cleavage sites (111, 115, 265). These differences, along with the distribution of cellular proteases capable of proteolytic activation of HA, separate the high pathogenic strains from low pathogenic strains. Any insertions or duplications of basic amino acids at the HA cleavage site could potentially transform a LPAI strain into a HPAI strain, allowing its HA to be activated by a greater number of ubiquitously expressed proteases, resulting in systemic dissemination. Ito et al. demonstrated that consecutive passaging of avirulent avian H5N3 resulted in the

production of a virulent strain and sequencing revealed the insertion of basic amino acids within the HA cleave site polybasic (102). Furthermore, the presence or absence of glycosylation near the HA cleavage site can influence pathogenicity. Specifically, a loss of glycosylation at position 22 of the HA1 subunit of an avian H5N2 enhanced its virulence by making the HA cleavage site more accessible to cellular proteases (49, 114).

The viral neuraminidase, along with hemagglutinin, is another well-established molecular pathogenicity determinant. The influenza neuraminidase is a type II membrane glycoprotein that acts as a sialidase responsible for removing terminal sialic acids from host cell surfaces and eliminating HA mediated sialic acid binding during virion release. There are approximately 100 NA glycoproteins on the virion surface of spherical laboratory strains (197). Mutations within the NA protein have been shown to influence pathogenicity. Wild bird AIV isolates from chickens have shown NA stalk deletions (19-20 amino acids of N1) that resulted in enhanced virulence in domestic flocks (31, 151, 161). Deletions of the NA stalk have been attributed to attenuation in viral replication. It has been proposed that the decrease in sialidase activity observed in these NA mutant viruses is apart of compensation mechanisms by which HA compensates for the NA defect through the introduction of mutations in its receptor binding site reducing its affinity for sialic acid receptors (31, 157). As observed in HA proteins, the loss of a NA glycosylation site can influence influenza pathogenicity. For example, Li et al. demonstrated the absence of a glycosylation site near the globular head (residue 146 in N2 numbering) attributed to the neurovirulence observed in A/WSN/33 virus in mice (135). It has been proposed that the loss of glycosylation enhances the sialidase activity of NA, allowing the virus to spread more efficiently throughout the

host. It is the neuraminidase N146R mutation present in the A/WSN/33 and not in its parental strain WS/33 that is responsible for the loss of NA glycosylation and confers plasminogen binding facilitating HA activation and infectivity in the absence of trypsin (73, 74, 253).

Other NA roles have been elucidated that could potentially augment virus pathogenicity. Influenza NA might play a part in the initial stages of virus infections. More recently, it has been shown that NA expression directly enhances HA fusion and virus entry when compared to a mutant, non-functional NA (237). Furthermore, NA activity has also been shown to facilitate virus spread in the upper respiratory tract by preventing viral inactivation by respiratory mucins (154). Also, NA from an avian H11N9 (A/Tern/Australia/G70C/75) exhibited hemagglutinin-like activity, where purified NA particles are able to agglutinate erythrocytes (131). The role of influenza NA in the viral life cycle is much more complex than originally proposed.

The influenza polymerase is a heterotrimeric complex made up of the PA, PB1 and PB2 viral proteins and each protein in the complex works together to form the RNA-dependent RNA polymerase (RdRp) that is responsible for the transcription of the viral mRNA and complementary RNA (cRNA) (16, 17, 216). Any mutation that enhances the viral polymerase could impact host range and pathogenicity. There are several cases where mutations in key amino acids of PB2 have been shown to enhance virulence in various species. A single mutation (E627K) in PB2 protein has been linked to enhanced pathogenicity and transmission of AIV strains in mammals (213). Sequence analysis of avian strains exhibits a glutamic acid (E) at position 627 of PB2, whereas all human strains express at lysine (K) at that position (243). Hoeven et al. have demonstrated that

1918 HA and PB2 proteins, which include lysine (K) at residue 627, allowed for airborne transmission of avian influenza in ferrets at 33°C, where most avian strains require higher temperatures (40 - 41°C) for replication (260). The PB2 627K was also shown to contribute to the enhanced virulence observed in HPAI Southeast Asian H5N1 strains in ferrets and mice (77, 84, 199, 213). While a change at position 701 of PB2, from aspartic acid (D) to asparagine (N), has been shown to increase the virulence of H5N1 in mice and guinea pigs (137, 231). Additional residues have been implicated in enhancing the viral polymerase activity and pathogenicity in mice, such as PB2 714R (arginine), PA 615R, NP 319K (lysine), and PB1-F2 66S (serine) (39, 61).

The nonstructural protein 1 (NS1) is a major component that contributes to viral pathogenicity, but through a different mechanism than the above mentioned viral proteins. NS1 works as a host type 1 interferon (IFN) antagonist, thus acting as a pathogenicity determinant by suppressing the host's antiviral immune response and facilitating viral replication and spread. It has been shown to contribute to the enhanced virulence observed in the HPAI H5N1 strains and the 1918 virus in humans (2, 12, 54, 65, 121, 122, 138, 175). The NS1 protein is made up of two distinct domains, an N-terminal RNA-binding (RBD) domain and a C-terminal effector domain (ED) (18, 140, 267). Each domain allows the NS1 protein to participate in protein-RNA and protein-protein interactions. The NS1 RBD binds double stranded RNA (dsRNA) and inhibits its entry into the antiviral oligo A synthetase/RNase L pathways that normally activate the host type I IFN response. Thus, the sequestering of dsRNA by NS1 reduces activation of INF-α/β induction pathways (38, 134, 144, 156, 166, 251, 252, 268). The NS1 ED has been shown to bind the cellular protein kinase R (PKR) preventing it from shutting down

cellular protein synthesis (144, 252). Another region of the NS1 ED has been shown to interact with cellular protein, CPSF30, whose function is to process the 3'end of pre-mRNA. The binding of NS1 to CPSF30 prevents processing of the IFN-β pre-mRNA into mature mRNA, thus interfering with the IFNα/β antiviral immune response (125, 166). Influenza virus pathogenicity is multi-factorial where no one viral determinant can account for the pathogenicity and transmission differences observed in avian and human strains.

Overview of Host Range and Restrictions

The transmission of AIV strains to humans is a complex network of interactions. The ability of influenza to establish an infection in a new host is largely determined through the interplay of host specific barriers and viral adaptation. There is not a defined list of pathogenicity markers for HPAI viruses that can be attributed to AIV transmission in mammals. There are several host specific determinants, such as host receptor distribution, optimal replication temperatures and cellular proteases, have been identified and possess a role in AIV virulence and pathogenicity.

As the previous discussion has illustrated, one of the primary host specific determinants is the presence of influenza sialic acid receptors on host cells and tissues. Avian and human influenza strains preferentially bind to two different 5-N-acetylneuraminic acid sialic acid receptors. AIV primarily binds to cells that contain α2,3 Gal-linked sialic acid receptors, while human influenza strains recognize α2,6 Gal-linked sialic acid receptors (9, 62, 63, 152, 193-195, 247). There is also a distinct difference between the types of cells infected by avian and human influenza strains, with

avian strains infecting predominantly ciliated cells and human strains infecting non-ciliated cells (153). The expression of the sialic acids varies between cells and tissues within the host can dictate the overall fitness and adaptation of the virus. Much work has been done to identify the sialic acid expression in various hosts and lectins have become an essential tool for examining sialic acid distribution in cells and tissues. They are naturally derived glycoproteins that bind to distinct cellular sialic acid oligosaccharides (143, 209). Two lectins, *Macckia amuresis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA), have been fundamental in identifying tissue specific expression of α2,3 Gal-linked and α2,6 Gal-linked sialic acids receptors, respectively (101, 109). In wild aquatic birds, AIV is predominately an asymptomatic gastrointestinal intestinal infection that spreads among birds via fecal-oral route. The avian gastrointestinal epithelial cells express predominately α2,3 Gal-linked sialic acids with only minute levels of α2,6 Gal-linked sialic acids moieties (101, 105). It has been shown that human upper respiratory tract epithelial cells express mostly α2,6 Gal-linked sialic acids moieties, while the lower respiratory tract epithelial cells express both of α2,3- and of α2,6 Gal-linked sialic acids (9, 41, 62, 153, 212). It is the sialic acid expression profiles and receptor distribution on host tissues and cells that dictates the ability of the virus to establish infections in potential hosts. It is the sialic acid species-specific restrictions that must be overcome for interspecies transmission of AIV strains.

Another host range restriction that reduces the ability of AIV to establish infections in a new host, specifically in humans, is temperature at the site of replication in susceptible tissues. AIV strains are adapted to replicate in the gastrointestinal tract of wild aquatic birds at 40 -41°C and are attenuated at lower temperatures (149, 204). The

work by Scull et al. demonstrated that the lower temperature of the human proximal airways (32°C) restricts AIV replication and they demonstrated that the avian specific hemagglutinin and neuraminidase glycoproteins were responsible for temperature restricted replication (204). It seems that AIV strains have evolved to replicate efficiently at 40 -41°C and it is this restriction that sets up another host barrier to reduce zoonotic transmission. If AIV strains became more adapted to replicate over a broader range of temperatures, this may increase its likelihood of infecting a wide array of species.

The distribution of cellular proteases and the structure of the HA cleavage sites influence the pathogenicity of AIV. In order for the virus to become infectious, HA cleavage by cellular proteases is a required process for the generation the infectious HA1 (328 residues) and HA2 (221 residues) subunits. The HA cleavage site and host protease distribution are essential factors for identifying pathogenicity in influenza. As discussed previously, LPAI strains usually contain a monobasic cleavage site, containing either a lysine (K) or in most cases an arginine (R), and are cleaved by localized, trypsin-like tissue specific proteases (i.e. plasmin, tryptase Clara, and TMPRSS2, HAT serine protease) (116, 118, 120). It is the limited tissue expression and distribution of these specific proteases that dictates proteolytic activation of HA and viral replication. Most LAPI and human influenza strains undergo extracellular HA cleavage but this is not the case for HPAI strains. Most of the tissues within the bird do not support viral replication but it is expression of localized trypsin-like proteases in the gastrointestinal and respiratory tracts that are capable of HA activation and viral replication in these tissues for LPAI strains. However for human influenza strains, it is the expression of trypsin-like proteases, such as tryptase Clara, HAT and TMPRSS family, in the respiratory tract

that are responsible for HA cleavage and infectivity remains largely undefined (116, 118, 120).

HPAI strains contain a polybasic cleavage site in HA with a series of basic amino acids prior to the final arginine of the HA1/HA2 cleavage site that serve to lengthen the cleavage site, making it more accessible to vast array of cellular proteases (19, 35, 36, 67, 118, 158, 273). Currently, only the H5 and H7 avian strains, with a polybasic cleavage site, are the only subtypes classified as HPAI strains. The consensus HA cleavage site for the H5 and H7 subtypes contains a derivative of the R-X-R/K-R sequence, that is generated by insertions or duplications at the HA cleavage site mediated by the error-prone viral polymerase (236, 263). One of the discerning features of these avian strains is they are capable of systemic viral spread, where proteolytic activation of HA achieved by a number of ubiquitously expressed proteases (19, 35, 36, 67, 118, 119, 158, 273). Typically, HPAI HA activation occurs intracellularly by ubiquitously expressed subtilisin-like proteases (i.e. furin and PC 5/6) within the trans-Golgi making the virion infectious upon virion release, thus broadening their tissue tropism and resulting in systemic dissemination (78, 98, 232, 236, 266, 284). As mention previously, the presence of absence of glycosylation in close proximity to the HA cleavage site can influence pathogenicity by making it more or less accessible to cellular proteases. Any changes that alter the HA cleavage site (mutations, basic amino acid insertions, and glycosylation modifications) can potentially broaden the tropism of the virus, making it more accessible to a wider array of cellular proteases.

Research Approaches For Examining Influenza Viruses

The propagation of influenza viruses typically employees the use of embryonated chicken eggs and/or cell culture. The use of eggs for studying influenza viruses has been a common laboratory practice since they were first recognized for effective virus propagation by Burnett et al. in the 1940's (25-27, 53, 60). Most commonly, viral inoculation of the allantoic compartment provides a sterile environment for virus growth and typically yields high viral titers making eggs the main source for the production of seasonal vaccines. One egg can produce 1 to 2 influenza vaccine doses (57). Viruses isolated from embryonated chicken eggs are considered infectious because of the prothrombin-like enzyme, similar to blood clotting factor x, provides proteolytic activation of HA during virus propagation (75). Limited passages in embryonated eggs are suggested for influenza viruses because the viruses become more adapted to growth in eggs and less like original virus (27). Egg adaptation of influenza viruses has been shown to alter receptor specificity and interfere with the agglutination of various species' erythrocytes (191).

The use of cell culture for the growth and characterization of influenza viruses is common practice. Immortalized cell lines, such as chicken embryo (DF-1), carcinoma human alveolar basal epithelial (A549), Madin-Darby canine kidney (MDCK), and Madin-Darby bovine kidney (MDBK), have been employed for *in vitro* studies of influenza viruses. Cell types, such as MDCK and MDBK, have become the gold standard for influenza research because of they are generally permissive to infection with most influenza viruses found in nature due to cell surface expression of both α2,3 Gal-linked and α2,6 Gal-linked sialic acid receptors (148). The MDCK and MDBK cell lines

are not only important in virus propagation but are also crucial for basic virology diagnostics of influenza strains (i.e. plaque assays and $TCID_{50}$) (68, 203). Most influenza viruses require the addition of exogenous trypsin to propagate in cell culture, but recently it has been shown that MDCK cells can be used to examine viruses that can replicate in a trypsin independent manner. These cells transiently express TMPRSS2 (transmembrane protease, serine S1 family member 2) and HAT (human airway trypsin-like proteases) and were able to facilitate HA activation and multiple rounds of replication in the absence of exogenous trypsin (20). The BHK-21 cell line has shown to be useful in the propagation of receptor-binding mutants that are unable to replicate in MDCK or MDBK cell lines (76). One of the favorable aspects influenza viruses grown in cell culture, they do not appear to undergo the adaptations observed in egg passaged viruses, such as altered receptor and erythrocyte binding preferences (106-108, 279).

Furthermore, the use of primary cell lines, such as normal human bronchial epithelial (NHBE) and human ciliated airway epithelial (HAE), are becoming more appealing because of their ability to fully differentiate into the epithelial cells as seen in the human airway. Fully differentiated NHBE, which contain polarized ciliated epithelium and mucus secreting goblet cells and Clara cells (48, 80). The use of primary cell lines is becoming the prefer method for evaluating respiratory illnesses, like AIV because they closely mimic what occurs in the human respiratory tract during infections (210). They have become particularly useful in examining influenza receptor specificity (7, 204). NHBE cells express varying levels of α2,6-linked and α2,3-linked sialic acids, where α2,6-linked sialic acids are more abundant than the α2,3-linked sialic acids (33, 178). Oshansky et al. demonstrated that LPAI isolates where able to infect and replicate

in NHBE despite a paucity of α2,3-linked sialic acids (178). Furthermore, NHBE cells can recapitulate human pathophysiology that occurs during virus infections, where cytokine, gene and protein expression levels can be analyzed in virus infected cells (3, 33, 51, 142). For example, Chan et al. demonstrated that H5N1 and H1N1 strains were able to infect and replicate in NHBE cells, where the state of differentiation influenced cytokine and chemokine levels (33). They have also become particularly beneficial in evaluating antiviral and drug efficacy during virus infection (32, 221, 257).

The use of erythrocytes to characterize influenza viruses was pioneered by Hirst et al. in the 1940's and is now a universal method for examining influenza receptor specificity (92). Many types of erythrocytes have been used to examine the agglutination properties of influenza viruses, such as chicken, turkey, mouse, rat, frog, human, guinea pig, cow, pig, and horse erythrocytes (104, 155, 194, 196). Human influenza viruses agglutinate chicken, human and guinea pig but not equine or cow erythrocytes, while avian strains generally agglutinate erythrocytes from all species (104, 155, 234). Two lectins, *Macckia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) have been fundamental in determining the sialic acid expression of erythrocytes (104, 234). Equine and cow erythrocytes express predominantly α2,3-linked sialic acids, while the remaining erythrocytes appear to express a combination of cell surface expressed α2,3- and α2,6-linked sialic acid moieties (104, 155, 234). Equine have been extremely useful for verifying the α2,3-linked sialic acid recognition of AIV strains. Erythrocytes can be modified to express a single type of sialic acid linkage through the use of neuraminidases (e.g. *Vibrio cholera* sialidase). The erythrocytes can be stripped of their sialyliogosaccharides and specific sialic acid linkages can then be added back by α2,3

and α2,6 designated sialyltransferases, generating erythrocytes that express predominantly α2,3- or α2,6-linked sialic acids (30, 194). These modified erythrocytes have been quite useful in examining sialic acid preference receptor of influenza viruses.

Another useful tool for understanding and characterizing the receptor specificity of influenza viruses is the glycan array. The advances made in glycan biology allow hundreds of glycan motifs (typically present on O- and N- linked glycoproteins) to be screened at one time, where the glycans are synthesized to mimic the carbohydrates present on tissues and cells. Virus receptor-mediated interactions with various linkages and complex carbohydrate modifications (e.g. fucosylation, sulfation, and sialylation) can be examined (172, 212, 214, 261, 262). Glycan length and branching have been shown to influence the HA recognition, where Chandrasekaran et al. illustrated that longer and branched α2,6 glycans, similar to those expressed on the apical side of upper respiratory epithelia, are a better approach for examining HA receptor specificity, instead of focusing primarily on α2,3 and α2,6 linkages (34). They also concluded that using higher concentrations of HA on the array can provide invalid receptor specificity designations and dose-dependent analysis needs to be determined to obtain accurate glycan binding affinities. Glycan arrays are extremely sensitive and allow for analysis of important HA residues that influence alter receptor specificity. For example, Stevens et al. evaluated two 1918 HA variants by glycan array and showed that the A/South Carolina/1/1918 HA preferentially bound α2,6-linked glycans, while the A/New York/1/1918 exhibited mixed α2,3 and α2,6, glycan recognition with a preference for sulfated oligosaccharides (235). The only difference between the two was residue 225, where the New York strain contained a glycine (G) rather than an aspartic acid (D) that was present in the North

Carolina strain. Additionally, a glutamic acid (E) at position 190 of the New York variant was sufficient to revert the receptor specificity to that recognized by AIV strains (235). The use of glycan arrays has allowed for a better understanding of the HA-sialic acid interactions, especially in uncharacterized and recombinant influenza strains.

Glycan arrays provide a more in depth examination of virus mediated binding properties than other binding assays, such as erythrocyte binding assays. Whereas erythrocyte binding assays are limited in the ability to identify only sialic acid linkages involved in agglutination and do not provide a true representation of receptors utilized by the virus to establish infection because the sialic acids on the surface of erythrocytes may not be the same type represented on cells at the site of infection (34). Conversely, glycan arrays are able to classify the complex carbohydrate structure of the HA-glycan interactions. Currently most glycan arrays contain synthesized glycan motifs, but there is a movement towards developing natural arrays by harvesting glycans from the surface of cells and assembling them on arrays (215, 225-227). The use of glycan arrays is a more precise approach and allows for definitive characterization of the receptor specificity of influenza viruses.

The use of animal models has become a vital tool for studying the virulence of influenza infection and understanding the molecular determinants shown to influence pathogenicity and zoonotic transmissibility of influenza strains, especially those that exhibit an extremely virulent phenotype. The ferret animal model system has become an indispensable approach examining the virus host interactions during influenza infections. They are highly susceptible to human and avian strains of influenza without prior virus adaptation. Instead of intranasal administration of viruses, which was the traditional

inoculation approach, the use of aerosol delivery systems is becoming the preferred virus inoculation method because it emulates what occurs during a natural influenza infection. Ferrets are considered an attractive animal model because they show similar clinical features present during influenza infections in humans, where they exhibit fevers, nasal discharge, sneezing, and lethargy (86, 146, 249, 285). Furthermore, the sialic acid distribution within the ferret respiratory tract is similar to that in humans. The upper respiratory expresses predominantly α2,6-linked sialic acid receptor, while the lower respiratory exhibits both α2,6-linked and α2,3-linked sialic moieties (100, 133, 250). They allow for examining the roles of viral gene segments and mutations know to influence the virulence and transmissibility of viruses (199, 285). Additionally, they have become critical in influenza transmission studies, where infected ferrets are able to transmit by direct and indirect contact to naïve ferrets (86, 146, 258, 260, 281). For example, Tumpey et al demonstrated that a two amino acid change (D190E and D225G) the 1918 HA glycoprotein switched the receptor specificity from α2,6-linked to avian α2,3-linked sialic acid receptor preference and rendered the virus incapable of aerosol transmission in the ferret model (258). Furthermore, Hoeven et al. demonstrated that the combination of 1918 HA, NA and PB2 proteins were required for airborne transmission in ferrets (260). The ferret model makes an ideal system for studying fundamental aspects of influenza infections, vaccination and antiviral drug efficacy because of its small size, available reagents, human-like clinical symptoms, and respiratory tract sialic acid distribution.

The application of reverse genetic systems to the study influenza has come a long way since its introduction in the 1980's (184). Preliminary approaches demonstrated that

vRNP complexes were sufficient for the generation of influenza viruses (97, 181). Reverse genetics approaches have allowed the development of a system to generate functional influenza viruses from cloned cDNAs without the use of helper viruses (56, 145, 168-170). It is the segmented nature of the RNA genome of influenza that makes it an ideal candidate for use in a reverse genetics system, where each segment can be cloned into its own plasmid. The transfection of all 8 cloned segments in one cell has been shown to rescue virus. With a clever plasmid genetic engineering approach, Hoffmann et al. have developed a bi-directional plasmid system containing an RNA polymerase I promoter and terminator sequence flanked by an RNA polymerase II promoter and polyadenylation sequence. The pol I/II system allows for the transcription of the vRNA and viral mRNA of all eight segments from an eight-plasmid transfection system (94, 95). The eight-plasmid transfection method generates the vRNA and viral proteins from the plasmid template and eliminates the need of a helper virus. Efforts to reduce the number of plasmids needed to reconstitute virus are ongoing. Neumann et al. have developed a one plasmid approach to reverse genetics, where all eight viral segments are juxtaposed on a single plasmid, although difficulties manipulating these large plasmids have reduced their utility (167). The use of reverse genetics has been a crucial part of the characterization of influenza viruses, where each segment can be examined for its individual role in viral replication and pathogenicity.

References:

1. 1998. From the Centers for Disease Control and Prevention. Update: isolation of avian influenza A(H5N1) viruses from humans--Hong Kong, 1997-1998. JAMA **279:**347-8.
2. **Abdel-Ghafar, A. N., T. Chotpitayasunondh, Z. Gao, F. G. Hayden, D. H. Nguyen, M. D. de Jong, A. Naghdaliyev, J. S. Peiris, N. Shindo, S. Soeroso, and T. M. Uyeki.** 2008. Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med **358:**261-73.
3. **Agarwal, A. R., J. Mih, and S. C. George.** 2003. Expression of matrix proteins in an in vitro model of airway remodeling in asthma. Allergy Asthma Proc **24:**35-42.
4. **Alexander, D. J.** 2000. A review of avian influenza in different bird species. Vet Microbiol **74:**3-13.
5. **Alford, R. H., J. A. Kasel, P. J. Gerone, and V. Knight.** 1966. Human influenza resulting from aerosol inhalation. Proc Soc Exp Biol Med **122:**800-4.
6. **Ali, A., R. T. Avalos, E. Ponimaskin, and D. P. Nayak.** 2000. Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. J Virol **74:**8709-19.
7. **Ayora-Talavera, G., H. Shelton, M. A. Scull, J. Ren, I. M. Jones, R. J. Pickles, and W. S. Barclay.** 2009. Mutations in H5N1 influenza virus hemagglutinin that confer binding to human tracheal airway epithelium. PLoS One **4:**e7836.
8. **Barman, S., A. Ali, E. K. Hui, L. Adhikary, and D. P. Nayak.** 2001. Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses. Virus Res **77:**61-9.
9. **Baum, L. G., and J. C. Paulson.** 1990. Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl **40:**35-8.
10. **Bean, B., B. M. Moore, B. Sterner, L. R. Peterson, D. N. Gerding, and H. H. Balfour, Jr.** 1982. Survival of influenza viruses on environmental surfaces. J Infect Dis **146:**47-51.
11. **Beaton, A. R., and R. M. Krug.** 1981. Selected host cell capped RNA fragments prime influenza viral RNA transcription in vivo. Nucleic Acids Res **9:**4423-36.
12. **Beigel, J. H., J. Farrar, A. M. Han, F. G. Hayden, R. Hyer, M. D. de Jong, S. Lochindarat, T. K. Nguyen, T. H. Nguyen, T. H. Tran, A. Nicoll, S. Touch, and K. Y. Yuen.** 2005. Avian influenza A (H5N1) infection in humans. N Engl J Med **353:**1374-85.
13. **Belser, J. A., T. R. Maines, T. M. Tumpey, and J. M. Katz.** 2010. Influenza A virus transmission: contributing factors and clinical implications. Expert Rev Mol Med **12:**e39.
14. **Belshe, R. B.** 2009. Implications of the emergence of a novel H1 influenza virus. N Engl J Med **360:**2667-8.
15. **Bertram, S., I. Glowacka, P. Blazejewska, E. Soilleux, P. Allen, S. Danisch, I. Steffen, S. Y. Choi, Y. Park, H. Schneider, K. Schughart, and S. Pohlmann.**

2010. TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells. J Virol **84:**10016-25.

16. **Bishop, D. H., J. F. Obijeski, and R. W. Simpson.** 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for in vitro activity of the ribonucleic acid-dependent ribonucleic acid polymerase. J Virol **8:**66-73.
17. **Bishop, D. H., J. F. Obijeski, and R. W. Simpson.** 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. II. Nature of the in vitro polymerase product. J Virol **8:**74-80.
18. **Bornholdt, Z. A., and B. V. Prasad.** 2006. X-ray structure of influenza virus NS1 effector domain. Nat Struct Mol Biol **13:**559-60.
19. **Bosch, F. X., W. Garten, H. D. Klenk, and R. Rott.** 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses. Virology **113:**725-35.
20. **Bottcher, E., C. Freuer, T. Steinmetzer, H. D. Klenk, and W. Garten.** 2009. MDCK cells that express proteases TMPRSS2 and HAT provide a cell system to propagate influenza viruses in the absence of trypsin and to study cleavage of HA and its inhibition. Vaccine **27:**6324-9.
21. **Bourmakina, S. V., and A. Garcia-Sastre.** 2003. Reverse genetics studies on the filamentous morphology of influenza A virus. J Gen Virol **84:**517-27.
22. **Brown, J. D., and D. E. Stallknecht.** 2008. Wild bird surveillance for the avian influenza virus. Methods Mol Biol **436:**85-97.
23. **Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley.** 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature **371:**37-43.
24. **Buonagurio, D. A., S. Nakada, J. D. Parvin, M. Krystal, P. Palese, and W. M. Fitch.** 1986. Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. Science **232:**980-2.
25. **Burnet, F. M., and K. B. Fraser.** 1952. Studies on recombination with influenza viruses in the chick embryo. I. Invasion of the chick embryo by influenza viruses. Aust J Exp Biol Med Sci **30:**447-58.
26. **Burnet, F. M., and P. E. Lind.** 1952. Studies on recombination with influenza viruses in the chick embryo. III. Reciprocal genetic interaction between two influenza virus strains. Aust J Exp Biol Med Sci **30:**469-77.
27. **Burnett, F. M. a. B., D.R.** 1943. Changes in influenza virus associated with adaptation to passage in chicken embryous. Aust. J. Exp. Biol. Med. Sci **21:**55-69.
28. **Bush, R. M., C. A. Bender, K. Subbarao, N. J. Cox, and W. M. Fitch.** 1999. Predicting the evolution of human influenza A. Science **286:**1921-5.
29. **Capua, I., S. Marangon, P. Cordioli, L. Bonfanti, and U. Santucci.** 2002. H7N3 avian influenza in Italy. Vet Rec **151:**743-4.
30. **Carroll, S. M., H. H. Higa, and J. C. Paulson.** 1981. Different cell-surface receptor determinants of antigenically similar influenza virus hemagglutinins. J Biol Chem **256:**8357-63.
31. **Castrucci, M. R., and Y. Kawaoka.** 1993. Biologic importance of neuraminidase stalk length in influenza A virus. J Virol **67:**759-64.

32. **Chan, R. W., M. C. Chan, A. C. Wong, R. Karamanska, A. Dell, S. M. Haslam, A. D. Sihoe, W. H. Chui, G. Triana-Baltzer, Q. Li, J. S. Peiris, F. Fang, and J. M. Nicholls.** 2009. DAS181 inhibits H5N1 influenza virus infection of human lung tissues. Antimicrob Agents Chemother **53:**3935-41.
33. **Chan, R. W., K. M. Yuen, W. C. Yu, C. C. Ho, J. M. Nicholls, J. S. Peiris, and M. C. Chan.** 2010. Influenza H5N1 and H1N1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. PLoS One **5:**e8713.
34. **Chandrasekaran, A., A. Srinivasan, R. Raman, K. Viswanathan, S. Raguram, T. M. Tumpey, V. Sasisekharan, and R. Sasisekharan.** 2008. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nat Biotechnol **26:**107-13.
35. **Chen, J., K. H. Lee, D. A. Steinhauer, D. J. Stevens, J. J. Skehel, and D. C. Wiley.** 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell **95:**409-17.
36. **Chen, J., J. J. Skehel, and D. C. Wiley.** 1998. A polar octapeptide fused to the N-terminal fusion peptide solubilizes the influenza virus HA2 subunit ectodomain. Biochemistry **37:**13643-9.
37. **Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell.** 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med **7:**1306-12.
38. **Chen, Z., Y. Li, and R. M. Krug.** 1999. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. EMBO J **18:**2273-83.
39. **Conenello, G. M., D. Zamarin, L. A. Perrone, T. Tumpey, and P. Palese.** 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog **3:**1414-21.
40. **Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:**17-23.
41. **Couceiro, J. N., J. C. Paulson, and L. G. Baum.** 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res **29:**155-65.
42. **Crispe, E., D. S. Finlaison, A. C. Hurt, and P. D. Kirkland.** 2011. Infection of dogs with equine influenza virus: evidence for transmission from horses during the Australian outbreak. Aust Vet J **89 Suppl 1:**27-8.
43. **Cross, K. J., W. A. Langley, R. J. Russell, J. J. Skehel, and D. A. Steinhauer.** 2009. Composition and functions of the influenza fusion peptide. Protein Pept Lett **16:**766-78.
44. **Daly, J. M.** 2006. Equine influenza in dogs: too late to bolt the stable door? Vet J **171:**7-8.
45. **Daniels, R. S., A. R. Douglas, J. J. Skehel, D. C. Wiley, C. W. Naeve, R. G. Webster, G. N. Rogers, and J. C. Paulson.** 1984. Antigenic analyses of

influenza virus haemagglutinins with different receptor-binding specificities. Virology **138:**174-7.

46. **De, B. K., G. G. Brownlee, A. P. Kendal, and M. W. Shaw.** 1988. Complete sequence of a cDNA clone of the hemagglutinin gene of influenza A/Chicken/Scotland/59 (H5N1) virus: comparison with contemporary North American and European strains. Nucleic Acids Res **16:**4181-2.
47. **de Jong, M. D., V. C. Bach, T. Q. Phan, M. H. Vo, T. T. Tran, B. H. Nguyen, M. Beld, T. P. Le, H. K. Truong, V. V. Nguyen, T. H. Tran, Q. H. Do, and J. Farrar.** 2005. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med **352:**686-91.
48. **de Jong, P. M., M. A. van Sterkenburg, S. C. Hesseling, J. A. Kempenaar, A. A. Mulder, A. M. Mommaas, J. H. Dijkman, and M. Ponec.** 1994. Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. Am J Respir Cell Mol Biol **10:**271-7.
49. **Deshpande, K. L., V. A. Fried, M. Ando, and R. G. Webster.** 1987. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. Proc Natl Acad Sci U S A **84:**36-40.
50. **Desselberger, U., V. R. Racaniello, J. J. Zazra, and P. Palese.** 1980. The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene **8:**315-28.
51. **Dinwiddie, D. L., and K. S. Harrod.** 2008. Human metapneumovirus inhibits IFN-alpha signaling through inhibition of STAT1 phosphorylation. Am J Respir Cell Mol Biol **38:**661-70.
52. **Domingo, E.** 2000. Quasispecies and RNA Virus Evolution: Principles and Consequences. Landes Bioscience.
53. **Edney, M., and F. M. Burnet.** 1952. Influence of ions on thermal inactivation and modification of an influenza virus. Aust J Exp Biol Med Sci **30:**129-38.
54. **Egorov, A., S. Brandt, S. Sereinig, J. Romanova, B. Ferko, D. Katinger, A. Grassauer, G. Alexandrova, H. Katinger, and T. Muster.** 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. J Virol **72:**6437-41.
55. **Elton, D., M. Simpson-Holley, K. Archer, L. Medcalf, R. Hallam, J. McCauley, and P. Digard.** 2001. Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway. J Virol **75:**408-19.
56. **Enami, M., and P. Palese.** 1991. High-efficiency formation of influenza virus transfectants. J Virol **65:**2711-3.
57. **Fields, B. N., D. M. Knipe, and P. M. Howley.** 2007. Fields virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.
58. **Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus.** 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol **79:**2814-22.
59. **Fouchier, R. A. M., P. M. Schneeberger, F. W. Rozendaal, J. M. Broekman, S. A. G. Kemink, V. Munster, T. Kuiken, G. F. Rimmelzwaan, M. Schutten, G. J. J. van Doornum, G. Koch, A. Bosman, M. Koopmans, and A. D. M. E. Osterhaus.** 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.

60. **Fraser, K. B., and F. M. Burnet.** 1952. Studies on recombination with influenza viruses in the chick embryo. II. Genetic interaction between influenza virus strains in the chick embryo. Aust J Exp Biol Med Sci **30:**459-68.
61. **Gabriel, G., B. Dauber, T. Wolff, O. Planz, H. D. Klenk, and J. Stech.** 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci U S A **102:**18590-5.
62. **Gambaryan, A. S., V. E. Piskarev, I. A. Yamskov, A. M. Sakharov, A. B. Tuzikov, N. V. Bovin, N. E. Nifant'ev, and M. N. Matrosovich.** 1995. Human influenza virus recognition of sialyloligosaccharides. FEBS Lett **366:**57-60.
63. **Gambaryan, A. S., A. B. Tuzikov, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, N. V. Bovin, and M. N. Matrosovich.** 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyllactosamine). Virology **232:**345-50.
64. **Gao, P., S. Watanabe, T. Ito, H. Goto, K. Wells, M. McGregor, A. J. Cooley, and Y. Kawaoka.** 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol **73:**3184-9.
65. **Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster.** 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology **252:**324-30.
66. **Garten, R. J., C. T. Davis, C. A. Russell, B. Shu, S. Lindstrom, A. Balish, W. M. Sessions, X. Xu, E. Skepner, V. Deyde, M. Okomo-Adhiambo, L. Gubareva, J. Barnes, C. B. Smith, S. L. Emery, M. J. Hillman, P. Rivailler, J. Smagala, M. de Graaf, D. F. Burke, R. A. Fouchier, C. Pappas, C. M. Alpuche-Aranda, H. Lopez-Gatell, H. Olivera, I. Lopez, C. A. Myers, D. Faix, P. J. Blair, C. Yu, K. M. Keene, P. D. Dotson, Jr., D. Boxrud, A. R. Sambol, S. H. Abid, K. St George, T. Bannerman, A. L. Moore, D. J. Stringer, P. Blevins, G. J. Demmler-Harrison, M. Ginsberg, P. Kriner, S. Waterman, S. Smole, H. F. Guevara, E. A. Belongia, P. A. Clark, S. T. Beatrice, R. Donis, J. Katz, L. Finelli, C. B. Bridges, M. Shaw, D. B. Jernigan, T. M. Uyeki, D. J. Smith, A. I. Klimov, and N. J. Cox.** 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **325:**197-201.
67. **Garten, W., F. X. Bosch, D. Linder, R. Rott, and H. D. Klenk.** 1981. Proteolytic activation of the influenza virus hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage. Virology **115:**361-74.
68. **Gaush, C. R., and T. F. Smith.** 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl Microbiol **16:**588-94.
69. **Glaser, L., J. Stevens, D. Zamarin, I. A. Wilson, A. Garcia-Sastre, T. M. Tumpey, C. F. Basler, J. K. Taubenberger, and P. Palese.** 2005. A single

amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol **79:**11533-6.

70. **Gorman, O. T., W. J. Bean, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of the nucleoprotein gene of influenza A virus. J Virol **64:**1487-97.
71. **Gorman, O. T., W. J. Bean, and R. G. Webster.** 1992. Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. Curr Top Microbiol Immunol **176:**75-97.
72. **Gorman, O. T., R. O. Donis, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of influenza A virus PB2 genes: implications for evolution of the ribonucleoprotein complex and origin of human influenza A virus. J Virol **64:**4893-902.
73. **Goto, H.** 2004. [Novel function of plasminogen-binding activity of the NA determines the pathogenicity of influenza A virus]. Uirusu **54:**83-91.
74. **Goto, H., and Y. Kawaoka.** 1998. A novel mechanism for the acquisition of virulence by a human influenza A virus. Proc Natl Acad Sci U S A **95:**10224-8.
75. **Gotoh, B., T. Ogasawara, T. Toyoda, N. M. Inocencio, M. Hamaguchi, and Y. Nagai.** 1990. An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. EMBO J **9:**4189-95.
76. **Govorkova, E. A., M. N. Matrosovich, A. B. Tuzikov, N. V. Bovin, C. Gerdil, B. Fanget, and R. G. Webster.** 1999. Selection of receptor-binding variants of human influenza A and B viruses in baby hamster kidney cells. Virology **262:**31-8.
77. **Govorkova, E. A., J. E. Rehg, S. Krauss, H. L. Yen, Y. Guan, M. Peiris, T. D. Nguyen, T. H. Hanh, P. Puthavathana, H. T. Long, C. Buranathai, W. Lim, R. G. Webster, and E. Hoffmann.** 2005. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol **79:**2191-8.
78. **Guo, X. L., L. Li, D. Q. Wei, Y. S. Zhu, and K. C. Chou.** 2008. Cleavage mechanism of the H5N1 hemagglutinin by trypsin and furin. Amino Acids **35:**375-82.
79. **Hagen, M., T. D. Chung, J. A. Butcher, and M. Krystal.** 1994. Recombinant influenza virus polymerase: requirement of both 5' and 3' viral ends for endonuclease activity. J Virol **68:**1509-15.
80. **Hanamure, Y., K. Deguchi, and M. Ohyama.** 1994. Ciliogenesis and mucus synthesis in cultured human respiratory epithelial cells. Ann Otol Rhinol Laryngol **103:**889-95.
81. **Hanson, B. A., M. P. Luttrell, V. H. Goekjian, L. Niles, D. E. Swayne, D. A. Senne, and D. E. Stallknecht.** 2008. Is the occurrence of avian influenza virus in Charadriiformes species and location dependent? J Wildl Dis **44:**351-61.
82. **Harder, T. C., and T. W. Vahlenkamp.** 2010. Influenza virus infections in dogs and cats. Vet Immunol Immunopathol **134:**54-60.
83. **Harrison, S. C.** 2008. Viral membrane fusion. Nat Struct Mol Biol **15:**690-8.
84. **Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840-2.
85. **Hawkins, M. G., B. M. Crossley, A. Osofsky, R. J. Webby, C. W. Lee, D. L. Suarez, and S. K. Hietala.** 2006. Avian influenza A virus subtype H5N2 in a red-lored Amazon parrot. J Am Vet Med Assoc **228:**236-41.

86. **Herlocher, M. L., S. Elias, R. Truscon, S. Harrison, D. Mindell, C. Simon, and A. S. Monto.** 2001. Ferrets as a transmission model for influenza: sequence changes in HA1 of type A (H3N2) virus. J Infect Dis **184:**542-6.
87. **Higa, H. H., G. N. Rogers, and J. C. Paulson.** 1985. Influenza virus hemagglutinins differentiate between receptor determinants bearing N-acetyl-, N-glycollyl-, and N,O-diacetylneuraminic acids. Virology **144:**279-82.
88. **Hinshaw, V. S., G. M. Air, A. J. Gibbs, L. Graves, B. Prescott, and D. Karunakaran.** 1982. Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J Virol **42:**865-72.
89. **Hinshaw, V. S., R. G. Webster, and R. J. Rodriguez.** 1979. Influenza A viruses: combinations of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. Brief review. Arch Virol **62:**281-90.
90. **Hinshaw, V. S., R. G. Webster, and B. Turner.** 1979. Water-bone transmission of influenza A viruses? Intervirology **11:**66-8.
91. **Hinshaw, V. S., J. M. Wood, R. G. Webster, R. Deibel, and B. Turner.** 1985. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bull World Health Organ **63:**711-9.
92. **Hirst, G. K.** 1941. The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected with Influenza Virus. Science **94:**22-3.
93. **Hirst, M., C. R. Astell, M. Griffith, S. M. Coughlin, M. Moksa, T. Zeng, D. E. Smailus, R. A. Holt, S. Jones, M. A. Marra, M. Petric, M. Krajden, D. Lawrence, A. Mak, R. Chow, D. M. Skowronski, S. A. Tweed, S. Goh, R. C. Brunham, J. Robinson, V. Bowes, K. Sojonky, S. K. Byrne, Y. Li, D. Kobasa, T. Booth, and M. Paetzel.** 2004. Novel avian influenza H7N3 strain outbreak, British Columbia. Emerg Infect Dis **10:**2192-5.
94. **Hoffmann, E., G. Neumann, G. Hobom, R. G. Webster, and Y. Kawaoka.** 2000. "Ambisense" approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. Virology **267:**310-7.
95. **Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A **97:**6108-13.
96. **Honda, A., A. Endo, K. Mizumoto, and A. Ishihama.** 2001. Differential roles of viral RNA and cRNA in functional modulation of the influenza virus RNA polymerase. J Biol Chem **276:**31179-85.
97. **Honda, A., J. Mukaigawa, A. Yokoiyama, A. Kato, S. Ueda, K. Nagata, M. Krystal, D. P. Nayak, and A. Ishihama.** 1990. Purification and molecular structure of RNA polymerase from influenza virus A/PR8. J Biochem **107:**624-8.
98. **Horimoto, T., K. Nakayama, S. P. Smeekens, and Y. Kawaoka.** 1994. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol **68:**6074-8.
99. **Horimoto, T., E. Rivera, J. Pearson, D. Senne, S. Krauss, Y. Kawaoka, and R. G. Webster.** 1995. Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology **213:**223-30.
100. **Husseini, R. H., C. Sweet, R. A. Bird, M. H. Collie, and H. Smith.** 1983. Distribution of viral antigen with the lower respiratory tract of ferrets infected

with a virulent influenza virus: production and release of virus from corresponding organ cultures. J Gen Virol **64 Pt 3:**589-98.

101. **Ito, T., J. N. Couceiro, S. Kelm, L. G. Baum, S. Krauss, M. R. Castrucci, I. Donatelli, H. Kida, J. C. Paulson, R. G. Webster, and Y. Kawaoka.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol **72:**7367-73.
102. **Ito, T., H. Goto, E. Yamamoto, H. Tanaka, M. Takeuchi, M. Kuwayama, Y. Kawaoka, and K. Otsuki.** 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol **75:**4439-43.
103. **Ito, T., and Y. Kawaoka.** 2000. Host-range barrier of influenza A viruses. Vet Microbiol **74:**71-5.
104. **Ito, T., Y. Suzuki, L. Mitnaul, A. Vines, H. Kida, and Y. Kawaoka.** 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology **227:**493-9.
105. **Ito, T., Y. Suzuki, T. Suzuki, A. Takada, T. Horimoto, K. Wells, H. Kida, K. Otsuki, M. Kiso, H. Ishida, and Y. Kawaoka.** 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. J Virol **74:**9300-5.
106. **Katz, J. M., and J. S. Robertson.** 1992. WHO-NIH meeting on host cell selection of influenza virus variants. 13-14 November 1991, National Institute for Biological Standards and Control, Hertfordshire, UK. Vaccine **10:**723-5.
107. **Katz, J. M., M. Wang, and R. G. Webster.** 1990. Direct sequencing of the HA gene of influenza (H3N2) virus in original clinical samples reveals sequence identity with mammalian cell-grown virus. J Virol **64:**1808-11.
108. **Katz, J. M., and R. G. Webster.** 1992. Amino acid sequence identity between the HA1 of influenza A (H3N2) viruses grown in mammalian and primary chick kidney cells. J Gen Virol **73 (Pt 5):**1159-65.
109. **Kawaguchi, T., I. Matsumoto, and T. Osawa.** 1974. Studies on hemagglutinins from Maackia amurensis seeds. J Biol Chem **249:**2786-92.
110. **Kawaoka, Y.** 2006. Influenza virology : current topics. Caister Academic Press, Wymondham.
111. **Kawaoka, Y.** 1991. Structural features influencing hemagglutinin cleavability in a human influenza A virus. J Virol **65:**1195-201.
112. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol **63:**4603-8.
113. **Kawaoka, Y., C. W. Naeve, and R. G. Webster.** 1984. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology **139:**303-16.
114. **Kawaoka, Y., and R. G. Webster.** 1989. Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin. J Virol **63:**3296-300.
115. **Kawaoka, Y., and R. G. Webster.** 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A **85:**324-8.

116. **Kido, H., Y. Yokogoshi, K. Sakai, M. Tashiro, Y. Kishino, A. Fukutomi, and N. Katunuma.** 1992. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem **267:**13573-9.
117. **Kingsbury, D. W., I. M. Jones, and K. G. Murti.** 1987. Assembly of influenza ribonucleoprotein in vitro using recombinant nucleoprotein. Virology **156:**396-403.
118. **Klenk, H. D., and W. Garten.** 1994. Host cell proteases controlling virus pathogenicity. Trends Microbiol **2:**39-43.
119. **Klenk, H. D., R. Rott, and M. Orlich.** 1977. Further studies on the activation of influenza virus by proteolytic cleavage of the haemagglutinin. J Gen Virol **36:**151-61.
120. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426-39.
121. **Kochs, G., A. Garcia-Sastre, and L. Martinez-Sobrido.** 2007. Multiple anti-interferon actions of the influenza A virus NS1 protein. J Virol **81:**7011-21.
122. **Kochs, G., I. Koerner, L. Thiel, S. Kothlow, B. Kaspers, N. Ruggli, A. Summerfield, J. Pavlovic, J. Stech, and P. Staeheli.** 2007. Properties of H7N7 influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens. J Gen Virol **88:**1403-9.
123. **Krauss, S., D. Walker, S. P. Pryor, L. Niles, L. Chenghong, V. S. Hinshaw, and R. G. Webster.** 2004. Influenza A viruses of migrating wild aquatic birds in North America. Vector Borne Zoonotic Dis **4:**177-89.
124. **Krug, R. M.** 1981. Priming of influenza viral RNA transcription by capped heterologous RNAs. Curr Top Microbiol Immunol **93:**125-49.
125. **Kuo, R. L., and R. M. Krug.** 2009. Influenza a virus polymerase is an integral component of the CPSF30-NS1A protein complex in infected cells. J Virol **83:**1611-6.
126. **Lakadamyali, M., M. J. Rust, and X. Zhuang.** 2004. Endocytosis of influenza viruses. Microbes Infect **6:**929-36.
127. **Lamb, R. A., S. L. Zebedee, and C. D. Richardson.** 1985. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell **40:**627-33.
128. **Langley, W. A., S. Thoennes, K. C. Bradley, S. E. Galloway, G. R. Talekar, S. F. Cummings, E. Vareckova, R. J. Russell, and D. A. Steinhauer.** 2009. Single residue deletions along the length of the influenza HA fusion peptide lead to inhibition of membrane fusion function. Virology **394:**321-30.
129. **Laver, W. G.** 1984. Antigenic variation and the structure of influenza virus glycoproteins. Microbiol Sci **1:**37-43.
130. **Laver, W. G., G. M. Air, R. G. Webster, W. Gerhard, C. W. Ward, and T. A. Dopheide.** 1980. The mechanism of antigenic drift in influenza virus: sequence changes in the haemagglutinin of variants selected with monoclonal hybridoma antibodies. Philos Trans R Soc Lond B Biol Sci **288:**313-26.
131. **Laver, W. G., P. M. Colman, R. G. Webster, V. S. Hinshaw, and G. M. Air.** 1984. Influenza virus neuraminidase with hemagglutinin activity. Virology **137:**314-23.

132. **Lee, M. T., K. Bishop, L. Medcalf, D. Elton, P. Digard, and L. Tiley.** 2002. Definition of the minimal viral components required for the initiation of unprimed RNA synthesis by influenza virus RNA polymerase. Nucleic Acids Res **30:**429-38.
133. **Leigh, M. W., R. J. Connor, S. Kelm, L. G. Baum, and J. C. Paulson.** 1995. Receptor specificity of influenza virus influences severity of illness in ferrets. Vaccine **13:**1468-73.
134. **Li, S., J. Y. Min, R. M. Krug, and G. C. Sen.** 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology **349:**13-21.
135. **Li, S., J. Schulman, S. Itamura, and P. Palese.** 1993. Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. J Virol **67:**6667-73.
136. **Li, X., and P. Palese.** 1992. Mutational analysis of the promoter required for influenza virus virion RNA synthesis. J Virol **66:**4331-8.
137. **Li, Z., H. Chen, P. Jiao, G. Deng, G. Tian, Y. Li, E. Hoffmann, R. G. Webster, Y. Matsuoka, and K. Yu.** 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol **79:**12058-64.
138. **Li, Z., Y. Jiang, P. Jiao, A. Wang, F. Zhao, G. Tian, X. Wang, K. Yu, Z. Bu, and H. Chen.** 2006. The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol **80:**11115-23.
139. **Liu, C., M. C. Eichelberger, R. W. Compans, and G. M. Air.** 1995. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol **69:**1099-106.
140. **Liu, J., P. A. Lynch, C. Y. Chien, G. T. Montelione, R. M. Krug, and H. M. Berman.** 1997. Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein. Nat Struct Biol **4:**896-9.
141. **Liu, J., H. Xiao, F. Lei, Q. Zhu, K. Qin, X. W. Zhang, X. L. Zhang, D. Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, and G. F. Gao.** 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science **309:**1206.
142. **Look, D. C., M. J. Walter, M. R. Williamson, L. Pang, Y. You, J. N. Sreshta, J. E. Johnson, D. S. Zander, and S. L. Brody.** 2001. Effects of paramyxoviral infection on airway epithelial cell Foxj1 expression, ciliogenesis, and mucociliary function. Am J Pathol **159:**2055-69.
143. **Loris, R.** 2002. Principles of structures of animal and plant lectins. Biochim Biophys Acta **1572:**198-208.
144. **Lu, Y., M. Wambach, M. G. Katze, and R. M. Krug.** 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. Virology **214:**222-8.
145. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1989. Amplification, expression, and packaging of foreign gene by influenza virus. Cell **59:**1107-13.
146. **Maines, T. R., L. M. Chen, Y. Matsuoka, H. Chen, T. Rowe, J. Ortin, A. Falcon, T. H. Nguyen, Q. Mai le, E. R. Sedyaningsih, S. Harun, T. M. Tumpey, R. O. Donis, N. J. Cox, K. Subbarao, and J. M. Katz.** 2006. Lack of

transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A **103:**12121-6.

147. **Manuguerra, J. C., C. Hannoun, F. Simon, E. Villar, and J. A. Cabezas.** 1993. Natural infection of dogs by influenza C virus: a serological survey in Spain. New Microbiol **16:**367-71.
148. **Markwell, M. A., P. Fredman, and L. Svennerholm.** 1984. Receptor ganglioside content of three hosts for Sendai virus. MDBK, HeLa, and MDCK cells. Biochim Biophys Acta **775:**7-16.
149. **Massin, P., S. van der Werf, and N. Naffakh.** 2001. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol **75:**5398-404.
150. **Matrosovich, M., A. Tuzikov, N. Bovin, A. Gambaryan, A. Klimov, M. R. Castrucci, I. Donatelli, and Y. Kawaoka.** 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol **74:**8502-12.
151. **Matrosovich, M., N. Zhou, Y. Kawaoka, and R. Webster.** 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol **73:**1146-55.
152. **Matrosovich, M. N., A. S. Gambaryan, S. Teneberg, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, and K. A. Karlsson.** 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:**224-34.
153. **Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk.** 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A **101:**4620-4.
154. **Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk.** 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J Virol **78:**12665-7.
155. **Medeiros, R., N. Escriou, N. Naffakh, J. C. Manuguerra, and S. van der Werf.** 2001. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology **289:**74-85.
156. **Min, J. Y., and R. M. Krug.** 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. Proc Natl Acad Sci U S A **103:**7100-5.
157. **Mitnaul, L. J., M. N. Matrosovich, M. R. Castrucci, A. B. Tuzikov, N. V. Bovin, D. Kobasa, and Y. Kawaoka.** 2000. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J Virol **74:**6015-20.
158. **Morsy, J., W. Garten, and R. Rott.** 1994. Activation of an influenza virus A/turkey/Oregon/71 HA insertion variant by the subtilisin-like endoprotease furin. Virology **202:**988-91.
159. **Mounts, A. W., H. Kwong, H. S. Izurieta, Y. Ho, T. Au, M. Lee, C. Buxton Bridges, S. W. Williams, K. H. Mak, J. M. Katz, W. W. Thompson, N. J. Cox,**

and K. Fukuda. 1999. Case-control study of risk factors for avian influenza A (H5N1) disease, Hong Kong, 1997. J Infect Dis **180:**505-8.

160. **Mukaigawa, J., and D. P. Nayak.** 1991. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. J Virol **65:**245-53.
161. **Munier, S., T. Larcher, F. Cormier-Aline, D. Soubieux, B. Su, L. Guigand, B. Labrosse, Y. Cherel, P. Quere, D. Marc, and N. Naffakh.** 2010. A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. J Virol **84:**940-52.
162. **Naeem, K., A. Ullah, R. J. Manvell, and D. J. Alexander.** 1999. Avian influenza A subtype H9N2 in poultry in Pakistan. Vet Rec **145:**560.
163. **Naeve, C. W., V. S. Hinshaw, and R. G. Webster.** 1984. Mutations in the hemagglutinin receptor-binding site can change the biological properties of an influenza virus. J Virol **51:**567-9.
164. **Nath, S. T., and D. P. Nayak.** 1990. Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1). Mol Cell Biol **10:**4139-45.
165. **Nayak, D. P., E. K. Hui, and S. Barman.** 2004. Assembly and budding of influenza virus. Virus Res **106:**147-65.
166. **Nemeroff, M. E., S. M. Barabino, Y. Li, W. Keller, and R. M. Krug.** 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. Mol Cell **1:**991-1000.
167. **Neumann, G., K. Fujii, Y. Kino, and Y. Kawaoka.** 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proc Natl Acad Sci U S A **102:**16825-9.
168. **Neumann, G., and Y. Kawaoka.** 1999. Genetic engineering of influenza and other negative-strand RNA viruses containing segmented genomes. Adv Virus Res **53:**265-300.
169. **Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A **96:**9345-50.
170. **Neumann, G., T. Watanabe, and Y. Kawaoka.** 2000. Plasmid-driven formation of influenza virus-like particles. J Virol **74:**547-51.
171. **Nfon, C., Y. Berhane, S. Zhang, K. Handel, O. Labrecque, and J. Pasick.** 2011. Molecular and Antigenic Characterization of Triple-Reassortant H3N2 Swine Influenza Viruses Isolated from Pigs, Turkey and Quail in Canada. Transbound Emerg Dis.
172. **Nicholls, J. M., R. W. Chan, R. J. Russell, G. M. Air, and J. S. Peiris.** 2008. Evolving complexities of influenza virus and its receptors. Trends Microbiol **16:**149-57.
173. **Nieto, A., S. de la Luna, J. Barcena, A. Portela, and J. Ortin.** 1994. Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit. J Gen Virol **75 (Pt 1):**29-36.
174. **Nobusawa, E., T. Aoyama, H. Kato, Y. Suzuki, Y. Tateno, and K. Nakajima.** 1991. Comparison of complete amino acid sequences and receptor-binding

properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology **182:**475-85.

175. **Obenauer, J. C., J. Denson, P. K. Mehta, X. Su, S. Mukatira, D. B. Finkelstein, X. Xu, J. Wang, J. Ma, Y. Fan, K. M. Rakestraw, R. G. Webster, E. Hoffmann, S. Krauss, J. Zheng, Z. Zhang, and C. W. Naeve.** 2006. Large-scale sequence analysis of avian influenza isolates. Science **311:**1576-80.
176. **Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier.** 2006. Global patterns of influenza a virus in wild birds. Science **312:**384-8.
177. **Ortega, J., J. Martin-Benito, T. Zurcher, J. M. Valpuesta, J. L. Carrascosa, and J. Ortin.** 2000. Ultrastructural and functional analyses of recombinant influenza virus ribonucleoproteins suggest dimerization of nucleoprotein during virus amplification. J Virol **74:**156-63.
178. **Oshansky, C. M., J. A. Pickens, K. C. Bradley, L. P. Jones, G. M. Saavedra-Ebner, J. P. Barber, J. M. Crabtree, D. A. Steinhauer, S. M. Tompkins, and R. A. Tripp.** 2011. Avian Influenza Viruses Infect Primary Human Bronchial Epithelial Cells Unconstrained by Sialic Acid alpha2,3 Residues. PLoS One **6:**e21183.
179. **Osterhaus, A. D., G. F. Rimmelzwaan, B. E. Martina, T. M. Bestebroer, and R. A. Fouchier.** 2000. Influenza B virus in seals. Science **288:**1051-3.
180. **Parvin, J. D., A. Moscona, W. T. Pan, J. M. Leider, and P. Palese.** 1986. Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. J Virol **59:**377-83.
181. **Parvin, J. D., P. Palese, A. Honda, A. Ishihama, and M. Krystal.** 1989. Promoter analysis of influenza virus RNA polymerase. J Virol **63:**5142-52.
182. **Patterson, S., J. Gross, and J. S. Oxford.** 1988. The intracellular distribution of influenza virus matrix protein and nucleoprotein in infected cells and their relationship to haemagglutinin in the plasma membrane. J Gen Virol **69 (Pt 8):**1859-72.
183. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** 1992. Influenza virus M2 protein has ion channel activity. Cell **69:**517-28.
184. **Plotch, S. J., M. Bouloy, I. Ulmanen, and R. M. Krug.** 1981. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23:**847-58.
185. **Poetranto, E. D., M. Yamaoka, A. M. Nastri, L. A. Krisna, M. H. Rahman, L. Wulandari, R. Yudhawati, T. E. Ginting, A. Makino, K. Shinya, and Y. Kawaoka.** 2011. An H5N1 highly pathogenic avian influenza virus isolated from a local tree sparrow in Indonesia. Microbiol Immunol.
186. **Poon, L. L., D. C. Pritlove, E. Fodor, and G. G. Brownlee.** 1999. Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. J Virol **73:**3473-6.
187. **Pritlove, D. C., L. L. Poon, L. J. Devenish, M. B. Leahy, and G. G. Brownlee.** 1999. A hairpin loop at the 5' end of influenza A virus virion RNA is required for synthesis of poly(A)+ mRNA in vitro. J Virol **73:**2109-14.

188. **Ray, R. K., and R. L. Simmons.** 1973. Differential release of sialic acid from normal and malignant cells by Vibrio cholerae neuraminidase or influenza virus neuraminidase. Cancer Res **33:**936-9.
189. **Reid, A. H., T. G. Fanning, J. V. Hultin, and J. K. Taubenberger.** 1999. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. Proc Natl Acad Sci U S A **96:**1651-6.
190. **Robertson, J. S.** 1979. 5' and 3' terminal nucleotide sequences of the RNA genome segments of influenza virus. Nucleic Acids Res **6:**3745-57.
191. **Robertson, J. S., C. Nicolson, D. Major, E. W. Robertson, and J. M. Wood.** 1993. The role of amniotic passage in the egg-adaptation of human influenza virus is revealed by haemagglutinin sequence analyses. J Gen Virol **74 (Pt 10):**2047-51.
192. **Robertson, J. S., M. Schubert, and R. A. Lazzarini.** 1981. Polyadenylation sites for influenza virus mRNA. J Virol **38:**157-63.
193. **Rogers, G. N., and B. L. D'Souza.** 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology **173:**317-22.
194. **Rogers, G. N., and J. C. Paulson.** 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:**361-73.
195. **Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature **304:**76-8.
196. **Rogers, G. N., T. J. Pritchett, J. L. Lane, and J. C. Paulson.** 1983. Differential sensitivity of human, avian, and equine influenza A viruses to a glycoprotein inhibitor of infection: selection of receptor specific variants. Virology **131:**394-408.
197. **Ruigrok, R. W., P. J. Andree, R. A. Hooft van Huysduynen, and J. E. Mellema.** 1984. Characterization of three highly purified influenza virus strains by electron microscopy. J Gen Virol **65 (Pt 4):**799-802.
198. **Rust, M. J., M. Lakadamyali, F. Zhang, and X. Zhuang.** 2004. Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat Struct Mol Biol **11:**567-73.
199. **Salomon, R., J. Franks, E. A. Govorkova, N. A. Ilyushina, H. L. Yen, D. J. Hulse-Post, J. Humberd, M. Trichet, J. E. Rehg, R. J. Webby, R. G. Webster, and E. Hoffmann.** 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med **203:**689-97.
200. **Scholtissek, C., and H. Becht.** 1971. Binding of ribonucleic acids to the RNP-antigen protein of influenza viruses. J Gen Virol **10:**11-6.
201. **Scholtissek, C., H. Burger, O. Kistner, and K. F. Shortridge.** 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology **147:**287-94.
202. **Scholtissek, C., W. Rohde, V. Von Hoyningen, and R. Rott.** 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87:**13-20.

203. **Schulman, J. L., and P. Palese.** 1977. Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. J Virol **24:**170-6.
204. **Scull, M. A., L. Gillim-Ross, C. Santos, K. L. Roberts, E. Bordonali, K. Subbarao, W. S. Barclay, and R. J. Pickles.** 2009. Avian Influenza virus glycoproteins restrict virus replication and spread through human airway epithelium at temperatures of the proximal airways. PLoS Pathog **5:**e1000424.
205. **Selleck, P. W., L. J. Gleeson, P. T. Hooper, H. A. Westbury, and E. Hansson.** 1997. Identification and characterisation of an H7N3 influenza A virus from an outbreak of virulent avian influenza in Victoria. Aust Vet J **75:**289-92.
206. **Senne, D. A., B. Panigrahy, Y. Kawaoka, J. E. Pearson, J. Suss, M. Lipkind, H. Kida, and R. G. Webster.** 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis **40:**425-37.
207. **Senne, D. A., D. L. Suarez, J. C. Pedersen, and B. Panigrahy.** 2003. Molecular and biological characteristics of H5 and H7 avian influenza viruses in live-bird markets of the northeastern United States, 1994-2001. Avian Dis **47:**898-904.
208. **Seto, J. T., and R. Rott.** 1966. Functional significance of sialidose during influenza virus multiplication. Virology **30:**731-7.
209. **Sharon, N.** 2007. Lectins: carbohydrate-specific reagents and biological recognition molecules. J Biol Chem **282:**2753-64.
210. **Shelton, H., G. Ayora-Talavera, J. Ren, S. Loureiro, R. J. Pickles, W. S. Barclay, and I. M. Jones.** 2011. Receptor binding profiles of avian influenza virus hemagglutinin subtypes on human cells as a predictor of pandemic potential. J Virol **85:**1875-80.
211. **Shinde, V., C. B. Bridges, T. M. Uyeki, B. Shu, A. Balish, X. Xu, S. Lindstrom, L. V. Gubareva, V. Deyde, R. J. Garten, M. Harris, S. Gerber, S. Vagasky, F. Smith, N. Pascoe, K. Martin, D. Dufficy, K. Ritger, C. Conover, P. Quinlisk, A. Klimov, J. S. Bresee, and L. Finelli.** 2009. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. N Engl J Med **360:**2616-25.
212. **Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka.** 2006. Avian flu: influenza virus receptors in the human airway. Nature **440:**435-6.
213. **Shinya, K., S. Hamm, M. Hatta, H. Ito, T. Ito, and Y. Kawaoka.** 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:**258-66.
214. **Shinya, K., and Y. Kawaoka.** 2006. [Influenza virus receptors in the human airway]. Uirusu **56:**85-9.
215. **Shriver, Z., R. Raman, K. Viswanathan, and R. Sasisekharan.** 2009. Context-specific target definition in influenza a virus hemagglutinin-glycan receptor interactions. Chem Biol **16:**803-14.
216. **Skehel, J. J.** 1971. RNA-dependent RNA polymerase activity of the influenza virus. Virology **45:**793-6.
217. **Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of

influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc Natl Acad Sci U S A **79:**968-72.

218. **Skehel, J. J., K. Cross, D. Steinhauer, and D. C. Wiley.** 2001. Influenza fusion peptides. Biochem Soc Trans **29:**623-6.
219. **Skehel, J. J., R. S. Daniels, A. R. Douglas, and D. C. Wiley.** 1983. Antigenic and amino acid sequence variations in the haemagglutinins of type A influenza viruses recently isolated from human subjects. Bull World Health Organ **61:**671-6.
220. **Skehel, J. J., and A. J. Hay.** 1978. Nucleotide sequences at the 5' termini of influenza virus RNAs and their transcripts. Nucleic Acids Res **5:**1207-19.
221. **Skevaki, C. L., I. Christodoulou, I. S. Spyridaki, I. Tiniakou, V. Georgiou, P. Xepapadaki, D. A. Kafetzis, and N. G. Papadopoulos.** 2009. Budesonide and formoterol inhibit inflammatory mediator production by bronchial epithelial cells infected with rhinovirus. Clin Exp Allergy **39:**1700-10.
222. **Smietanka, K., Z. Minta, K. Wyrostek, M. Jozwiak, M. Olszewska, A. K. Domanska-Blicharz, A. M. Reichert, A. Pikula, A. Habyarimana, and T. van den Berg.** 2011. Susceptibility of pigeons to clade 1 and 2.2 high pathogenicity avian influenza H5N1 virus. Avian Dis **55:**106-12.
223. **Smith, D. J., A. S. Lapedes, J. C. de Jong, T. M. Bestebroer, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier.** 2004. Mapping the antigenic and genetic evolution of influenza virus. Science **305:**371-6.
224. **Smith, F. I., J. D. Parvin, and P. Palese.** 1986. Detection of single base substitutions in influenza virus RNA molecules by denaturing gradient gel electrophoresis of RNA-RNA or DNA-RNA heteroduplexes. Virology **150:**55-64.
225. **Song, X., Y. Lasanajak, L. J. Olson, M. Boonen, N. M. Dahms, S. Kornfeld, R. D. Cummings, and D. F. Smith.** 2009. Glycan microarray analysis of P-type lectins reveals distinct phosphomannose glycan recognition. J Biol Chem **284:**35201-14.
226. **Song, X., Y. Lasanajak, C. Rivera-Marrero, A. Luyai, M. Willard, D. F. Smith, and R. D. Cummings.** 2009. Generation of a natural glycan microarray using 9-fluorenylmethyl chloroformate (FmocCl) as a cleavable fluorescent tag. Anal Biochem **395:**151-60.
227. **Song, X., B. Xia, S. R. Stowell, Y. Lasanajak, D. F. Smith, and R. D. Cummings.** 2009. Novel fluorescent glycan microarray strategy reveals ligands for galectins. Chem Biol **16:**36-47.
228. **Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank.** 1990. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Dis **34:**412-8.
229. **Stallknecht, D. E., and S. M. Shane.** 1988. Host range of avian influenza virus in free-living birds. Vet Res Commun **12:**125-41.
230. **Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank.** 1990. Persistence of avian influenza viruses in water. Avian Dis **34:**406-11.
231. **Steel, J., A. C. Lowen, S. Mubareka, and P. Palese.** 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog **5:**e1000252.

232. **Steinhauer, D. A.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.
233. **Steinhauer, D. A., and J. J. Skehel.** 2002. Genetics of influenza viruses. Annu Rev Genet **36:**305-32.
234. **Stephenson, I., J. M. Wood, K. G. Nicholson, and M. C. Zambon.** 2003. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Med Virol **70:**391-8.
235. **Stevens, J., O. Blixt, L. Glaser, J. K. Taubenberger, P. Palese, J. C. Paulson, and I. A. Wilson.** 2006. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J Mol Biol **355:**1143-55.
236. **Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J **11:**2407-14.
237. **Su, B., S. Wurtzer, M. A. Rameix-Welti, D. Dwyer, S. van der Werf, N. Naffakh, F. Clavel, and B. Labrosse.** 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One **4:**e8495.
238. **Suarez, D. L.** 2000. Evolution of avian influenza viruses. Vet Microbiol **74:**15-27.
239. **Suarez, D. L., M. Garcia, J. Latimer, D. Senne, and M. Perdue.** 1999. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. J Virol **73:**3567-73.
240. **Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol **72:**6678-88.
241. **Suarez, D. L., and D. A. Senne.** 2000. Sequence analysis of related low-pathogenic and highly pathogenic H5N2 avian influenza isolates from United States live bird markets and poultry farms from 1983 to 1989. Avian Dis **44:**356-64.
242. **Suarez, D. L., P. R. Woolcock, A. J. Bermudez, and D. A. Senne.** 2002. Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis **46:**111-21.
243. **Subbarao, E. K., W. London, and B. R. Murphy.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol **67:**1761-4.
244. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox.** 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science **279:**393-6.
245. **Sugrue, R. J., and A. J. Hay.** 1991. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology **180:**617-24.
246. **Suzuki, T., G. Horiike, Y. Yamazaki, K. Kawabe, H. Masuda, D. Miyamoto, M. Matsuda, S. I. Nishimura, T. Yamagata, T. Ito, H. Kida, Y. Kawaoka, and**

Y. Suzuki. 1997. Swine influenza virus strains recognize sialylsugar chains containing the molecular species of sialic acid predominantly present in the swine tracheal epithelium. FEBS Lett **404:**192-6.

247. **Suzuki, Y.** 2005. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull **28:**399-408.
248. **Swayne, D. E.** 2008. Avian influenza, 1st ed. Blackwell Pub., Ames, Iowa.
249. **Sweet, C., R. A. Bird, D. Cavanagh, G. L. Toms, M. H. Collie, and H. Smith.** 1979. The local origin of the febrile response induced in ferrets during respiratory infection with a virulent influenza virus. Br J Exp Pathol **60:**300-8.
250. **Sweet, C., J. C. Macartney, R. A. Bird, D. Cavanagh, M. H. Collie, R. H. Husseini, and H. Smith.** 1981. Differential distribution of virus and histological damage in the lower respiratory tract of ferrets infected with influenza viruses of differing virulence. J Gen Virol **54:**103-14.
251. **Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre.** 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J Virol **74:**7989-96.
252. **Tan, S. L., and M. G. Katze.** 1998. Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. J Interferon Cytokine Res **18:**757-66.
253. **Taubenberger, J. K.** 1998. Influenza virus hemagglutinin cleavage into HA1, HA2: no laughing matter. Proc Natl Acad Sci U S A **95:**9713-5.
254. **Taubenberger, J. K., A. H. Reid, A. E. Krafft, K. E. Bijwaard, and T. G. Fanning.** 1997. Initial genetic characterization of the 1918 "Spanish" influenza virus. Science **275:**1793-6.
255. **Tiley, L. S., M. Hagen, J. T. Matthews, and M. Krystal.** 1994. Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5' ends of the viral RNAs. J Virol **68:**5108-16.
256. **Trebbien, R., L. E. Larsen, and B. M. Viuff.** 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virol J **8:**434.
257. **Triana-Baltzer, G. B., M. Babizki, M. C. Chan, A. C. Wong, L. M. Aschenbrenner, E. R. Campbell, Q. X. Li, R. W. Chan, J. S. Peiris, J. M. Nicholls, and F. Fang.** 2010. DAS181, a sialidase fusion protein, protects human airway epithelium against influenza virus infection: an in vitro pharmacodynamic analysis. J Antimicrob Chemother **65:**275-84.
258. **Tumpey, T. M., T. R. Maines, N. Van Hoeven, L. Glaser, A. Solorzano, C. Pappas, N. J. Cox, D. E. Swayne, P. Palese, J. M. Katz, and A. Garcia-Sastre.** 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science **315:**655-9.
259. **Vahlenkamp, T. W., and T. C. Harder.** 2006. Influenza virus infections in mammals. Berl Munch Tierarztl Wochenschr **119:**123-31.
260. **Van Hoeven, N., C. Pappas, J. A. Belser, T. R. Maines, H. Zeng, A. Garcia-Sastre, R. Sasisekharan, J. M. Katz, and T. M. Tumpey.** 2009. Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A **106:**3366-71.

261. **Varki, A.** 2007. Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature **446:**1023-9.
262. **Varki, N. M., and A. Varki.** 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest **87:**851-7.
263. **Vey, M., M. Orlich, S. Adler, H. D. Klenk, R. Rott, and W. Garten.** 1992. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. Virology **188:**408-13.
264. **Vines, A., K. Wells, M. Matrosovich, M. R. Castrucci, T. Ito, and Y. Kawaoka.** 1998. The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J Virol **72:**7626-31.
265. **Walker, J. A., and Y. Kawaoka.** 1993. Importance of conserved amino acids at the cleavage site of the haemagglutinin of a virulent avian influenza A virus. J Gen Virol **74 (Pt 2):**311-4.
266. **Walker, J. A., S. S. Molloy, G. Thomas, T. Sakaguchi, T. Yoshida, T. M. Chambers, and Y. Kawaoka.** 1994. Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus. J Virol **68:**1213-8.
267. **Wang, Q., and Y. J. Tao.** 2010. Influenza : molecular virology. Caister Academic, Norfolk, UK.
268. **Wang, X., M. Li, H. Zheng, T. Muster, P. Palese, A. A. Beg, and A. Garcia-Sastre.** 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J Virol **74:**11566-73.
269. **Watanabe, K., N. Takizawa, M. Katoh, K. Hoshida, N. Kobayashi, and K. Nagata.** 2001. Inhibition of nuclear export of ribonucleoprotein complexes of influenza virus by leptomycin B. Virus Res **77:**31-42.
270. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-79.
271. **Webster, R. G., and W. G. Laver.** 1980. Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology **104:**139-48.
272. **Webster, R. G., W. G. Laver, G. M. Air, C. Ward, W. Gerhard, and K. L. van Wyke.** 1980. The mechanism of antigenic drift in influenza viruses: analysis of Hong Kong (H3N2) variants with monoclonal antibodies to the hemagglutinin molecule. Ann N Y Acad Sci **354:**142-61.
273. **Webster, R. G., and R. Rott.** 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell **50:**665-6.
274. **White, J., J. Kartenbeck, and A. Helenius.** 1982. Membrane fusion activity of influenza virus. EMBO J **1:**217-22.
275. **WHO.** 2011. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011.
276. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu Rev Biochem **56:**365-94.

277. **Woolcock, P. R., D. L. Suarez, and D. Kuney.** 2003. Low-pathogenicity avian influenza virus (H6N2) in chickens in California, 2000-02. Avian Dis **47:**872-81.
278. **Xu, X., S. E. Lindstrom, M. W. Shaw, C. B. Smith, H. E. Hall, B. A. Mungall, K. Subbarao, N. J. Cox, and A. Klimov.** 2004. Reassortment and evolution of current human influenza A and B viruses. Virus Res **103:**55-60.
279. **Yates, P. J., J. S. Bootman, and J. S. Robertson.** 1990. The antigenic structure of a human influenza A (H1N1) virus isolate grown exclusively in MDCK cells. J Gen Virol **71 (Pt 8):**1683-8.
280. **Yen, H. L., J. R. Aldridge, A. C. Boon, N. A. Ilyushina, R. Salomon, D. J. Hulse-Post, H. Marjuki, J. Franks, D. A. Boltz, D. Bush, A. S. Lipatov, R. J. Webby, J. E. Rehg, and R. G. Webster.** 2009. Changes in H5N1 influenza virus hemagglutinin receptor binding domain affect systemic spread. Proc Natl Acad Sci U S A **106:**286-91.
281. **Yen, H. L., L. M. Herlocher, E. Hoffmann, M. N. Matrosovich, A. S. Monto, R. G. Webster, and E. A. Govorkova.** 2005. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother **49:**4075-84.
282. **Youzbashi, E., M. Marschall, I. Chaloupka, and H. Meier-Ewert.** 1996. [Distribution of influenza C virus infection in dogs and pigs in Bavaria]. Tierarztl Prax **24:**337-42.
283. **Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351:**467-71.
284. **Zhirnov, O. P., I. V. Vorobjeva, A. V. Ovcharenko, and H. D. Klenk.** 2003. Intracellular cleavage of human influenza a virus hemagglutinin and its inhibition. Biochemistry (Mosc) **68:**1020-6.
285. **Zitzow, L. A., T. Rowe, T. Morken, W. J. Shieh, S. Zaki, and J. M. Katz.** 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol **76:**4420-9.

CHAPTER 3

REPLICATION AND TRANSMISSION OF LOW PATHOGENIC AVIAN INFLUENZA FIELD ISOLATES IN MAMMALIAN SYSTEMS [1],[2]

[1] Pickens J.A., E.A. Driskell, 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. In Revision at PLoS One.

[2] Oshansky CM, Pickens JA, Bradley KC, Jones LP, Saavedra-Ebner GM, Barber JP, Crabtree JM, Steinhauer DA, Tompkins SM, Tripp RA. PLoS One. 2011;6(6):e21183. Reprinted here with permission from the publisher.

Abstract

Wild aquatic birds are the natural reservoir of avian influenza viruses and direct transmission of these viruses to mammals has become an important research initiative, especially since the 1997 HPAI outbreaks that exhibited high fatality rates in humans, even though they lacked human to human transmission. However, there is minimal insight into the potential of low pathogenic avian influenza (LPAI) viruses from the wild aquatic reservoir to infect and cause disease in mammals. There are host range restrictions that reduce the zoonotic transmission of avian viruses. Previously, we demonstrated that a subset of LPAI isolates were able to replicate and induced lesions in the respiratory tract in mice (10). In this study, we were interested in examining the zoonotic potential of LPAI isolates using a better representations of human infection; the fully differentiated, primary normal human bronchial epithelial (NHBE) cell model and the ferret animal model. We show that the LPAI isolates replicated in both the NHBE cells and the ferret model despite having the classical avian influenza receptor specificity, where all isolates had an affinity for α2,3-linked sialic acid glycans. One LPAI (H1N9) isolate was able to transmit to naïve ferrets by direct contact, despite an α2,3-linked sialic acid receptor preference. Furthermore, LPAI isolates were able to infect and replicate in neuraminidase-treated NHBE cells. Collectively, these results provide evidence that LPAI isolates can potentially infect mammals without prior adaptation and are unimpeded by expression of α2,3-linked sialic acid receptors. This work emphasizes the need for a more in depth examination of the potential of LPAI from the wild aquatic reservoir as a source of the next pandemic.

Introduction

Influenza A viruses have posed a constant threat since long before the emergence of the 2009 H1N1 pandemic strain, with the most devastating influenza pandemic in history being the "1918 Spanish influenza" that resulted in greater than 40 million deaths worldwide. Annually, there are an average 200,000 hospitalizations and 41,000 deaths caused by seasonal influenza epidemics (11). Influenza viruses belong to the *Orthomyxoviridae* family and are comprised of a segmented, negative sense RNA genome that encodes up to eleven viral proteins. They are subtyped based on the antigenically diverse primary glycoproteins hemagglutinin (HA) and neuraminidase (NA), where currently there are 16 HA and 9 NA subtypes identified in nature. Wild aquatic birds are the natural hosts for influenza viruses, where all HA and NA subtypes are perpetuated (74). It is this reservoir that has been hypothesized as the source of all human influenza strains (15, 19). The H1, H2, H3, N1 and N2 subtypes are the only subtypes currently circulating in the human population, but there is growing concern that the introduction of a novel influenza subtype from the avian reservoir into the immunologically naive population would result in substantial morbidity and mortality. Avian influenza viruses (AIV) are more adapted to their natural host and exhibit poor human to human spread because of host range restrictions that reduce zoonotic transmission. Only highly pathogenic avian influenza (HPAI) virus strains of the H5 and H7 subtypes have been shown to infect and cause severe, even fatal, disease in humans (59, 61). As of 2003, there have been over 550 cases of HPAI virus infection in humans and greater than 330 deaths as a result of these infections (75).

Viral contributors to host range restriction and virulence of AIVs in mammals are multifactorial. The interaction between the major viral glycoprotein, HA and the host cell sialic acid receptors is considered critical for establishing influenza infection, where species specific binding restrictions have been identified. It has been previously established that avian viruses preferentially bind terminal sialic acids with an 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 (8, 14, 25, 38, 47, 49). Conversely, human influenza viruses exhibit preferential binding to terminal sialic acids with an α2,6-linked sialic acid located most prominently on non-ciliated cells of the human upper respiratory tract (nasopharynx and trachea) (8, 13, 36, 41, 42, 47, 51, 57). It is thought that the receptor specificity of influenza viruses is a large factor limiting transmission, where in some AIV cases (H5, H7, and H9 subtypes) the viruses were able to infect and cause disease in humans yet exhibit poor human to human transmission (7, 26, 30, 69, 77).

The amino acid residues of viral HA influence α2,3 versus α2,6-linked sialic acid binding specificity. Through mutagenesis studies of avian and human influenza viruses, several residues are directly linked to altering viral receptor specificity. In human H3 strains, conserved amino acids leucine (L) at 226 and serine (S) at 228 (H3 numbering) result in α2,6-linked sialic acid recognition, while avian strains that preferentially bind α2,3-linked sialic acid receptors contain a glutamine (Q) at position 226 and a glycine (G) at 228 of the HA1 subunit (8, 36, 47, 72). Amino acid residues 138, 190, 194, and 225 (H3 numbering) have also been shown to be differentially conserved in avian and human influenza viruses (36). However, while experimental and ecological evidence supports

the species specific tropism based upon α2,3- versus α2,6-linked sialic acid binding preference, there is mounting evidence that our understanding of these mechanisms(s) of tissue and species tropism and transmission is incomplete (14, 36, 40, 43).

Here we further examine the potential of LPAI viruses to infect mammals, using a primary cell line, normal human bronchial epithelial (NHBE) cells and ferret model systems. Fully differentiate NHBE cells are an ideal approach for studying the human response to influenza infections because they emulate the human respiratory epithelium with polarized ciliated epithelial cell, mucus secreting goblet cells and Clara cells present in the human respiratory tract. More importantly for influenza research, they also express α2,3- and α2,6-linked sialic acids (6, 24, 29, 44). The ferret animal model system has become an indispensable approach for examining the potential for influenza viruses to infect and transmit in humans. They are highly susceptible to human and avian strains of influenza without prior virus adaptation and exhibit the same clinical symptoms observed in humans, such as sneezing, fever, and nasal discharge (5, 22, 34, 62, 80). Ferrets also exhibit similar sialic acid distribution as observed in the human respiratory tract, with α2,6-linked sialic acids predominantly expressed in the upper respiratory tract, while a mixture of α2,3- and α2,6-linked sialic acids are present in the lower respiratory tract (23, 32, 64, 66).

Using primary cells, sequencing, *in vitro* binding, and glycan array analysis, we examined receptor specificity and tropism of these viruses. In these studies, we demonstrate that low pathogenic H5N1, H5N2 and H5N3 AIV viruses of chicken or wild bird origin could infect and replicate in fully differentiated NHBE cells independent of α2,3- or α2,6-linked sialic acid moieties. Strikingly, every virus maintained the prototypic

α2,3-linked sialic acid binding preference by sequence analysis, glycan microarray binding and agglutination of equine erythrocytes. These viruses also infected ferrets and were shed from the upper respiratory tract, although they did not cause clinical disease.

In a separate study we examined the capacity of non-H5 LPAI isolates to infect, cause disease and transmit in ferrets. Previously, we demonstrated a number of wild bird LPAI viruses were able to infect and induce lesions in a mouse model without prior adaptation (10). Here, we examine the potential for infection and transmission of an H1N9 (A/Ruddy Turnstone/DE/650645/02, abbreviated H1N9) and an H6N1 (A/Ruddy Turnstone/DE/650625/02, abbreviated H6N1) AIV in the ferret model and assess the genetic features contributing to the phenotype. Interestingly, we demonstrate that the H1N9 and H6N1 viruses replicate in both the upper and lower respiratory tract of ferrets, despite a dominant α2,3-linked sialic acid avian binding specificity. Furthermore, we demonstrate that one of these viruses (H1N9) is able to transmit to naïve ferrets via contact, despite its dominant avian α2,3-linked sialic acid binding preference. These findings suggest that transmission of wild bird LPAI to mammals is not restricted to specific subtypes, where alternative mechanism(s) may be in place to enhance the potential of LPAI to infect and cause disease in mammals.

Materials and Methods

Viruses

The low pathogenic AIV (LPAI) strains from domestic birds A/Mute Swan/MI/06/451072-2/2006 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), and A/chicken/Texas/167280-4/02 (H5N3) were kindly provided by Dr. David Suarez, USDA-

Southeast Poultry Research Laboratory, Athens, GA. The wild aquatic North American LPAI strains, A/Ruddy Turnstone/DE/650645/02 (H1N9) and A/Ruddy Turnstone/DE/650625/02 (H6N1) were acquired from Southeastern Cooperative Wildlife Disease Study, Athens GA. The A/New York/55/2004 (H3N2) virus was kindly provided by Dr. Richard Webby, St. Jude Children's Research Hospital, Memphis, TN. Virus stocks were prepared in 9 day old specific pathogen-free (SPF) eggs and harvesting the allantoic fluid 48h post-inoculation. Each LPAI isolate was minimally passaged (3 or fewer passages) in ECEs. Viral titers were obtained by serial dilution on Madin-Darby canine kidney (MDCK) cells in the presence of 1 μg/mL TPCK-trypsin (Sigma), and 50% egg infectious doses (EID_{50}) were performed in 9-day old SPF chicken embryos and calculated according to the method of Reed and Muench (46).

The H1N9 and H6N1 viruses examined by glycan microarray were cultured on MDCK cells for 72 hours in 1x Minimal Essential Medium (MEM) supplemented with 1μg/mLTPCK-treated trypsin. The viral supernatant was collected and centrifuged at 5,000 RPM for 5 minutes to remove cell debris before viral purification. All viral stocks were stored at - 80°C.

NHBE cells

Normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) from a single 17 year old healthy male donor were expanded, cryopreserved, and cultured in an air-liquid interface system as previously described (31). The cells from the same donor were used in all assays for assay consistency. The apical surface of the cells was

exposed to a humidified 95% air / 5% CO_2 environment, and the basolateral medium was changed every two days.

Viral infection of NHBE cells

Human and LPAI viruses were diluted in BEBM (Lonza, Portsmouth, NH) to equal titers as determined by MDCK plaque assay. NHBE cells were washed three times with PBS to remove excess mucus secretion on the apical surface prior to infection. Viruses were allowed to adsorb for 1h at 37°C, the virus dilutions were removed by aspiration and washed again with PBS 3 times. NHBE cells were incubated for the indicated times post infection (pi) at 37°C. Viruses released apically were harvested by the apical addition and collection of 300 µl of 0.05% BSA-BEBM allowed to equilibrate at 37°C for 30 min. Samples were stored at –80°C until assayed.

Neuraminidase treatment and influenza infection of NHBE cells

To remove sialic acid moieties from the cell surface, and to confirm the specificity of lectin binding, NHBE cells were apically treated with the indicated concentration of neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, MO) in PBS for 1 hour at 37°C as previously described. Following sialidase incubation, cells were washed three times with PBS. NHBE cells were apically mock infected or infected with A/Mute Swan/MI/06/451072-2/2006 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), A/chicken/TX/167280-4/02 (H5N3), or NY/04/55/2004 (H3N2) at the indicated multiplicities of infection (MOI). Cells were fixed in 3.7% formaldehyde for 30 min or harvested in triplicate at the times indicated post-infection.

Confocal microscopy

NHBE cells were fixed for 30 minutes in 3.7% formaldehyde at the times indicated post-infection. Sialic acid staining was performed as previously described (78). Briefly, to stain for sialic acids, cells were incubated with 20 μg/mL biotinylated MAA-II (Vector Laboratories) to detect α2,3, or 20 μg/mL biotinylated SNA (Vector Laboratories) to detect α2,6-linked sialic acids for 1 hour at room temperature, washed with PBS, and incubated with 15 μg/mL Texas Red streptavidin (Vector Laboratories). MAA-II was specifically chosen because it preferentially binds to sialic acids α2-3Galβ1-3(Siaα2-6)GalNAc and not to non-sialic acid residues as do other isoforms of MAA (28). Following washing, cells were permeabilized in PBS containing 0.5% TX-100, washed in PBS-0.05%TWEEN and incubated with mouse anti-NP IgG2a diluted in 3% bovine serum albumin (BSA) in PBS-T. The cells were then washed with PBS-T, incubated for one hour with anti-mouse IgG AlexaFluor488 (Molecular Probes, Carlsbad, California) and anti-β-tubulin directly conjugated to FITC (cilia stain). Cells were rapid stained with DAPI (1μg/mL). After washing with PBS-T, membranes were excised from their culture inserts and mounted on glass slides.

Ferrets

Castrated male Fitch ferrets, 3 months old, were purchased from Triple F Farms (Sayre, PA). Prior to infection, ferrets were housed in a BSL2 facility in HEPA filtered isolator caging (Allentown) and monitored for four days to establish baseline temperature. A subcutaneous temperature transponder (BMDS) was implanted in each ferret for identification and temperature measurement. Temperatures were recorded every

other day. One week prior to infection, blood was collected and serum tested for antibodies against circulating H1 and H3 influenza viruses using the hemagglutination inhibition (HI) assay. All ferrets had less than 1:20 titer against circulating H1 and H3 via HI assay. Studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia.

Infection and direct transmission study of the H1N9 and H6N1 wild bird isolates

For each of the two viruses examined, 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. One direct contact ferret was housed with one inoculated ferret as paired cagemates. 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 first anesthetized with isoflurane and then intranasally inoculated with 5 X 10^5 PFU in 500 uL of sterile PBS (250 uL of inoculum per nostril) with either A/Ruddy Turnstone/DE/650645/02 (H1N9) and A/Ruddy Turnstone/DE/650625/02 (H6N1). Direct contact ferrets were then placed with an inoculated ferret twenty-four hours post inoculation. An additional study was performed with RT/645 as described for the first study, using three inoculated and three direct contact ferrets. Repeat study ferrets were all housed in open caging (Allentown) in ABSL-3 facilities. Nasal washes were sampled from ferrets on days 1, 3, 5, 7, 10 pi or days 2, 4, 6, 9, 13, and 17 pc to monitor for viral infection. For nasal washes, ferrets were anesthetized with ketamine via intramuscular injection and 1 mL of sterile PBS with antibiotics was introduced into the nostrils to induce sneezing and

collected in specimen cups. Nasal washes were titrated in MDCK cells with serial 1:10 dilutions with a limit of detection 1.3 $\log_{10}$ $TCID_{50}$/mL.

Infection of ferret with H5 LPAI isolates

Four ferrets were inoculated intranasally with of 10^6 EID_{50}/mL (500 uL of inoculum per nostril) of A/MuteSwan/MI/06/451072-2/200 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), and A/chicken/Texas/167280-4/02 (H5N3). An additional three ferrets were mock infected with allantoic fluid in PBS for negative controls for nasal washes, serology, and complete blood counts (CBC). Nasal washes and blood samples were from ferrets on days 1, 3, 5, 7, 14 pi infection to monitor for viral infection. For nasal washes, ferrets were anesthetized with ketamine via intramuscular injection and 1 mL of sterile PBS with antibiotics was introduced into the nostrils to induce sneezing and collected in specimen cups. Nasal washes were titrated in 50% egg infectious doses (EID_{50}) were performed in 9-day old SPF chicken embryos and calculated according to the method of Reed and Muench (46).

Sequencing hemagglutinin from Wild Bird LPAI isolates

Total viral RNA was extracted from AIV infected allantoic fluid using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One-step RT-PCR was performed on viral RNA to using a universal primer set as previously described by Zhou et al. (79).

Primer Name	Primer Sequence (5' – 3')
Uni12/Inf-1	GGGGGGAGCAAAAGCAGG
Uni13/Inf-1	CGGGTTATTAGTAGAAACAAGG

All 8 segments were generated, the HA segment was excised and gel purified using the QIAquick Gel Extraction kit (Qiagen, Valenica, CA). The HA was sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA) 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/0.5%BSA. Each AIV strain was serially diluted in 1X PBS and standard hemagglutination assay was performed, where 50 μL of 1% erythrocytes were added to each 50 μL of diluted virus and incubated at 4°C for 1 hour. Each plate was scored for the appearance of agglutination.

AIV receptor specificity determined by glycan array

Glycan arrays were used to examine the receptor specificity of the LPAI viruses. The A/Mute Swan/MI/06/451072-2/2006 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), A/chicken/TX/167280-4/02 (H5N3), NY/55/2004 (H3N2), and A/Pennsylvania/08/2008 (H1N1) viruses were purified from allantoic fluid on a 25/60 percent sucrose gradient by ultracentrifugation and resuspended in 1mM EDTA/PBS. The remaining viruses, A/Ruddy Turnstone/DE/650645/02 (H1N9) and A/Ruddy Turnstone/DE/650625/02 (H6N1) were cultured in MDCK cells before being purified on a 25% sucrose cushion and resuspended in 1xPBS + 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 in1X PBS + 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 NaHCO3 (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 1X PBS + 1mM EDTA overnight. The labeled viruses were analyzed via glycan array by the Core H of the Consortium of Functional Glycomics (www.functionalglycomics.org), where 70 μL of labeled virus was added to glycan array slide and incubated at 4°C for 1 hour. Each array was scanned by Perkin-Elmer ProScanAray that detected sialic acid binding peaks designated as relative fluorescent units (RFUs).

Results

Domestic LPAI strains replicate and are shed apically from NHBE cells

To determine if LPAI viruses were capable of infecting NHBE cells, the cells were apically infected with A/chicken/Pennsylvania/13609/1993 (H5N2; PA/93), A/chicken/TX/167280-4/02 (H5N3; TX/02), or NY/04/55/04 (H3N2) (Figure. 3) at a multiplicity of infection (MOI) of 0.001 (equivalent to $10^{4.38}$ EID_{50}/mL for PA/93, $10^{3.86}$ EID_{50}/mL for TX/02, or $10^{4.99}$ EID_{50}/mL for NY/04/55/04). This low MOI was chosen to allow for better detection of virus replication in subsequent apical cell washings at the time-points indicated. Within 24h post infection, NHBE cells infected with PA/93 had apical wash virus titers of 10^{5} EID_{50}/mL, which peaked by 48h post infection (pi) to $10^{5.8}$ EID_{50}/mL (Figure. 3.1). NHBE cells infected with TX/02 had apical wash titers that increased slightly at 24h pi to $10^{4.3}$ EID_{50}/mL, subsequently increased to $10^{5.8}$ EID_{50}/mL at 48h pi, and peaked at $10^{6.1}$ EID_{50}/mL at 72h pi (Figure. 3.1). As the EID_{50} values were

determined from apical washes, the results suggest that both PA/93 and TX/02 replicate and are shed apically from NHBE cells, however we cannot exclude the possibility that virus shed from the basolateral side of the culture did not leak upward toward the apical side. As expected, NHBE cells infected with human influenza NY/04/55/04 (H3N2) supported a productive infection in the first 24h pi ($10^{5.9}$ EID_{50}/mL), but due to considerable cell death related to virus replication, the apical wash titers were decreased by 48h pi ($10^{4.8}$ EID_{50}/mL), and few cells remained at 72h pi.

LPAI strains infect neuraminidase-treated NHBE cells

Even though NHBE cells express a higher abundance of α2,6-linked sialic acids than α2,3-linked sialic acids, they are susceptible to infection and can undergo multiple rounds of replication despite reduced levels of α2,3-linked sialic acid receptors (6, 45). Since NHBE cells were permissive to LPAI strains, we were interested in examining the potential of LPAI viruses from domestic birds to infect NHBE cells pretreated with a neuraminidase, designed to remove all sialic acids. Recent findings suggest that neuraminidase treatment can reduce influenza virus infection, but total inhibition does not occur (58, 65). Similar to previous findings, neuraminidase treatment of NHBE cells had little effect on AIV or human influenza virus infection, as determined by NP staining (Figure 3.2).

Low pathogenic H5 avian influenza viruses capable of infecting ferrets

Since these H5 LPAI viruses were capable of infecting and replicating in neuraminidase-treated NHBE cells, we were interested in examining the potential of these viruses to infect and cause disease in the ferret, the best model for human infection. Naïve ferrets were intranasally inoculated with 10^6 EID_{50} of A/MuteSwan/MI/06/451072-2/200 (H5N1), A/chicken/Pennsylvania/13609/1993 (H5N2), or A/chicken/Texas/167280-4/02 (H5N3). Infection in all inoculated ferrets was demonstrated with presence of virus in nasal washes and seroconversion, however there were minimal clinical signs.

Wild bird H1N9 avian influenza viruses transmit between ferrets

In the previous experiments H5 LPAI viruses isolated from poultry or a wild bird (mute swan) were tested, as H5 influenza viruses can mutant to a highly pathogenic phenotype and have also infected humans. However, we questioned the potential of non-H5 LPAI viruses from the natural reservoir, wild aquatic birds, to infect and transmit in the ferret model. Naïve ferrets were inoculated with 5 X 10^5 PFU in 500 μL of sterile PBS (250 μL of per nostril) with either H6N1 or H1N9 virus. Direct contact ferrets were placed with inoculated ferrets twenty-four hours post inoculation. Infection in all inoculated ferrets was demonstrated with presence of virus in nasal washes and seroconversion despite minimal clinical signs (Table 3.1). Most interestingly, the H1N9 virus consistently transmitted to naïve contact ferrets, however the H6N1 virus did not transmit (Table 3.1). None of the aerosol-exposed ferrets became infected as measured by nasal washes and seroconversion (data not shown). All three direct contact ferrets that became infected with H1N9 virus had similar peak viral titers with similar length of

shedding time 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 matches 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. Direct contact transmission of H1N9 was repeated with a second study, and subsequently confirmed by two out of three direct contact ferrets becoming infected (data not shown).

Wild bird LPAI viruses exhibited avian-specific HA residues and erythrocyte binding

Effective transmission by the H1N9 virus as well as efficient upper respiratory tract infection of both LPAI viruses raised questions regarding potential genotypes associated with infection and transmission. Since the viral hemagglutinin is an established determinant of infection and transmission, we sequenced the HA gene from the viruses for comparison with previously sequenced and published viruses. Each LPAI HA sequence was analyzed at specific residues known to influence receptor specificity as defined in the literature. The amino acids at positions 190, 225, 226, and 228 of the HA1 subunit are distinct for avian and human receptor recognition. Avian strains typically contain the α2,3-linked sialic acid genotype, including a glutamic acid (E) at 190, glycine (G) at 225, a glutamine (Q) at position 226, and glycine (G) at position 228. All of the LPAI viruses maintained the HA amino acid residues most commonly present in

avian influenza viruses and are associated with α2,3-linked sialic acid binding specificity (Table 3.2). In contrast, human influenza viruses, A/New York/55/2004 and A/Pennsylvania/08/2008 contain aspartic acid (D) at both the 190 and 225 positions (Table 3.2), which are associated with to α2,6-linked sialic acid receptor specificity (18, 68).

To functionally assess the sialic acid receptor specificity of the H1N9 and H6N1 viruses, we examined the erythrocyte binding of the avian influenza strains. Both the H1N9 and H6N1 isolates were able to agglutinate equine erythrocytes to a 512 HAU/mL titer (Table 3.3), while the human influenza strains (A/New Caledonia/20/1999 and A/California/04/2009) generated no detectable titer. Erythrocytes from most species express both α2,3- and α2,6-linked sialic acids, but equine erythrocytes are unique in that they exhibit predominantly α2,3-linked sialic acid receptors. Both the H1N9 and H6N1 viruses agglutinated guinea pig and turkey erythrocytes to similar levels as compared to human influenza strains. Turkey erythrocytes contain a mixture of α2,3- and α2,6-linked sialic acid linked receptors and guinea pig erythrocytes express largely α2,6-linked sialic acids with lower levels of α2,3-linked sialic acid receptors (4, 39). While the mixed α2,3 and α2,6 linkages on these erythrocytes 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.

LPAI strains preferentially recognize α2,3-linked sialic acids

To further define the receptor specificity of the viruses, glycan microarrays were utilized to determine the precise sialyl-oligosaccharide binding profile for all the LPAI strains as compared to previously defined seasonal H1N1 human influenza virus A/ Pennsylvania/08/2008 and H3N2 A/New York/55/2004 (4). Purified and fluorescently labeled viruses were submitted to Core H of the Consortium for Functional Glycomics and binding was assessed against an array that contained up to 611 glycans. The binding motif for LPAI strains exhibited similar binding preferences, where they predominantly bind oligosaccharides that contain N-acetylneuraminic acid α2,3-linked moieties with little binding observed for the α2,6- and α2,8-linked sialic acid containing glycans (Figure 3.4). In contrast, the human control strain, A/Pennsylvania/08/2008 and A/New York/55/2004, demonstrated the predicted α2,6-linked sialic acid receptor binding properties (Figure 3.4 C) with minor α2,3-linked sialic acid binding, which is common among human influenza viruses (4).

Discussion

A complete understanding of the potential of LPAI isolates to cross species specific barriers and infect mammals still remains elusive. One of the primary host range restrictions is the sialic acid binding preference of the HA from AIVs compared to human influenza strains, where AIV HA preferentially recognize α2,3-linked sialic acid receptors, while human influenza HA binds α2,6-linked sialic acids. It is the distribution of sialic acid receptors within the host that is hypothesized to reduce the potential of zoonotic transmission. The α2,6-linked sialic acids are found throughout the human respiratory tract, while α2,3-linked sialic acids are found predominantly within the lower

respiratory tract (1, 9, 37). With the α2,6-linked sialic acids being the dominant receptor found throughout the human respiratory tract, AIVs would need to overcome this barrier to establish infections in humans. This led us to employ a human primary cell line, NHBE cells, and the ferret model of infection and transmission as complementary approaches to examine the zoonotic potential of LPAI viruses. The rationale for this approach centers on the hypothesis that α2,3- and α2,6-linked sialic acids on human airway epithelium are key receptors for avian and human viruses, and that reducing sialic acid levels on the airway surface would have significant impact on influenza virus infectivity.

In this study, we confirmed using NHBE cells that human bronchial epithelial cells express both forms of sialic acid, and that α2,6-linked sialic acids are more abundant than α2,3-linked sialic acids. Furthermore, neuraminidase treated NHBE cells were readily infected despite the paucity of detectable sialic acids (Figures 3.1 - 3.2). These findings are consistent with similar studies that have demonstrated the infectivity of H3N2 viruses in neuraminidase-treated human airway epithelium cells, as well as infection of neuraminidase-treated MDCK cells with reassortment H1 influenza viruses (58). It is important to emphasize that despite a >95% reduction of the α2,3-linked sialic acid expression in the neuraminidase-treated NHBE cells, AIV had the same level of infection as compared to mock-treated cells. The AIV strains predictably exhibit α2,3 receptor specificity as illustrated in the glycan array with minimal recognition of α2,6-linked sialic acid glycans. It is possible that glycan arrays are not a conclusive means for identifying viral receptor binding. The microarray contains approximately 100 influenza-specific sialic acid targets with 32 glycans representing the α2,6-linked sialic acid

repertoire, which is a minute representation of the α2,6 oligosaccharides present in nature. The α2,3-linked moieties included in the array contained complex modifications (i.e. fucosylation, sulfation) that were excluded from the α2,6-linked glycans, so with the limited number of α2,6-linked sialic acids on the glycan array it would be difficult to definitively conclude that these avian strains do not bind α2,6-linked sialic acid receptors. A more comprehensive array, potentially using naturally derived glycolipids might more fully characterize the receptor specificity of these AIV strains and identify alternative receptor specificities located ubiquitously throughout the respiratory tract (54, 55).

Ferrets have demonstrated both upper and lower respiratory infections when inoculated with a various subtyped AIVs, notably low pathogenicity isolates (3, 17, 20, 35, 53, 73). We have further supported that current circulating low pathogenicity North American wild bird avian viruses do have a capacity to infect and replicate in mammals. We have demonstrated that subtypes of lesser "concern" can also directly infect and replicate in mammals, even with the potential for direct contact mammal to mammal transmission (Figure 3.3). 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 (data not shown). Despite the presence of viral replication and pathology, there was poor seroconversion for both viruses in both inoculated and direct contact animals (Table 3.1). Similar observations of efficient infection and replication with minimal morbidity have been observed in experimental infections of ferrets with other low pathogenicity avian influenza viruses (3, 17, 35, 73). 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) (17). This study provides additional evidence that the source of virus may not be an important 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.

Infectivity of AIVs in mammals, and humans in particular, is thought to be reliant on the viral hemagglutinin recognition and binding sialic acid receptor present 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 (8, 32, 49, 71). It is thought that the avian influenza preference for binding α2,3-linked sialic acid receptors, compared to the human influenza preference for binding α2,6-linked sialic acid receptors, provides a partial barrier to transmission in ferrets and humans due to the paucity of α2,3-linked sialic acid receptors in the upper respiratory tract. However, α2,3-linked sialic receptors are present in the lower respiratory tract of both these species and it has been proposed that if enough influenza can be deposited in the lower respiratory tract, pulmonary infection will predominate in these species (27, 76). We did observe infection and replication in the lung of these ferrets for both the H1N9 and H6N1 viruses supporting viral attachment in the lung (bronchioles, but not alveoli; data not shown); however, we also observed robust upper respiratory tract infections that were more productive with higher viral titers compared to the lung despite the α2,3-linked sialic acid binding preference. We saw similar results with ferrets inoculated with LPAI H5 viruses, although virus titers from the upper respiratory tracts were generally lower than those seen with the H1 and H6 infections. We and other groups have similarly observed that

ferrets infected with human influenza viruses have viral replication restricted to the upper respiratory tract (48, 52, 63). The absence of pulmonary infection is confirmed by negative virus isolation, absence of lesions on histopathology, and absence of viral antigen on immunohistochemistry. In this study, neither the H6N1 nor H1N9 virus appeared to infect alveolar epithelial cells, yet both viruses did infect mouse and feline alveolar epithelial cells in other 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 (3, 17, 35, 53). Clearly, cellular tropism in an influenza infected host is complex, and while cellular sialic acid ligands for HA binding are certainly an important component, additional mechanisms are involved.

In our study, the H1N9 subtype AIV exhibited direct contact transmission between ferrets. Aerosol transmission was tested in this study, but the lack of a validated aerosol transmission model makes the absence of transmission inconclusive. 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 sialic acids over α2,6-linked sialic acids (2, 67, 73). In contrast, recent work has demonstrated that an H7N2 low pathogenicity avian influenza virus with avian-like receptor specificity (α2,3-linked sialic acid binding) can transmit via direct contact in ferrets (53). Similarly, the H1N9 virus studied here did transmit to naïve cage mates despite its clear avian-like receptor specificity. Direct contact H6N1 ferrets did not become infected and did not seroconvert. Perhaps this is due to lower levels of viral shedding for a shorter period of time in H6N1-inoculated ferrets as compared to

H1N9-inoculated ferrets. Both the H1 and H6 viruses have avian specific α 2,3-linked sialic acid 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 H1N9 virus dominantly bound glycans having α2,3 sialic acid linkages. There may be other unidentified amino acids in the HA or other viral gene segments that mediated the direct transmission of the H1 virus in ferrets.

The viral polymerase has also been suggested to have a potential 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 (12, 16, 21, 50, 56, 60, 70). Avian influenza viruses having a lysine (K) at position 627 of PB2 show improved replication in mammalian culture and in vivo, improved replication at lower temperatures, and improved transmission compared to avian viruses having a glutamic acid (E) at this position (21, 50, 56). Also, an aspartic acid (D) found in avian viruses to asparagine (N, found in human viruses) mutation at position 701 of PB2 has been shown to enhance replication, virulence, or transmission in mice and guinea pigs (16, 33). Sequence analysis of H1N9 PB2 found avian specific E627 and N701 residues (data not shown), suggesting that there may be other genetic features contributing to the robust upper respiratory replication and transmission of the H1N9 virus in ferrets. Clearly, mechanisms of localization of AIV infection in the mammalian respiratory tract are multifactorial. Pulmonary replication of these two AIVs was rapid in onset and rapid to resolve regarding 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 previously discussed transmission experiments (53, 73) 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.

Our studies, 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 viable AIV reassortant viruses underscoring the potential pandemic risk of AIVs from the reservoir host. 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 poor 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 is flawed.

References:

1. **Baum, L. G., and J. C. Paulson.** 1990. Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl **40:**35-8.
2. **Belser, J. A., O. Blixt, L. M. Chen, C. Pappas, T. R. Maines, N. Van Hoeven, R. Donis, J. Busch, R. McBride, J. C. Paulson, J. M. Katz, and T. M. Tumpey.** 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:**7558-63.
3. **Belser, J. A., X. Lu, T. R. Maines, C. Smith, Y. Li, R. O. Donis, J. M. Katz, and T. M. Tumpey.** 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:**11139-47.
4. **Bradley, K. C., S. E. Galloway, Y. Lasanajak, X. Song, J. Heimburg-Molinaro, G. R. Talekar, D. F. Smith, R. D. Cummings, and D. A. Steinhauer.** 2011. Analysis of influenza HA receptor binding mutants with limited receptor recognition properties and conditional replication characteristics. J Virol.
5. **Cavanagh, D., F. Mitkis, C. Sweet, M. H. Collie, and H. Smith.** 1979. The localization of influenza virus in the respiratory tract of ferrets: susceptible nasal mucosa cells produce and release more virus than susceptible lung cells. J Gen Virol **44:**505-14.
6. **Chan, R. W., K. M. Yuen, W. C. Yu, C. C. Ho, J. M. Nicholls, J. S. Peiris, and M. C. Chan.** 2010. Influenza H5N1 and H1N1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. PLoS One **5:**e8713.
7. **Claas, E. C., J. C. de Jong, R. van Beek, G. F. Rimmelzwaan, and A. D. Osterhaus.** 1998. Human influenza virus A/HongKong/156/97 (H5N1) infection. Vaccine **16:**977-8.
8. **Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:**17-23.
9. **Couceiro, J. N., J. C. Paulson, and L. G. Baum.** 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res **29:**155-65.
10. **Driskell, E. A., C. A. Jones, D. E. Stallknecht, E. W. Howerth, and S. M. Tompkins.** 2010. Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. Virology **399:**280-9.
11. **Dushoff, J., J. B. Plotkin, C. Viboud, D. J. Earn, and L. Simonsen.** 2006. Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. Am J Epidemiol **163:**181-7.
12. **Gabriel, G., A. Herwig, and H. D. Klenk.** 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.

13. **Gambaryan, A., S. Yamnikova, D. Lvov, A. Tuzikov, A. Chinarev, G. Pazynina, R. Webster, M. Matrosovich, and N. Bovin.** 2005. Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virology **334:**276-83.
14. **Gambaryan, A. S., A. B. Tuzikov, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, N. V. Bovin, and M. N. Matrosovich.** 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyllactosamine). Virology **232:**345-50.
15. **Gammelin, M., A. Altmuller, U. Reinhardt, J. Mandler, V. R. Harley, P. J. Hudson, W. M. Fitch, and C. Scholtissek.** 1990. Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol **7:**194-200.
16. **Gao, Y., Y. Zhang, K. Shinya, G. Deng, Y. Jiang, Z. Li, Y. Guan, G. Tian, Y. Li, J. Shi, L. Liu, X. Zeng, Z. Bu, X. Xia, Y. Kawaoka, and H. Chen.** 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.
17. **Gillim-Ross, L., C. Santos, Z. Chen, A. Aspelund, C. F. Yang, D. Ye, H. Jin, G. Kemble, and K. Subbarao.** 2008. Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. Journal Of Virology **82:**10854-10863.
18. **Glaser, L., J. Stevens, D. Zamarin, I. A. Wilson, A. Garcia-Sastre, T. M. Tumpey, C. F. Basler, J. K. Taubenberger, and P. Palese.** 2005. A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. Journal Of Virology **79:**11533-6.
19. **Gorman, O. T., W. J. Bean, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of the nucleoprotein gene of influenza A virus. J Virol **64:**1487-97.
20. **Govorkova, E. A., J. E. Rehg, S. Krauss, H. L. Yen, Y. Guan, M. Peiris, T. D. Nguyen, T. H. Hanh, P. Puthavathana, H. T. Long, C. Buranathai, W. Lim, R. G. Webster, and E. Hoffmann.** 2005. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. Journal Of Virology **79:**2191-8.
21. **Hatta, M., Y. Hatta, J. H. Kim, S. Watanabe, K. Shinya, T. Nguyen, P. S. Lien, Q. M. Le, and Y. Kawaoka.** 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathogens **3:**1374-9.
22. **Herlocher, M. L., S. Elias, R. Truscon, S. Harrison, D. Mindell, C. Simon, and A. S. Monto.** 2001. Ferrets as a transmission model for influenza: sequence changes in HA1 of type A (H3N2) virus. J Infect Dis **184:**542-6.
23. **Husseini, R. H., C. Sweet, R. A. Bird, M. H. Collie, and H. Smith.** 1983. Distribution of viral antigen with the lower respiratory tract of ferrets infected with a virulent influenza virus: production and release of virus from corresponding organ cultures. J Gen Virol **64 Pt 3:**589-98.
24. **Ilyushina, N. A., E. A. Govorkova, T. E. Gray, N. V. Bovin, and R. G. Webster.** 2008. Human-like receptor specificity does not affect the

neuraminidase-inhibitor susceptibility of H5N1 influenza viruses. PLoS Pathog **4:**e1000043.

25. **Ito, T., Y. Suzuki, L. Mitnaul, A. Vines, H. Kida, and Y. Kawaoka.** 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology **227:**493-9.
26. **Kemink, S. A., R. A. Fouchier, F. W. Rozendaal, J. M. Broekman, M. Koopmans, A. D. Osterhaus, and P. M. Schneeberger.** 2004. [A fatal infection due to avian influenza-A (H7N7) virus and adjustment of the preventive measures]. Nederlands Tijdschrift voor Geneeskunde **148:**2190-4.
27. **Kirkeby, S., C. J. Martel, and B. Aasted.** 2009. Infection with human H1N1 influenza virus affects the expression of sialic acids of metaplastic mucous cells in the ferret airways. Virus Research **144:**225-32.
28. **Knibbs, R. N., I. J. Goldstein, R. M. Ratcliffe, and N. Shibuya.** 1991. Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem **266:**83-8.
29. **Kogure, T., T. Suzuki, T. Takahashi, D. Miyamoto, K. I. Hidari, C. T. Guo, T. Ito, Y. Kawaoka, and Y. Suzuki.** 2006. Human trachea primary epithelial cells express both sialyl(alpha2-3)Gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and sialyl(alpha2-6)Gal receptor for human influenza viruses. Glycoconj J **23:**101-6.
30. **Koopmans, M., B. Wilbrink, M. Conyn, G. Natrop, H. van der Nat, H. Vennema, A. Meijer, J. van Steenbergen, R. Fouchier, A. Osterhaus, and A. Bosman.** 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet **363:**587-93.
31. **Krunkosky, T. M., B. M. Fischer, L. D. Martin, N. Jones, N. J. Akley, and K. B. Adler.** 2000. Effects of TNF-alpha on expression of ICAM-1 in human airway epithelial cells in vitro. Signaling pathways controlling surface and gene expression. Am J Respir Cell Mol Biol **22:**685-92.
32. **Leigh, M. W., R. J. Connor, S. Kelm, L. G. Baum, and J. C. Paulson.** 1995. Receptor specificity of influenza virus influences severity of illness in ferrets. Vaccine **13:**1468-73.
33. **Li, Z., H. Chen, P. Jiao, G. Deng, G. Tian, Y. Li, E. Hoffmann, R. G. Webster, Y. Matsuoka, and K. Yu.** 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. Journal Of Virology **79:**12058-64.
34. **Maines, T. R., L. M. Chen, Y. Matsuoka, H. Chen, T. Rowe, J. Ortin, A. Falcon, T. H. Nguyen, Q. Mai le, E. R. Sedyaningsih, S. Harun, T. M. Tumpey, R. O. Donis, N. J. Cox, K. Subbarao, and J. M. Katz.** 2006. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A **103:**12121-6.
35. **Maines, T. R., X. H. Lu, S. M. Erb, L. Edwards, J. Guarner, P. W. Greer, D. C. Nguyen, K. J. Szretter, L. M. Chen, P. Thawatsupha, M. Chittaganpitch, S. Waicharoen, D. T. Nguyen, T. Nguyen, H. H. Nguyen, J. H. Kim, L. T. Hoang, C. Kang, L. S. Phuong, W. Lim, S. Zaki, R. O. Donis, N. J. Cox, J. M.**

Katz, and T. M. Tumpey. 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. Journal Of Virology **79:**11788-800.

36. **Matrosovich, M. N., A. S. Gambaryan, S. Teneberg, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, and K. A. Karlsson.** 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:**224-34.
37. **Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk.** 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A **101:**4620-4.
38. **Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk.** 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:**4620-4.
39. **Medeiros, R., N. Escriou, N. Naffakh, J. C. Manuguerra, and S. van der Werf.** 2001. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology **289:**74-85.
40. **Nakajima, K., E. Nobusawa, A. Nagy, and S. Nakajima.** 2005. Accumulation of amino acid substitutions promotes irreversible structural changes in the hemagglutinin of human influenza AH3 virus during evolution. J Virol **79:**6472-7.
41. **Nicholls, J. M., A. J. Bourne, H. Chen, Y. Guan, and J. S. Peiris.** 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.
42. **Nicholls, J. M., M. C. Chan, W. Y. Chan, H. K. Wong, C. Y. Cheung, D. L. Kwong, M. P. Wong, W. H. Chui, L. L. Poon, S. W. Tsao, Y. Guan, and J. S. Peiris.** 2007. Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nature Medicine **13:**147-9.
43. **Nobusawa, E., T. Aoyama, H. Kato, Y. Suzuki, Y. Tateno, and K. Nakajima.** 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology **182:**475-85.
44. **Oshansky, C. M., T. M. Krunkosky, J. Barber, L. P. Jones, and R. A. Tripp.** 2009. Respiratory syncytial virus proteins modulate suppressors of cytokine signaling 1 and 3 and the type I interferon response to infection by a toll-like receptor pathway. Viral Immunol **22:**147-61.
45. **Oshansky, C. M., J. A. Pickens, K. C. Bradley, L. P. Jones, G. M. Saavedra-Ebner, J. P. Barber, J. M. Crabtree, D. A. Steinhauer, S. M. Tompkins, and R. A. Tripp.** 2011. Avian Influenza Viruses Infect Primary Human Bronchial Epithelial Cells Unconstrained by Sialic Acid alpha2,3 Residues. PLoS One **6:**e21183.
46. **Reed, L. J. M., H.** 1938. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene **27:**493-497.

47. **Rogers, G. N., and J. C. Paulson.** 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:**361-73.
48. **Rowe, T., A. J. Leon, C. J. Crevar, D. M. Carter, L. Xu, L. Ran, Y. Fang, C. M. Cameron, M. J. Cameron, D. Banner, D. C. Ng, R. Ran, H. K. Weirback, C. A. Wiley, D. J. Kelvin, and T. M. Ross.** 2010. Modeling host responses in ferrets during A/California/07/2009 influenza infection. Virology **401:**257-65.
49. **Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka.** 2006. Avian flu: influenza virus receptors in the human airway. Nature **440:**435-6.
50. **Shinya, K., S. Hamm, M. Hatta, H. Ito, T. Ito, and Y. Kawaoka.** 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:**258-66.
51. **Skehel, J. J., and D. C. Wiley.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Review of Biochemistry **69:**531-69.
52. **Smith, J. H., T. Nagy, E. Driskell, P. Brooks, S. M. Tompkins, and R. A. Tripp.** 2011. Comparative Pathology in Ferrets Infected with H1N1 Influenza A Viruses Isolated from Different Hosts. J. Virol.**:**JVI.00512-11.
53. **Song, H., H. Wan, Y. Araya, and D. R. Perez.** 2009. Partial direct contact transmission in ferrets of a mallard H7N3 influenza virus with typical avian-like receptor specificity. Virology journal **6:**12.
54. **Song, X., Y. Lasanajak, B. Xia, J. Heimburg-Molinaro, J. M. Rhea, H. Ju, C. Zhao, R. J. Molinaro, R. D. Cummings, and D. F. Smith.** 2011. Shotgun glycomics: a microarray strategy for functional glycomics. Nat Methods **8:**85-90.
55. **Song, X., H. Yu, X. Chen, Y. Lasanajak, M. M. Tappert, G. M. Air, V. K. Tiwari, H. Cao, H. A. Chokhawala, H. Zheng, R. D. Cummings, and D. F. Smith.** 2011. A sialylated glycan microarray reveals novel interactions of modified sialic acids with proteins and viruses. J Biol Chem **286:**31610-22.
56. **Steel, J., A. C. Lowen, S. Mubareka, and P. Palese.** 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathogens **5:**e1000252.
57. **Stevens, J., O. Blixt, L. Glaser, J. K. Taubenberger, P. Palese, J. C. Paulson, and I. A. Wilson.** 2006. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. Journal Of Molecular Biology **355:**1143-55.
58. **Stray, S. J., R. D. Cummings, and G. M. Air.** 2000. Influenza virus infection of desialylated cells. Glycobiology **10:**649-58.
59. **Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol **72:**6678-88.
60. **Subbarao, E. K., W. London, and B. R. Murphy.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. Journal Of Virology **67:**1761-4.
61. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K.**

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

62. **Sweet, C., R. A. Bird, D. Cavanagh, G. L. Toms, M. H. Collie, and H. Smith.** 1979. The local origin of the febrile response induced in ferrets during respiratory infection with a virulent influenza virus. Br J Exp Pathol **60:**300-8.
63. **Sweet, C., R. A. Bird, D. M. Coates, H. A. Overton, and H. Smith.** 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:**305-11.
64. **Sweet, C., J. C. Macartney, R. A. Bird, D. Cavanagh, M. H. Collie, R. H. Husseini, and H. Smith.** 1981. Differential distribution of virus and histological damage in the lower respiratory tract of ferrets infected with influenza viruses of differing virulence. J Gen Virol **54:**103-14.
65. **Thompson, C. I., W. S. Barclay, M. C. Zambon, and R. J. Pickles.** 2006. Infection of human airway epithelium by human and avian strains of influenza a virus. J Virol **80:**8060-8.
66. **Tripp, R. A., and S. M. Tompkins.** 2009. Animal models for evaluation of influenza vaccines. Curr Top Microbiol Immunol **333:**397-412.
67. **Tumpey, T. M., T. R. Maines, N. Van Hoeven, L. Glaser, A. Solorzano, C. Pappas, N. J. Cox, D. E. Swayne, P. Palese, J. M. Katz, and A. Garcia-Sastre.** 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science (New York, N Y) **315:**655-9.
68. **Tumpey, T. M., T. R. Maines, N. Van Hoeven, L. Glaser, A. Solorzano, C. Pappas, N. J. Cox, D. E. Swayne, P. Palese, J. M. Katz, and A. Garcia-Sastre.** 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science **315:**655-9.
69. **Uyeki, T. M., Y. H. Chong, J. M. Katz, W. Lim, Y. Y. Ho, S. S. Wang, T. H. Tsang, W. W. Au, S. C. Chan, T. Rowe, J. Hu-Primmer, J. C. Bell, W. W. Thompson, C. B. Bridges, N. J. Cox, K. H. Mak, and K. Fukuda.** 2002. Lack of evidence for human-to-human transmission of avian influenza A (H9N2) viruses in Hong Kong, China 1999. Emerging Infectious Diseases **8:**154-9.
70. **Van Hoeven, N., J. A. Belser, K. J. Szretter, H. Zeng, P. Staeheli, D. E. Swayne, J. M. Katz, and T. M. Tumpey.** 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:**2851-61.
71. **van Riel, D., V. J. Munster, E. de Wit, G. F. Rimmelzwaan, R. A. Fouchier, A. D. Osterhaus, and T. Kuiken.** 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:**1215-23.
72. **Vines, A., K. Wells, M. Matrosovich, M. R. Castrucci, T. Ito, and Y. Kawaoka.** 1998. The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. Journal Of Virology **72:**7626-31.
73. **Wan, H., E. M. Sorrell, H. Song, M. J. Hossain, G. Ramirez-Nieto, I. Monne, J. Stevens, G. Cattoli, I. Capua, L. M. Chen, R. O. Donis, J. Busch, J. C.**

Paulson, C. Brockwell, R. Webby, J. Blanco, M. Q. Al-Natour, and D. R. Perez. 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One **3:**e2923.

74. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-79.
75. **WHO.** 2011. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011.
76. **Xu, Q., W. Wang, X. Cheng, J. Zengel, and H. Jin.** 2010. Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. Journal Of Virology **84:**4936-45.
77. **Yen, H. L., A. S. Lipatov, N. A. Ilyushina, E. A. Govorkova, J. Franks, N. Yilmaz, A. Douglas, A. Hay, S. Krauss, J. E. Rehg, E. Hoffmann, and R. G. Webster.** 2007. Inefficient transmission of H5N1 influenza viruses in a ferret contact model. Journal Of Virology **81:**6890-8.
78. **Zeng, H., C. Goldsmith, P. Thawatsupha, M. Chittaganpitch, S. Waicharoen, S. Zaki, T. M. Tumpey, and J. M. Katz.** 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type i interferon response in polarized human bronchial epithelial cells. J Virol **81:**12439-49.
79. **Zhou, B., M. E. Donnelly, D. T. Scholes, K. St George, M. Hatta, Y. Kawaoka, and D. E. Wentworth.** 2009. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. J Virol **83:**10309-13.
80. **Zitzow, L. A., T. Rowe, T. Morken, W. J. Shieh, S. Zaki, and J. M. Katz.** 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol **76:**4420-9.

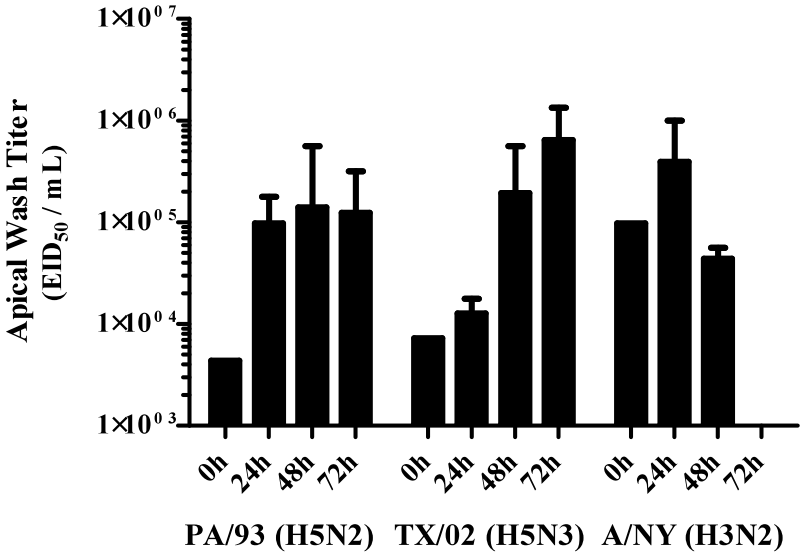


Figure 3.1 Avian influenza viruses replicate and are shed apically from NHBE cells. NHBE cells were infected with PA/93 (H5N2), TX/02 (H5N3), or A/NY/55/04 at MOI=0.001. At the times indicated post-infection, BEBM-0.05% BSA was added to the apical surface of the cells and incubated for 30 minutes at 37°C. EID_{50} titers were determined according the method of Reed and Meunch (46). Data are shown as means ± from two independent experiments.

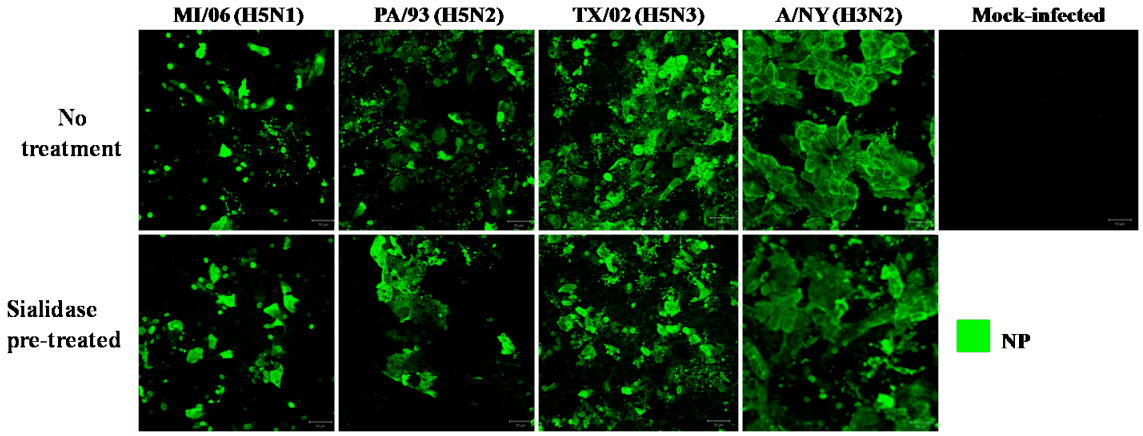


Figure 3.2 Neuraminidase (sialidase)-treated NHBE cells are robustly infected by LPAI strains. NHBE cells were mock-treated (top panels) or treated with 25mU/mL neuraminidase (bottom panels) for 1 hour at 37°C, washed with PBS, and infected with the indicated viruses at a MOI of 0.5. Cells were fixed in 3.7% formaldehyde at 24h pi and immunostained for influenza NP expression. Results shown are representative of two experiments.

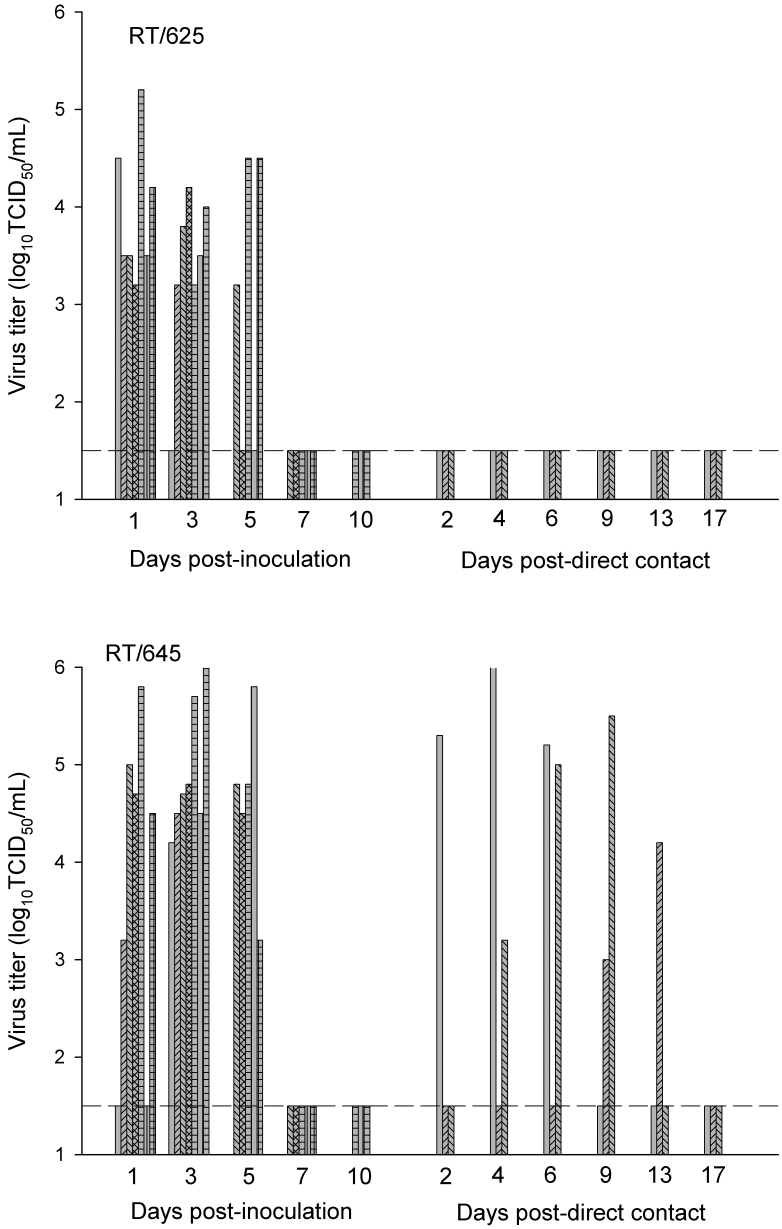
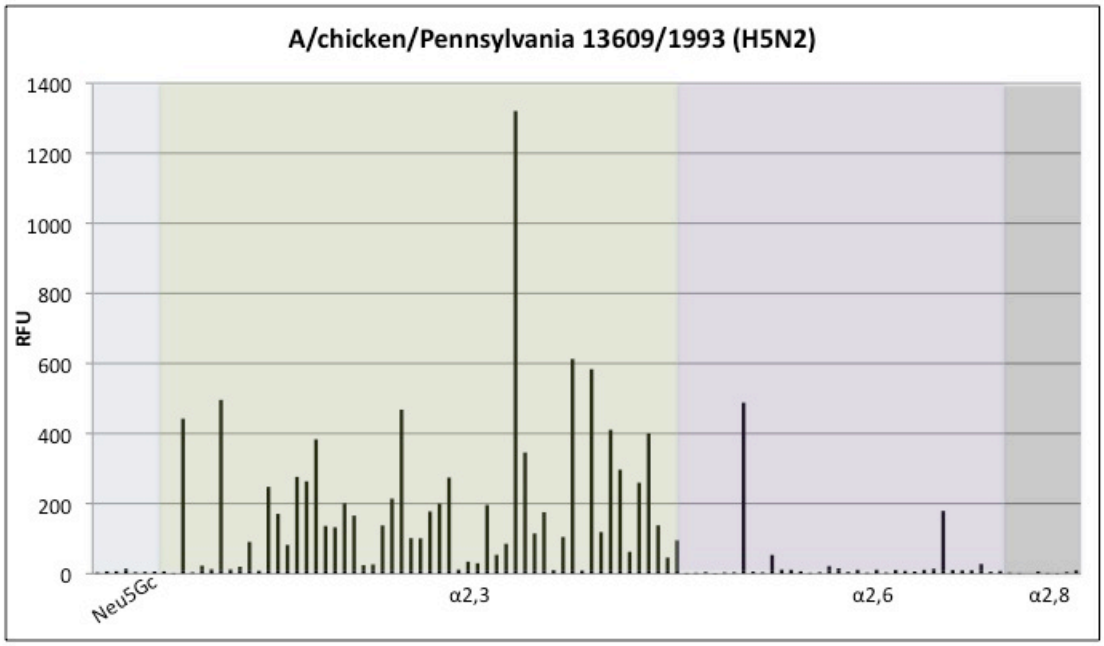
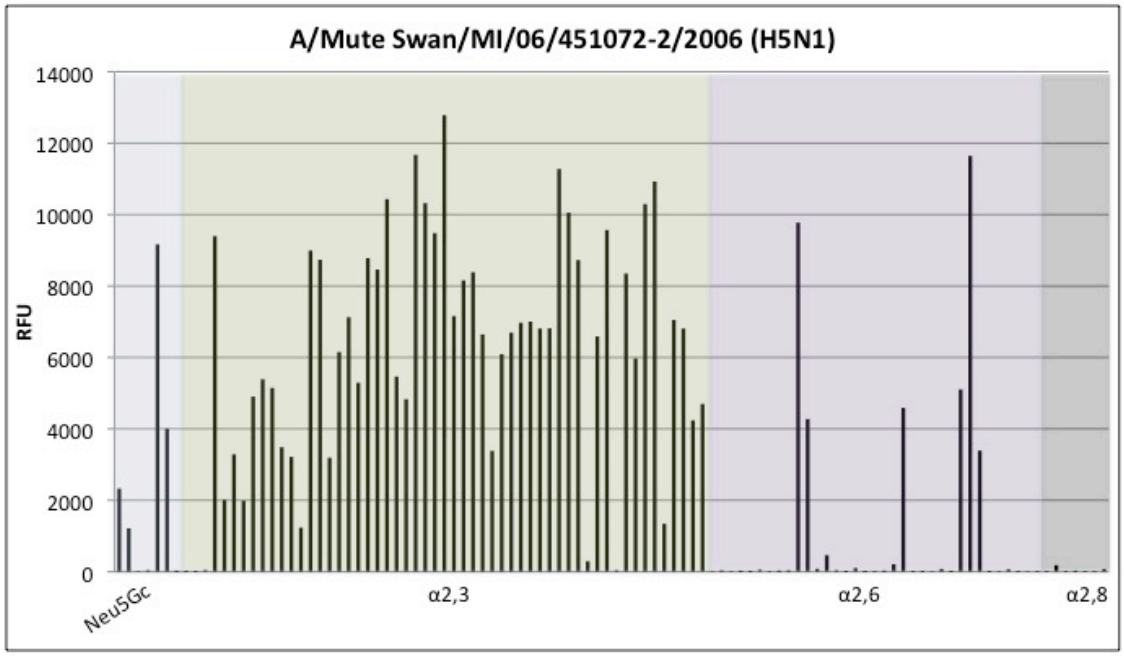


Figure 3.3 Replication and direct contact transmission of wild bird avian influenza viruses RT/625 (H6N1) and RT/645 (H9N1) in the upper respiratory tract of ferrets. Seven ferrets were intranasally inoculated with $5X10^5$ PFUs of either RT/625 or RT/645 and nasal washes were collected and titered on MDCK cells (days post-inoculation portion of graph). Three naïve ferrets were paired with three of the inoculated ferrets twenty four 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.

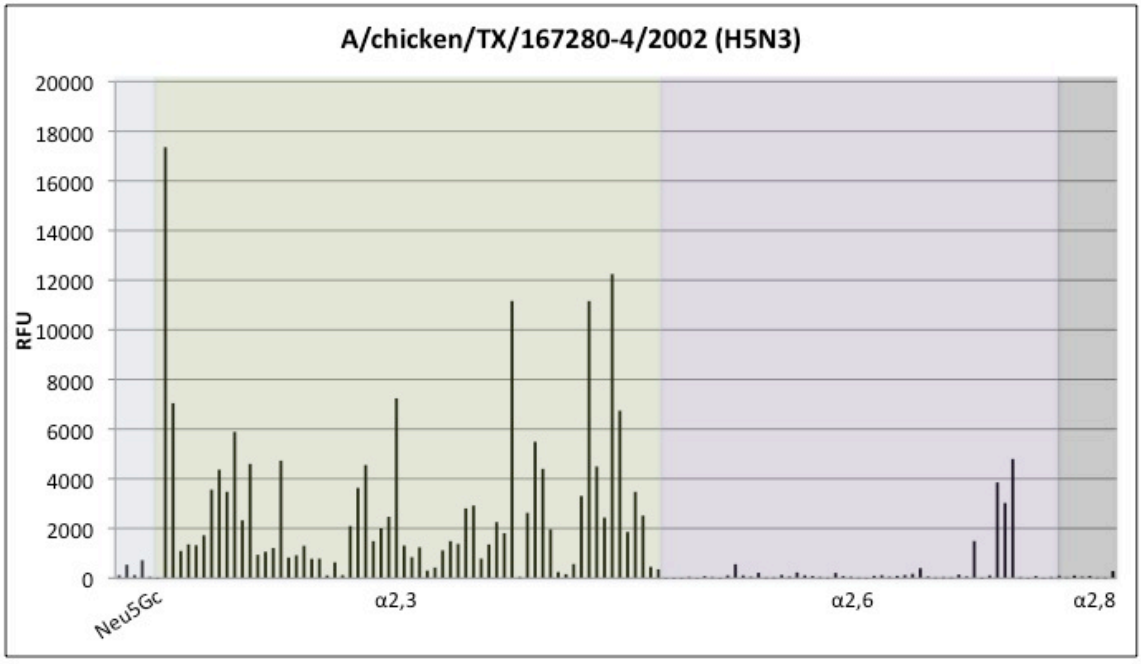
A


A/chicken/Pennsylvania 13609/1993 (H5N2)
RFU
1400
1200
1000
800
600
400
200
0
Neu5Gc
α2,3
α2,6
α2,8

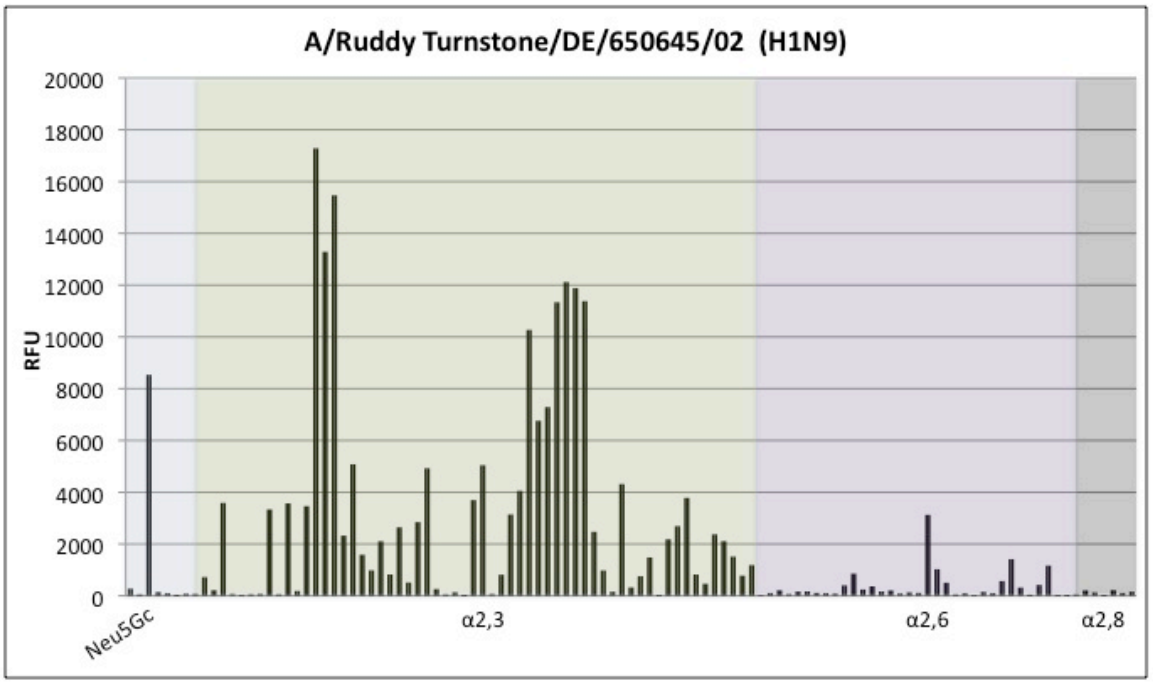
B.


A/Mute Swan/MI/06/451072-2/2006 (H5N1)
RFU
14000
12000
10000
8000
6000
4000
2000
0
Neu5Gc
α2,3
α2,6
α2,8

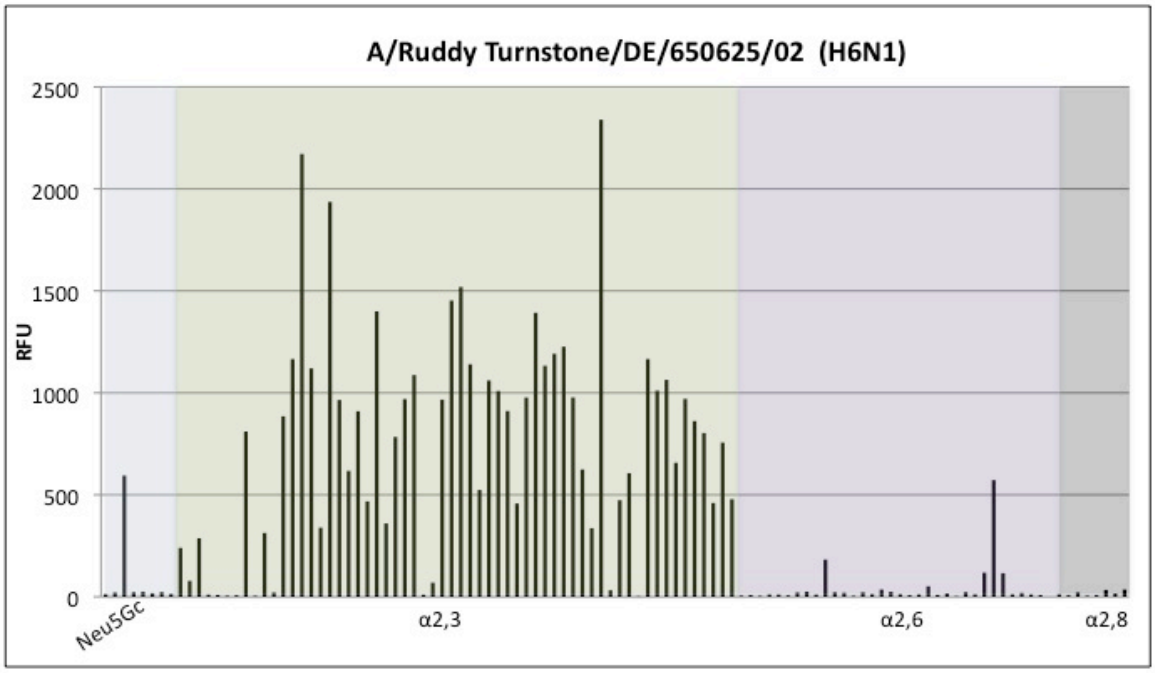
C.


A/chicken/TX/167280-4/2002 (H5N3)
RFU
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Neu5Gc
α2,3
α2,6
α2,8

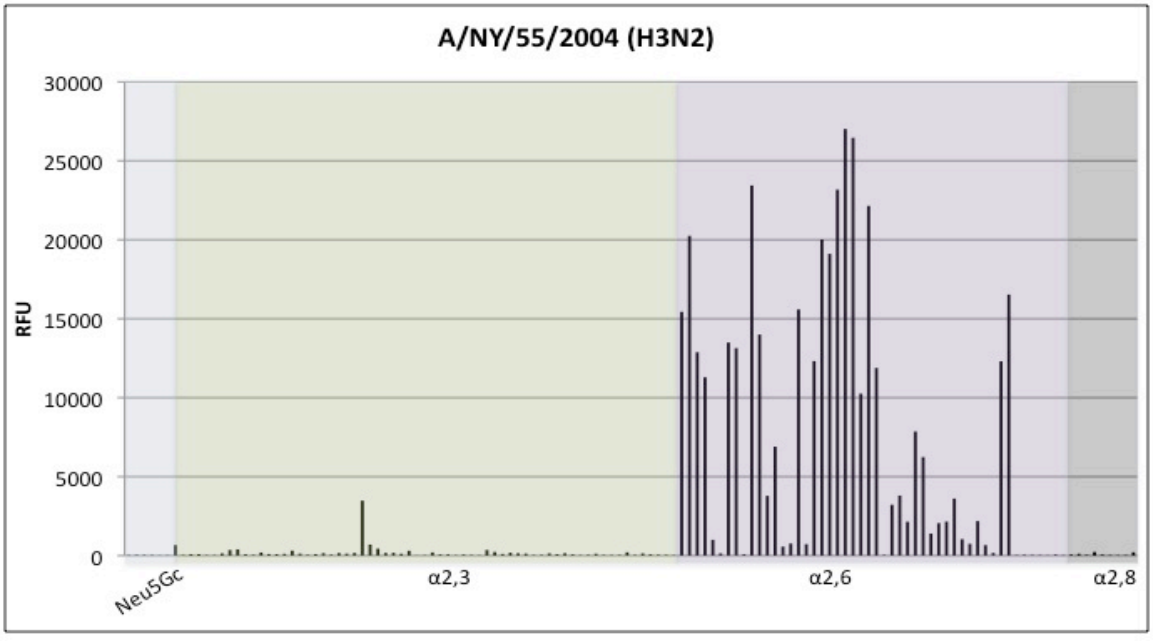
D.


A/Ruddy Turnstone/DE/650645/02 (H1N9)
RFU
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Neu5Gc
α2,3
α2,6
α2,8

E.


A/Ruddy Turnstone/DE/650625/02 (H6N1)
RFU
2500
2000
1500
1000
500
0
Neu5Gc
α2,3
α2,6
α2,8

F.


A/NY/55/2004 (H3N2)
RFU
30000
25000
20000
15000
10000
5000
0
Neu5Gc
α2,3
α2,6
α2,8

G.

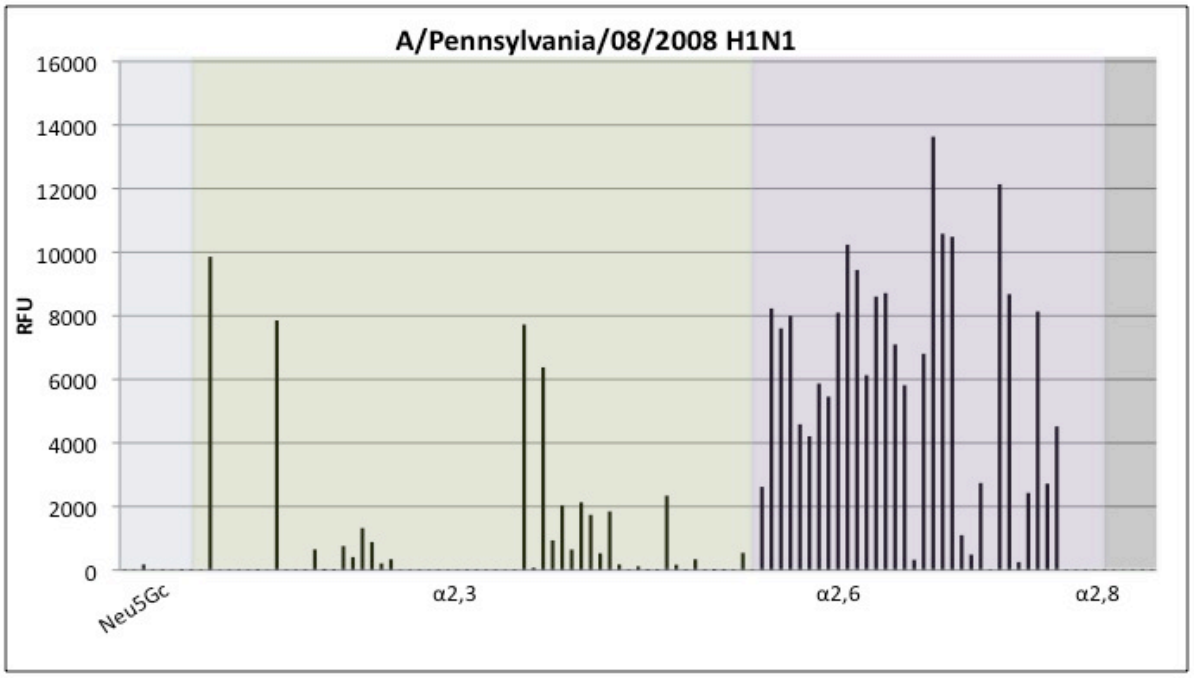


Figure 3.4 Domestic and wild aquatic LPAI strains preferentially bind α2,3 glycan moieties. Purified viral stocks (>10^5pfu/ml) were labeled with 25 µg of Alexa488 dye in 1M $NaHCO_3$ (pH 9) for 1 hour and dialyzed against 1mM EDTA/PBS overnight. The Core H of the Consortium of Functional Glycomics analyzed the labeled viruses via glycan array. The PA/93 (A), MI/06 (B), RT/645 (D), RT/625 (E), and PA/08 (G) were analyzed against 511 glycans (version 4.2), while the TX/02 (C) and A/NY/55 (F) were evaluated on the most recent enhanced Mammalian Printed Array Version 5.0 that contained 611 glycans. The graph represents the N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) α2,3, α2,6, and α2,8 glycans. RFU: relative fluorescent units.

Table 3.1 Morbidity, seroconversion, and respiratory viral replication of ferrets inoculated with H1N9 and H6N1 wild bird avian influenza viruses. Minimal morbidity was present in the H1N9 and H6N1 isolates infected ferrets, despite upper and lower respiratory viral replication. Direct contact transmission was observed with RT/645 but not in RT/625, with presence of upper respiratory shedding of virus and seroconversion. Seroconversion in both directly inoculated ferrets and infected transmission ferrets was poor.

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)**
RT/625 (H6N1)	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)
RT/645 (H1N9)	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)
Allantoic fluid	0/3	0.3 (1)	0/3	0/3	ND[d]	0/3[e]

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)**
RT/625	1/3	0.4-1.4	0/3	0/3	ND	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)

[a]Temperature is in degrees Celsius
[b]Limit of detection for nasal wash 1.5 $TCID_{50}$/mL
[c]Limit of detection for lung day 7 pi both viruses and day 3 pi for RT/625 is 1.2 $TCID_{50}$/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 3.2 Comparison of critical HA amino acids involved in receptor specificity.

Virus	Isolate ID	HA Subtype	Host	Hemagglutinin Amino Acids[a] 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/chicken/Pennsylvania /13609/1993	PA/93	H5	Avian	E	G	Q	G	AAF89540.1
A/chicken/Texas/167280-4/2002	TX/02	H5	Avian	E	G	Q	G	ACH88844.1
A/Mute Swan/MI/06/451072-2/2006	MI/06	H5	Avian	E	G	Q	G	ABV25968.1
A/Pennsylvania/08/2008	PA/08	H1	Human	D	D	Q	G	ACM51308.1
A/New York/55/2004	NY/04	H3	Human	D	D	V	S	AAY18564.1

[a] Hemagglutinin residues using H3 numbering

Table 3.3 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/Mute Swan/MI/06/451072-2/2006	MI/06	H5	Avian	64	128	NB
A/Pennsylvania/08/2008		H1	Human	128	0	32

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

CHAPTER 4

CLEAVAGE AND ACTIVATION OF LOW PATHOGENIC AVIAN INFLUENZA HEMAGGLUTININ BY ALTERNATIVE CELLULAR PROTEASES [1]

[1] Pickens, J.A., Galloway, S.E., Stalknecht, D.E., Steinhauer, D.A., Tompkins, S.M. To be submitted.

Abstract

Proteolytic activation of influenza virus hemagglutinin by host cell proteases is crucial for infectivity, where only a small number of proteases have been identified as facilitating HA cleavage. It has been well established that most low pathogenic avian influenza (LPAI) strains require exogenous trypsin to replicate in cell culture. We have identified a subset of LPAI viruses from the wild aquatic bird reservoir that replicate in culture independent of exogenous trypsin and are able to infect and replicate in mammals (i.e. mice and ferrets), where one isolate exhibited direct contact transmission. Moreover, both viruses exhibited limited dissemination to extrapulmonary tissues during infection. In this study, the two LPAI isolates (H1N9 and H6N1) that were examined in mice and ferrets were molecularly characterized. We show that these isolates replicate in multiple cell types independent of exogenous trypsin and that proteolytic activation of HA occurs regardless of trypsin in cell culture. Furthermore, we show that the trypsin-independent phenotype is a characteristic of whole virus, where the phenotype could not be reproduced in presence of the hemagglutinin and neuraminidase glycoproteins alone. Using a protease inhibitor screen, it appears that in the absence of exogenous trypsin, some of the LPAI isolates are capable of inducing novel proteases to facilitate hemagglutinin cleavage and infectivity. It is not clear what features of the virus enable this phenotype. Sequence analysis did not reveal a unique protease cleavage site and protease screens failed to identify a novel class of proteases responsible for cleavage. This study shows that there is still much to be elucidated about the mechanism by which avian influenza viruses are able to activate their HA proteins and infect outside of their normal tissue range.

Introduction

Influenza viruses are responsible for seasonal epidemics that account for significant morbidity and mortality, especially in young children and the elderly. Annually, they are linked to an average 41,000 deaths and 200,000 hospitalizations in the United States (8, 11). Influenza viruses belong to the *Orthomyxoviridae* family and are comprised of a segmented, negative sense RNA genomes that are capable of infecting a wide array of species. Wild aquatic birds are considered the primordial reservoir of all influenza A viruses and a contributing source of human influenza strains, where all human influenza A viruses contain one or more avian derived genetic factors (14, 15, 18). All 16 hemagglutinin (HA) and 9 neuraminidase (NA) have been identified in aquatic bird populations, providing a potential source for novel HA subtypes to which an immunologically naïve human population would be susceptible (12, 58). Avian influenza viruses (AIV) are classified as either high pathogenic (LPAI) or high pathogenic (HPAI) in birds based on *in vivo* examination in chickens and sequence of the HA proteolytic cleavage site. Currently, only H5 and H7 subtypes have been defined as HPAI in that they cause greater than 75% mortality in chickens infected intravenously and contain a polybasic cleavage site in the HA protein (40). It was originally proposed that avian influenza viruses were apathogenic in humans (1). However, this was not the case with the 1997 outbreak of HPAI (H5N1) in humans (24, 48, 49, 60). Even though it exhibited poor human to human transmission, it was extremely virulent in humans and subsequent outbreaks of H5N1 have been linked to greater than 330 deaths out of 550 cases (60). Our understanding of the viral and host determinants that dictate species and

tissue tropism is incomplete. It is critical to evaluate the mechanisms by which AIV are able to enhance their ability to infect and establish disease in mammals.

One of the primary determinants of pathogenicity of influenza viruses is the HA glycoprotein that facilitates infection by viral attachment and fusion with host cells. The receptor specificity for AIV and human influenza viruses is distinct, where AIV strains preferentially recognize α2,3-linked sialic acids while human strains predominantly bind α2,6-linked sialic acids. The HA protein is synthesized as a precursor HA0 that must undergo cleavage by cellular proteases, generating HA1 and HA2 subunits, liberating the HA fusion peptide enabling the viral fusion process and rendering the virus infectious (7, 34, 44, 53). It is the presence and localization (i.e. extracellular or subcellular) of these cellular proteases that contribute to the tissue tropism of influenza viruses. The amino acid sequence at the HA cleavage site also influences protease susceptibility and HA processing. Typically, LPAI viruses contain a monobasic (i.e. single arginine (R) or lysine (K)) HA cleavage site, making them susceptible to localized, extracellular trypsin-like proteases (2, 7, 16, 25, 31, 34). Several proteases have been shown to cleave HA glycoproteins, including mini-plasmin, tryptase Clara, and bacterial proteases (32, 35, 36, 39, 51). LPAI virus infections are typically asymptomatic in birds and it is localized by trypsin-like proteases of the gastrointestinal tract and respiratory tract (28, 29, 50). In contrast, HPAI viruses contain polybasic HA cleavage sites that are readily cleaved intracellularly by ubiquitously expressed substilin-proteases (e.g. furin, PC5/6) found in the Golgi, eliminating the requirement for respiratory or gastrointestinal proteases and making these viruses capable of systemic spread within the host (7, 26, 45, 56, 57). In human influenza virus infections the role of human airway respiratory proteases in HA

processing has been inadequately defined. Recent demonstrations of HA cleavage by human airway trypsin-like (HAT) protease and transmembrane protease serine S1 member 2 (TMPRSS2) have begun to shed light on this process, though it remains unclear what other uncharacterized cellular proteases may be capable of facilitating influenza infections in humans.

It is well established that LPAI isolates studied in cell culture require addition of trypsin for propagation (35). We have identified a proportion of North American LPAI viruses that undergo multiple rounds of replication and generate high viral titers in mammalian cell culture in the absence of exogenous trypsin. To confirm the trypsin-independent phenotype, 23 LPAI isolates were evaluated in various cell types including Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), African green monkey kidney (Vero), and chicken embryo fibroblast (DF-1). Moreover, we have shown that many of these LPAI viruses replicate and induce lesions in both the mouse and ferret model systems (in one case transmitting to naïve ferrets) without prior adaptation. Furthermore, some of these viruses were detected in fecal swabs or found in multiple organs, suggesting extrapulmonary spread (10). Here, we examine the HA cleavage and fusion of these trypsin-independent viruses and their potential activation by alternative cellular proteases to facilitate viral spread. We show that the trypsin-independent cleavage of the HA is not due to the presence of a polybasic cleavage site and that the viral NA does not facilitate HA cleavage. However, we demonstrate that virus infection induces proteolytic cleavage (not expressed during HA transfection studies) that is capable of activation of the specific HA, where only certain HA proteins are susceptible to cleavage. Thus, activation of HA by alternative protease cleavage is

HA restricted, but dependent upon viral infection for induction of protease expression. These studies have implications for identification of proteases responsible for HA cleavage, and thus determinants of virus tropism.

Material and Methods

Viruses and cells

The Southeastern Cooperative Wildlife Disease Study generously provided all avian influenza isolates and were collected from cloacal swabs collected as part of a North American wild aquatic bird surveillance initiative. The H6N1 AIV isolate, A/Ruddy Turnstone/DE/650625/02 (abbreviated RT/625), the H1N9 AIV isolate and A/Ruddy Turnstone/DE/650645/02 (abbreviated RT/645) were minimally passaged (> 3 total) in specific pathogen free 9 day old embryonated chicken eggs (ECE) at 37°C for 72 hours. The A/X-31 (H3N2) strain was propagated in specific pathogen free 9 day old embryonated chicken eggs (ECE) for 72 hours. All virus stocks were stored at -80°C. Viral titers (PFU/mL) were determined via plaque assays or tissue culture infectious dose 50% ($TCID_{50}$) on Madin-Darby canine kidney cells (MDCK) (17). All virus infections were completed at the indicated MOI in Dulbecco's modified Eagle medium (DMEM) or minimal essential medium (MEM) supplemented with 1μg/mL of TPCK-treated trypsin.

The Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), chicken embryo fibroblast (DF-1), and African green monkey kidney cells (Vero) were cultured in DMEM supplemented with 5% fetal bovine serum (FBS) at 37°C with 5% CO_2 until confluent. The BSR-T7 (BHK-21 cell line stably transfected with the T7 RNA expression plasmid) was maintained in DMEM supplemented with 10% FBS

and penicillin and streptomycin and alternating passages of 0.6mg/mL of geneticin (G-418) to maintain the stably transfected T7 RNA polymerase plasmid.

Two-step infection assay

The MDCK, MDBK, and DF-1 cells were infected with RT/645 and RT/625 at a MOI 0.001 in MEM supplemented with 1μg/mL of TPCK-trypsin for 2 hours at 37°C. The infection medium was washed off before the addition of MEM only for the (-) trypsin samples and the (+) trypsin samples received MEM supplemented with 1μg/mL of TPCK-trypsin. The cells incubated for 48 hours and time points were collected 4, 24, and 48 hours. All samples were titrated in MDCK cells by $TCID_{50}$ assay.

Plasmids and ligation independent cloning

The ligation independent cloning (LIC) plasmid, pCAGGS -LIC, was graciously provided by David Steinhauer (Microbiology and Immunology Department, Emory University School of Medicine) and contained LIC priming sequences flanking the cloning site. Subtype specific primers were generated to include the LIC priming sequence. Each forward primer contained the LIC priming sequence 5'-TACTTCCAATCCATTTGCCACC -3', while the reverse primer included the LIC priming sequence 5'TTATCCACTTCCATTTGTCA- 3'. The pCAGGS-LIC plasmid was cut with *SwaI* (New England Biosystems, Ipswich, MA) restriction enzymes at 25°C overnight, where the linear product (~4.5 kbp) was gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) per manufacturing instructions and dialyzed against deionized water before being quantified by spectrophotometry.

Total viral RNA was extracted from AIV infected allantoic fluid using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The HA and NA cDNA was generated by 2-step RT-PCR, where each amplification primers contained the corresponding LIC priming sequence and subtype specific start and stop sequences. The viral cDNA was generated using Superscript III First Strand synthesis kit (Invitrogen, Carlsbad, CA) using 4μL of total RNA and the gene specific forward primer as per the manufacture's protocol, allowing the cDNA reaction to incubate at 50°C for 50 minutes and terminating the reaction at 95°C for 5 minutes. The RNA template was eliminated following RNase H treatment. The HA and NA cDNA were PCR amplified using the Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) and subtype specific primers as per the manufacture's instructions. The HA and NA PCR amplified segments were gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) per manufacturing instructions and dialyzed against deionized water before being quantified by spectrophotometry.

The assembling of the *SwaI* (New England Biosystems, Ipswich, MA) cut pCAGGS-LIC plasmid with the RT/645 and RT/625 HA and NA PCR products was mediated by treatment of each with T4 DNA polymerase that was responsible for the generation of the single stranded ends. The pCAGGS-LIC (200ng) vector was treated with 3U/μL T4 DNA polymerase in the presence of dGTP (2.5 mM), while the HA and NA PCR products (200ng each) were treated separately with the same concentration on T4 DNA polymerase combined with of dCTP (2.5 mM). The T4 DNA polymerase treated plasmid and HA/NA PCR products were incubated on ice for 30 minutes to allow complementary single strand ends to interact and bind, resulting in the generation of a

pCAGGS –HA and pCAGGS –NA plasmids for the RT/645 and RT/625 isolates. The plasmids were transformed using chemically competent DH5α or Top Shot Top10 *E.coli* (Invitrogen, Carlsbad, CA) cells as per manufacture instructions. Each clone was verified by PCR and restriction digestion. Each plasmid was sequenced as stated below.

Sequencing HA cleavage site and cloning targets

The sequence of the HA cleavage site was determined on total viral RNA extracted from RT/645 and RT/625 infected allantoic fluid using the RNeasy kit (Qiagen) according to the manufacturer's protocol. One-step RT-PCR was performed on viral RNA using a set of HA0 primers as previously described by Gall *et al* (13).

HA0 Primer Name	**5' – 3' Sequence**
HA-1057.1-F	GGR GAA TGC CCC AAA TAY GT
HA-1057.2-F	GGR ARA TGC CCC AGR TAT GT
HA-1057.3-F	GGR GAA TGC CCC AAR TAY AT
HA-1232.1(555)-R	*CTG AGT CCG AAC ATT GAG* TTG CTA TGV TGR TAW CCA TAC CA
HA-1232.2(555)-R	*CTG AGT CCG AAC ATT GAG* TTY TGA TGY CTG AAD CCR TAC CA

The HA product was excised and gel purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). In addition, the cloned HA and NA segments in the pCAGGS vector were sequenced using LIC priming sequences.

LIC Primer Name	5' - 3' Sequence
pCAGGS-LIC-FWD	TACTTCCAATCCATTTGCCACC
pCAGGS-LIC-REV	TTATCCACTTCCATTTGTCA

All sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA) using the primers listed above.

Transfections

Transfections were completed using Vero cell lines. The RT/645 and RT/625 pCAGGS-HA and pCAGGS- NA plasmids were transfected individually or in combination using Lipofectamine and PLUS reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The pCAGGS-HA plasmid was transfected at a concentration of 1μg/reaction depending on cell type, while the pCAGGS- NA plasmid was used at a concentration of 500ng/reaction. Each transfection reaction was incubated at 37°C for 4 hours in Opti-MEM before being washed with 1x PBS and allowed to incubate for 24 hours in DMEM supplemented with 10% FBS.

Metabolic labeling

Metabolic labeling was used to examine HA cleavage in virus infected or transfected cells. Briefly, the cells were starved with MEM (-MET) medium that lacked methionine at 37°C for 30 minutes followed by labeling medium that contained 25μCi [^{35}S]-methionine at 37°C for 15 minutes. The labeling medium was removed and replaced with chase medium that contained 2mM cold methionine in DMEM supplemented with 5% FBS at 37°C for 3 hours for transfected cells and 90 minutes for

virus infected cells. Following the metabolic labeling, the trypsin (+) subset of cells was treated with 5μg/mL of TPCK-treated trypsin at 37°C for 20 minutes and the trypsin (-) subset received DMEM only. The radiolabeled cells were lysed in RIPA buffer, cell debris was removed by centrifugation and samples were stored at -20°C overnight. The radiolabeled samples were immunoprecipitated with subtype specific polyclonal antisera on protein G dynabeads (Invitrogen, Carlsbad, CA) . The HA immunoprecipitated proteins were analyzed by SDS-PAGE and visualized on autoradiography screens.

T7 luciferase fusion assay

For the T7 luciferase fusion assay, the T7-luciferase plasmid was transfected in Vero cells at a concentration of 1μg/reaction along with 1μg/reaction of pCAGGS- HA plasmid alone or co-transfected with 500 ng/reaction of pCAGGS-NA plasmid. At 24 hours post-transfection, the (+) trypsin subset of cells was treated with 5μg/mL of TPCK-treated trypsin at 37°C for 30 minutes, while the (-) trypsin samples were incubated with DMEM only. The cells were subsequently treated with 20μg/mL of soybean trypsin inhibitor at 37°C for 20 minutes. The Vero cells were washed and the BSR-T7 cells, in DMEM with 10%FBS, were added as an overlay on top of the Vero cells and allowed to incubate at 37°C for 1 hour. The cells were pH pulsed with pH-adjusted PBS at pH5 and pH7 for 5 minutes at 37°C, where after the pH pulse the cells were washed with PBS and incubated in DMEM/10%FBS medium for 6 hours at 37°C. The luciferase activities of cellular lysates were determined by a commercially available luciferase reporter kit (Promega, Madison, WI).

Protease Inhibitors

Protease inhibitor (Roche, Indianapolis, IN) screen was conducted in MDCK cells infected with RT/625, RT/645, X-31 infection at an MOI of 0.1 for 2 hours at 37°C either in the presence of the inhibitor (pretreatment) or following (post treatment) the 2 hour infection. Final concentrations of antipain-dihydrochloride, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, EDTA-NA_2, aprotinin, and pefabloc were added as indicated to the DMEM or MEM at specified times. Each sample was metabolic labeled and immunoprecipitated as previously stated.

Results

Avirulent avian influenza replicates independent of exogenous trypsin

One of the criteria for definition of a LPAI virus or de-selection of a HPAI virus is the requirement for an exogenous protease source, i.e. addition of trypsin to enable replication in cell culture; while HPAI viruses can replicate independent of exogenous trypsin (35). With this being an established phenotype for LPAI designation, we asked whether individual LPAI viruses isolated from wild aquatic and shore birds were capable of replicating without exogenous trypsin in cell culture. We screened over 400 LPAI isolates collected throughout North American that included a range of HA and NA subtypes isolated from a variety of species. We found that greater than 10% of the AIV isolates replicated to high titers in MDCK cells in the absence of exogenous trypsin, regardless of avian species, collection location, or HA and NA subtypes (data shown in the Appendix). We selected 2 AIV isolates, an H1N9 (A/Ruddy Turnstone/DE/650645 or RT/645) and an H6N1 (A/Ruddy Turnstone/DE/650625 or RT/625) that replicated to

high viral titers in MDCK cells and established infections in the mouse and ferret model system. Similar viral titers were observed under (+) and (-) trypsin conditions by plaque assay for the RT/645 and RT/625 isolates, while the trypsin dependent control virus, X-31, generated viral titers only in the presence of exogenous trypsin (Figure 4.1A). Furthermore, sequencing analysis confirmed that the RT/645 and RT/625 isolates grown in the presence or absence of trypsin maintained the prototypical monobasic, arginine (R), HA cleavage site (Table 4.1). There were no insertions or mutations at the HA cleavage site to serve as a possible explanation as to why the RT/645 and RT/625 isolates were able to replicate to high titer independent of trypsin. To further examine the trypsin independent replication of the RT/645 and RT/625 isolates in various cell lines, a two-step infection approach was employed using MDCK, MDBK and DF-1 cell lines. Viral replication was observed in the presence of trypsin for all cell types. Interestingly, the RT/645 and RT/625 isolates were able to replicate absence of trypsin in the MDCK and MDBK cell lines while viral replication in the DF-1 cell lines were trypsin dependent for all the isolates (Figure 4.1B). Two separate infection approaches confirmed the trypsin independent phenotype for the RT/645 and RT/625 isolates in MDCK and MDBK (mammalian) cell lines, regardless of HA or NA subtype.

Trypsin independent hemagglutinin cleavage observed in MDCK and Vero cells

Since these AIV isolates exhibited trypsin independent replication by plaque assay and two-step infection assay, we were interested in examining proteolytic HA cleavage in MDCK and Vero cells, two cell lines extensively used for the study of HA cleavage and fusion [(22, 23, 42, 61). Both isolates appeared to undergo HA cleavage

independent of the exogenous trypsin in both MDCK and Vero cells (Figure 4.2A). As expected, the negative control, X-31 strain, did not undergo HA cleavage in the absence of trypsin in either of the two cell lines (Figure 4.2A). To confirm viral replication under these experimental conditions, plaque assays were completed on the supernatants from RT/645 and RT/625 grown in MDCK and Vero cells under (+) and (-) trypsin conditions (Figure 4.2B). The RT/645 and RT/625 isolates replicated in the presence and absence of trypsin in MDCK cells, as exhibited in the original screen. Moreover, robust viral replication was also detected from virus infected Vero cells regardless of exogenous trypsin. This was particularly striking as Vero cells are known to inactivate trypsin and impair trypsin-dependent replication of influenza virus in culture (30). Therefore, the trypsin independent viral replication was associated with proteolytic activation of HA in MDCK and Vero cells.

Neuraminidase does not enhance proteolytic activation of HA or fusion in the absence of exogenous trypsin

The trypsin independent replication of RT/645 and RT/625 in various cell lines led us to investigate the viral components employed by the virus to mediate trypsin independent replication. While most influenza strains require exogenous trypsin to propagate in cell culture, there have been examples where other viral proteins facilitate replication. Goto et al. demonstrated a novel mechanism by which A/WSN/33 was able to achieve HA cleavage in trypsin negative systems, where the viral neuraminidase facilitated HA cleavage by sequestering plasminogen present in serum (20). As serum was not present in the trypsin-negative cultures for RT/645 and RT/625, this was not

likely to be the mechanism specific mechanism of HA cleavage; however we sequenced the NA genes from each virus to look for the mutation in NA residue 146 (N2 numbering) from an asparagine (N) to a tyrosine (Y) or arginine (R), which would result in a loss of glycosylation allowing sequestering of plasminogen that could mediate HA cleavage (19, 21, 36, 37). For all the isolates, the NA maintained a N146 residue. This did not rule out a contribution by NA of RT/645 and RT/625 NA in mediating HA cleavage independent of exogenous trypsin, thus we assayed their function using an *in vitro* transfection system. The RT/645 and RT/625 neuraminidase genes were cloned into the pCAGGS expression vector, where each clone was transfected into Vero cells to verify protein expression by SDS-PAGE. To determine that the expressed NA proteins were enzymatically active, neuraminidase activity was verified using a fluorescently tagged neuraminidase substrate, MUNANA (Figure 4.3A). Both RT/645 and RT/625 expressed NA proteins that were enzymatically active as compared to the previously examined N1, N4, and N9 positive controls. Next, HA cleavage was examined as a function of NA expression. Vero cells were transfected with either RT/645 or RT/625 HA alone or co-transfected with congruent NA or mismatched NA under (+) or (-) trypsin conditions (Figure 4.3B). Although minimal HA cleavage observed in the (-) trypsin samples, those in the presence of trypsin exhibited efficient cleavage. However, the addition of the NA did not improve HA proteolytic activation for either isolate as no apparent enhancement was observed in the cells that were co-transfected with the pCAGGS-HA and pCAGGS-NA plasmids as compared to cells transfected with pCAGGS-HA only.

There was a modest amount of HA proteolytic cleavage observed in the Vero cells in the absence of trypsin and we tested whether the low levels of cleaved HA were sufficient to mediate viral fusion. To address this possibility, we employed a T7 luciferase approach to examine the fusiogenic capabilities of the isolates under (-) and (+) trypsin conditions. Vero cells were transfected with a plasmid that encoded the firefly luciferase gene under the control of a T7 promoter along with a pCAGGS-HA plasmid alone or with congruent or mismatched pCAGGS-NA plasmids. Only Vero cells that display cleaved HA are capable of undergoing fusion with the target BSR-T7 cells. Upon fusion, the T7 luciferase plasmid is transported into the BSR-T7 cell and transcribed by the T7 polymerase, and fusion is quantified by luciferase expression. The level of membrane fusion can be positively correlated with luciferase expression levels. In the absence of trypsin, no fusion was observed for either isolate, though fusion was demonstrated in the presence of trypsin (Figure 4.4). Interestingly, Vero cells that were co-transfected with pCAGGS-HA and pCAGGS-NA underwent higher levels of fusion than Vero cells transfected with pCAGGS-HA alone, suggesting that NA is contributing to fusion efficiency. The minor amount of HA cleavage demonstrated in the absence of trypsin was not sufficient to mediate viral fusion.

Avian influenza recruits alternative cellular proteases in the absence of exogenous trypsin

The trypsin independent phenotype was only observed during virus infection. This led us to evaluate the contribution of specific classes of cellular proteases during infection with trypsin independent LPAI strains. A protease inhibitor screen was

performed on influenza infected MDCK cells (without exogenous trypsin, except where noted) to identify cellular proteases involved in infectivity and HA cleavage. Initially, the cells were pretreated with a series of 10 protease inhibitors that were known to interfere with a wide array of cellular proteases (i.e. trypsin, cysteine, serine, etc.) (Figure 4.5A). Infectivity was examined for MDCK cells pretreated with each inhibitor prior to a 2 hour infection in presence of the inhibitor. The infected cells were washed and infectivity was detected 24 hour post infection by $TCID_{50}$ and only one serine protease inhibitor, Pefabloc, repressed viral replication for both RT/645 and RT/625 isolates. All other inhibitors examined did not obstruct viral replication, generating viral titers similar to the untreated virus only controls.

At the same time, we analyzed whether or not treatment with the protease inhibitors, interfered with proteolytic HA cleavage. Pefabloc and aprotinin inhibit serine proteases, while pepstatin targets aspartyl proteases. Leupeptin affects a wide arrangement of proteases, such as cysteine, serine and threonine peptidases, however among all of these inhibitors, pefabloc is membrane permeable and irreversible inhibitor (9). In the initial protease inhibitor screen, we pretreated MDCK cells with each inhibitor prior to infection and continued treatment throughout the 5 hour infection before examining HA cleavage (Figure 4.5B). The virus infected cells treated with the leupeptin, aprotinin, and pepstatin all underwent HA cleavage, but the cells that received pefabloc treatment exhibited no HA signal for either the HA0 precursor or the HA1 and HA2 cleavage products for both isolates. The lack of any HA protein in infected cells pretreated with either concentration (250 or 500μg/mL) of pefabloc lead us to examine the toxicity of the protease inhibitor. To address the possibility of cytotoxicity induced

by the pefabloc protease inhibitor treatment, cell viability was analyzed for protease inhibitor treated MDCK cells by trypan blue staining. No substantial difference in cytotoxicity was observed between the treated cells and untreated cells at the 5 hour time point (data not shown). However, there was significant cell damage observed in the initial protease inhibitor screen at the 24 hour time point. The 500 μg/mL (2mM) or 250 μg/mL (1mM) pefabloc concentration were suggested concentrations from the manufacturer and were substantially higher than what has been used in the literature and appeared to be potentially toxic (4, 6).

Even though pefabloc was potentially toxic to the cells at high concentrations, we were interested in further examining the role of serine proteases on the infectivity and HA cleavage of the trypsin independent RT/645 and RT/625 isolates. Recently, it has been shown that serine proteases, TMPRSS2 and HAT, were capable of HA proteolytic activation in the absence of trypsin and that inhibiting TMPRSS2 and HAT with the serine protease inhibitor pefabloc abolishes influenza replication (4, 6). We questioned the potential of the RT/645 and RT/625 isolates to induce serine proteases capable of mediating HA cleavage in the absence of trypsin. The serine inhibitors, aprotinin, leupeptin, and pefabloc were examined and the concentrations of the protease inhibitors were adjusted to match what was previously shown in the literature to inhibit influenza replication and HA cleavage (4, 6). The pefabloc concentrations 50μM, 200μM, and 1mM were used, while the aprotinin and leupeptin concentration were increased to 50μM. Viral replication was not inhibited in the cells that received the lower concentrations of each of the serine inhibitors during (pre-treated) or following (post-treated) virus infection (Figure 4.6A and B). These results were confirmed by qRT-PCR

of viral M gene from cell lysates, where all samples exhibited similar threshold cycles (Ct) as the virus only (+trypsin) positive control (data not shown). As expected, no virus replication was detected in either virus pre- or post-treatment with 1mM of pefabloc (Figure 4.6A and B). Since pefabloc is known to be a potent serine protease inhibitor and has been shown to directly influence viral replication, we wanted to examine HA cleavage in the presence of increasing concentrations of pefabloc at 2 and 5 hours post infection. There was no difference in HA cleavage for both isolates at the 50μM or 200μM pefabloc concentrations at 2 or 5 hours post infection, while the trypsin dependent X-31 only underwent HA cleavage in the presence of trypsin (Figure 4.7A and B). The RT/645 and RT/625 isolates exhibited trypsin independent HA cleavage regardless of pefabloc treatment. It does not appear that serine proteases are contributing to the trypsin independent phenotype observed in the RT/645 and RT/625 isolates.

Discussion

Wild aquatic birds are the established reservoir for influenza viruses and little is known about the ability of these AIV strains to cross species-specific barriers and cause disease in mammals. Our previous study demonstrated the ability of these two isolates to replicate and cause histopathologic lesions in the mouse model without prior adaptation (10). Furthermore, the RT/645 and RT/625 were shown to readily infect and replicate in the ferret model without adaptation, and the (H1N9) RT/645 was transmitted to naïve ferrets by direct contact (Chapter 3). The objective of this study was to characterize the proteolytic HA activation and fusion properties of the wild bird LPAI RT/645 and RT/625 isolates that exhibit the capacity to establish infection independent of exogenous

trypsin, which may enhance their potential to infect mammals. One of the defining features of LPAI strains is the trypsin requirement for viral replication, which is not case for HPAI strains, where HA cleavage occurs intracellularly by ubiquitously expressed proteases (i.e. furin and PC5/6) producing infectious progeny virions (27, 38, 45, 57). However, this is not typically the case for LPAI strains that undergo extracellular HA cleavage by localized, trypsin-like proteases (33, 35). We have identified several LPAI isolates that do not adhere to the trypsin requirement for replication in culture, where they replicate to high viral titers in the absence of trypsin (Figure 4.1A). In the initial screen, the RT/645 and RT/625 isolates generated similar titers as when grown in the presence of trypsin (Figure 4.1A). In addition, the RT/645 and RT/625 isolates were was able to undergo multiple rounds of replication (as shown in Figure 4.1B), where over 48 hours there was trypsin independent replication in the absence of exogenously added trypsin in the MDCK and MDBK cell lines. Similar results were seen with Vero cell infections (Figure 4.2B), despite the ability of Vero cells to inactivate trypsin (30). Interestingly, both of these viruses disseminated to extrapulmonary tissues during infection in ferret and mouse models.

Since LPAI isolates normally require proteolytic activation of HA to facilitate viral entry and fusion, we wanted to assess if there were alterations to HA cleavage in the absence of trypsin (3, 16, 31, 43, 59). We first examined the HA cleavage site sequence for viruses grown in the presence and absence of exogenous trypsin. Sequence analysis of the HA cleavage site revealed that each isolate maintained the prototypic monobasic arginine (R) (Table 4.1) and that there were no basic amino acid insertions or mutations present at the cleavage site making it more accessible to intracellular proteolytic

activation. The HA cleavage site sequence was preserved regardless of trypsin treatment. Next, we confirmed HA cleavage for each isolate in MDCK and Vero cell lines. Each isolate exhibited similar levels of HA cleavage regardless of trypsin treatment in these cells (Figure 4.2A). While, the trypsin dependent X-31 strain exhibited HA cleavage only in the presence of trypsin in both cell lines (Figure 4.2A). Furthermore, each isolate exhibited high viral titers following infection in MDCK and Vero cells regardless of trypsin treatment (Figure 4.2B). The trypsin independent phenotype was confirmed in multiple mammalian cell lines, where the general permissiveness of the cell contributed to the replication capacity of the virus.

The viral NA glycoprotein has multiple functions to facilitate influenza spread. In A/WSN/33, the NA mediates HA cleavage in the absence of trypsin by sequestering plasminogen (19, 21, 36, 47). Furthermore, Tumpey et al. have shown that the HA and NA glycoproteins from the 1918 influenza virus contributed to the enhanced virulence observed in mammals (54, 55). While, Su et al. demonstrated that NA mediated HA cell-to-cell fusion and enhanced infectivity in avian H5 and H7 subtypes in the presence and absence of trypsin (46). We hypothesized that trypsin independent replication was a result of the NA glycoprotein enhancing HA cleavage for the RT/645 and RT/625 isolates. Vero cells transfected with HA only or in combination with enzymatically active NA did not improve HA cleavage regardless of trypsin treatment (Figure 4.3). The minor level of HA cleavage observed in the absence of trypsin was not sufficient to mediate viral fusion, where a threshold of HA cleavage must be overcome for the virus to achieve fusion and infectivity (Figure 4.4). Therefore, NA glycoprotein did not appear to enhance HA cleavage during trypsin independent viral replication. However in the

presence of trypsin, there was an apparent enhancement in fusion for cells transfected with both the HA and NA plasmids as compared to cells that received HA only. This trend was shown in both isolates and while we did not explore the mechanism of enhanced fusion here, it does support the results shown by Su et al, where the presence of the NA improved HA fusion and viral entry (46). The NA primary function is the removal of sialic acids to eliminate HA mediated binding during virion release. Thus, the NA mediated removal of sialic acids from HA receptor binding site may enhance the fusogenic and infectivity properties of the virus, which is not possible in the cells that express HA alone.

The cleavage of HA by proteases with trypsin-like specificities is an established requirement for propagation of influenza LPAI strains in cell culture. We have over 40 LPAI isolates that do not abide by trypsin dependent constraints. These wild bird AIV isolates appear to be utilizing alternative pathways for achieving HA cleavage in the absence of exogenous trypsin. Recent work has demonstrated that the family of type II transmembrane serine proteases, transmembrane protease S1 member 2 (TMPRSS2), TMPRSS4, TMPRSS13, human airway trypsin-like protease (HAT), are capable of cleaving HA that contain a single arginine at the cleavage site and facilitating trypsin independent viral spread (4-6). Bottcher et al. has shown that MDCK cells that transiently expressed membrane bound HAT and TMPRSS2 were capable of HA cleavage in the absence of trypsin (5). Furthermore, the HAT protease was responsible for HA cleavage of incoming virions at the cell surface and newly synthesized HA before and during progeny virion release, while TMPRSS2 cleaved newly synthesized HA within the cell. We hypothesized that the trypsin independent replication phenotype resulted from virus

induction of serine proteases that were capable of HA activation and infectivity. The trypsin independent phenotype is only present in virus infected cells and could not be reproduced in any of the transfection experiments. This shifted our focus away from examining viral genetic factors contributing to the trypsin independent replication and led to investigate the induction, activation, and/or recruitment of alternative serine proteases during virus infection to enable influenza replication. In the initial screen, no detectable viral titers or HA cleavage was present when the cells were treated with the potent serine protease inhibitor, pefabloc, which lead us to purpose that the RT/645 and RT/625 isolates were inducing serine proteases to facilitate trypsin independent replication and HA activation. Since the mechanism of action for the pefabloc is distinct from the leupeptin and aprotinin serine protease inhibitors. Pefabloc is an irreversible serine inhibitor that chemically modifies the proteases' active site by acylation, while leupeptin and aprotinin are reversible competitive inhibitors that bind and block the serine active site (9, 41, 52, 63). As a starting point, half of the highest suggested starting concentration was used, where high concentrations (up to 500 μg/mL at 2mM) of pefabloc were used as compared to lower concentrations of leupeptin (2.5 μg/mL at 5.2μM) and aprotinin (1μg/mL at 150 nM), which are at the lower end of the range used by others for similar studies (6, 62). At the 2mM and 1mM concentrations, the pefabloc appeared to be toxic to the cells during the pre- and post-treatment, shutting down all cellular functions and offering an explanation as to why no HA protein or viral titers was present in the original screen (Figure 4.5A and B). In contrast, other serine protease inhibitors used in the original screen, such as aprotinin and leupeptin, did not inhibit AIV replication or HA cleavage (Figure 4.5A & B). The replication and HA cleavage

observed in cell treated with leupeptin and aprotinin was a result of using minimal inhibitor concentrations. The effective concentration of the inhibitors is especially important for the reversible competitive inhibitors, leupeptin and aprotinin, where lower concentrations are less effective at blocking the active site, especially in the presence of the increasing concentrations of substrate. In the second approach, the concentrations of each serine protease inhibitors were adjusted to 50μM and 200μM for the pefabloc and increased to 50μM for aprotinin and leupeptin. At the lower protease inhibitor concentrations, there were negligible effects on virus replication and HA cleavage as compared to the virus only positive controls (Figure 4.6 and 4.7). The AIV isolates were still able to undergo replication and proteolytic HA activation in the presence of the serine protease inhibitor regardless of trypsin treatment (Figure 4.6A). It does not appear from the result proved here that the trypsin independent replication of the RT/645 and RT/625 isolates is through the induction of a serine protease, but rather through a serine and trypsin independent mechanism. To be able to deduce the exact interaction of the protease inhibitors during LPAI infection and HA activation, the optimal protease inhibitor concentration and establishing the proper controls would need to be determined to ensure that the inhibitors are working accurately to eliminate protease activity. Furthermore to fully explore the induction of alternative proteases, we are interested in utilizing mRNA arrays to look for increases in levels of cellular protease mRNA in virus infected cells.

The recruitment and induction of cellular proteases responsible for influenza infectivity remain to be elucidated but we have shown that select LPAI isolates can induce or activate alternative proteases to achieve infectivity in culture in the absence of

trypsin. However, our understanding of proteolytic activation of LPAI is incomplete and there are other mechanism(s) in place to help facilitate infection. This may provide some explanation for variable dissemination of LPAI viruses in organs and tissues outside of the respiratory and gastrointestinal tracts seen during experimental infection in animal models. To fully understand the cellular protease expression and activation during influenza infection, a complete characterization of influenza-stimulated protease expression would need to be conducted, and compared to specific protease activities, while we did not attempt that here, we do show the understanding of proteases responsible for HA activation during influenza virus infection remains undefined.

References:

1. **Beare, A. S., and R. G. Webster.** 1991. Replication of avian influenza viruses in humans. Arch Virol **119:**37-42.
2. **Bosch, F. X., W. Garten, H. D. Klenk, and R. Rott.** 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses. Virology **113:**725-35.
3. **Bosch, F. X., M. Orlich, H. D. Klenk, and R. Rott.** 1979. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology **95:**197-207.
4. **Bottcher, E., C. Freuer, T. Steinmetzer, H. D. Klenk, and W. Garten.** 2009. MDCK cells that express proteases TMPRSS2 and HAT provide a cell system to propagate influenza viruses in the absence of trypsin and to study cleavage of HA and its inhibition. Vaccine **27:**6324-9.
5. **Bottcher, E., T. Matrosovich, M. Beyerle, H. D. Klenk, W. Garten, and M. Matrosovich.** 2006. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J Virol **80:**9896-8.
6. **Bottcher-Friebertshauser, E., C. Freuer, F. Sielaff, S. Schmidt, M. Eickmann, J. Uhlendorff, T. Steinmetzer, H. D. Klenk, and W. Garten.** 2010. Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors. J Virol **84:**5605-14.
7. **Chen, J., K. H. Lee, D. A. Steinhauer, D. J. Stevens, J. J. Skehel, and D. C. Wiley.** 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell **95:**409-17.
8. **Cox, N. J., and K. Subbarao.** 2000. Global epidemiology of influenza: past and present. Annu Rev Med **51:**407-21.
9. **Dentan, C., A. D. Tselepis, M. J. Chapman, and E. Ninio.** 1996. Pefabloc, 4-[2-aminoethyl]benzenesulfonyl fluoride, is a new, potent nontoxic and irreversible inhibitor of PAF-degrading acetylhydrolase. Biochim Biophys Acta **1299:**353-7.
10. **Driskell, E. A., C. A. Jones, D. E. Stallknecht, E. W. Howerth, and S. M. Tompkins.** 2010. Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. Virology **399:**280-9.
11. **Dushoff, J., J. B. Plotkin, C. Viboud, D. J. Earn, and L. Simonsen.** 2006. Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. Am J Epidemiol **163:**181-7.
12. **Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus.** 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol **79:**2814-22.
13. **Gall, A., B. Hoffmann, T. Harder, C. Grund, and M. Beer.** 2008. Universal primer set for amplification and sequencing of HA0 cleavage sites of all influenza A viruses. J Clin Microbiol **46:**2561-7.

14. **Gammelin, M., A. Altmuller, U. Reinhardt, J. Mandler, V. R. Harley, P. J. Hudson, W. M. Fitch, and C. Scholtissek.** 1990. Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol **7:**194-200.
15. **Garten, R. J., C. T. Davis, C. A. Russell, B. Shu, S. Lindstrom, A. Balish, W. M. Sessions, X. Xu, E. Skepner, V. Deyde, M. Okomo-Adhiambo, L. Gubareva, J. Barnes, C. B. Smith, S. L. Emery, M. J. Hillman, P. Rivailler, J. Smagala, M. de Graaf, D. F. Burke, R. A. Fouchier, C. Pappas, C. M. Alpuche-Aranda, H. Lopez-Gatell, H. Olivera, I. Lopez, C. A. Myers, D. Faix, P. J. Blair, C. Yu, K. M. Keene, P. D. Dotson, Jr., D. Boxrud, A. R. Sambol, S. H. Abid, K. St George, T. Bannerman, A. L. Moore, D. J. Stringer, P. Blevins, G. J. Demmler-Harrison, M. Ginsberg, P. Kriner, S. Waterman, S. Smole, H. F. Guevara, E. A. Belongia, P. A. Clark, S. T. Beatrice, R. Donis, J. Katz, L. Finelli, C. B. Bridges, M. Shaw, D. B. Jernigan, T. M. Uyeki, D. J. Smith, A. I. Klimov, and N. J. Cox.** 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **325:**197-201.
16. **Garten, W., F. X. Bosch, D. Linder, R. Rott, and H. D. Klenk.** 1981. Proteolytic activation of the influenza virus hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage. Virology **115:**361-74.
17. **Gaush, C. R., and T. F. Smith.** 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl Microbiol **16:**588-94.
18. **Gorman, O. T., W. J. Bean, and R. G. Webster.** 1992. Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. Curr Top Microbiol Immunol **176:**75-97.
19. **Goto, H.** 2004. [Novel function of plasminogen-binding activity of the NA determines the pathogenicity of influenza A virus]. Uirusu **54:**83-91.
20. **Goto, H., and Y. Kawaoka.** 1998. A novel mechanism for the acquisition of virulence by a human influenza A virus. Proc Natl Acad Sci U S A **95:**10224-8.
21. **Goto, H., K. Wells, A. Takada, and Y. Kawaoka.** 2001. Plasminogen-binding activity of neuraminidase determines the pathogenicity of influenza A virus. J Virol **75:**9297-301.
22. **Govorkova, E. A., S. Kodihalli, I. V. Alymova, B. Fanget, and R. G. Webster.** 1999. Growth and immunogenicity of influenza viruses cultivated in Vero or MDCK cells and in embryonated chicken eggs. Dev Biol Stand **98:**39-51; discussion 73-4.
23. **Govorkova, E. A., G. Murti, B. Meignier, C. de Taisne, and R. G. Webster.** 1996. African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. J Virol **70:**5519-24.
24. **Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840-2.
25. **Hayashi, T.** 1985. [A study on the causes of an epidemic of influenza, especially an analysis of relative humidity as a main cause]. Nihon Ika Daigaku Zasshi **52:**272-80.

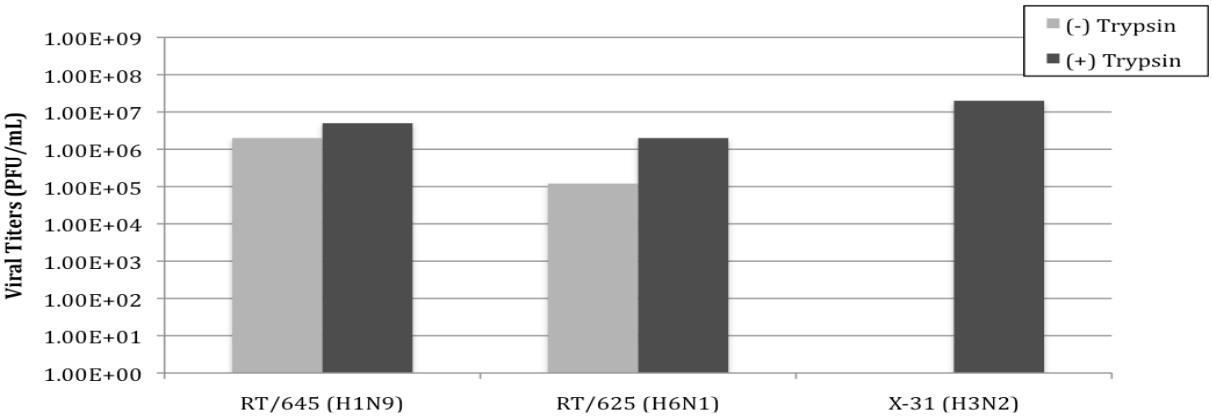
26. **Horimoto, T., and Y. Kawaoka.** 1995. The hemagglutinin cleavability of a virulent avian influenza virus by subtilisin-like endoproteases is influenced by the amino acid immediately downstream of the cleavage site. Virology **210:**466-70.
27. **Horimoto, T., K. Nakayama, S. P. Smeekens, and Y. Kawaoka.** 1994. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol **68:**6074-8.
28. **Ito, T., and Y. Kawaoka.** 2000. Host-range barrier of influenza A viruses. Vet Microbiol **74:**71-5.
29. **Ito, T., Y. Suzuki, T. Suzuki, A. Takada, T. Horimoto, K. Wells, H. Kida, K. Otsuki, M. Kiso, H. Ishida, and Y. Kawaoka.** 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. J Virol **74:**9300-5.
30. **Kaverin, N. V., and R. G. Webster.** 1995. Impairment of multicycle influenza virus growth in Vero (WHO) cells by loss of trypsin activity. J Virol **69:**2700-3.
31. **Kawaoka, Y., and R. G. Webster.** 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A **85:**324-8.
32. **Kido, H., M. Murakami, K. Oba, Y. Chen, and T. Towatari.** 1999. Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. Mol Cells **9:**235-44.
33. **Kido, H., Y. Yokogoshi, K. Sakai, M. Tashiro, Y. Kishino, A. Fukutomi, and N. Katunuma.** 1992. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem **267:**13573-9.
34. **Klenk, H. D., R. Rott, and M. Orlich.** 1977. Further studies on the activation of influenza virus by proteolytic cleavage of the haemagglutinin. J Gen Virol **36:**151-61.
35. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426-39.
36. **Lazarowitz, S. G., A. R. Goldberg, and P. W. Choppin.** 1973. Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: host cell activation of serum plasminogen. Virology **56:**172-80.
37. **Li, S., J. Schulman, S. Itamura, and P. Palese.** 1993. Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. J Virol **67:**6667-73.
38. **Morsy, J., W. Garten, and R. Rott.** 1994. Activation of an influenza virus A/turkey/Oregon/71 HA insertion variant by the subtilisin-like endoprotease furin. Virology **202:**988-91.
39. **Murakami, M., T. Towatari, M. Ohuchi, M. Shiota, M. Akao, Y. Okumura, M. A. Parry, and H. Kido.** 2001. Mini-plasmin found in the epithelial cells of bronchioles triggers infection by broad-spectrum influenza A viruses and Sendai virus. Eur J Biochem **268:**2847-55.
40. **OIE.** 2003. Manual of standards for diagnostic tests and vaccines. . Office of International des Epizooties.

41. **Ovcharenko, A. V., and O. P. Zhirnov.** 1994. Aprotinin aerosol treatment of influenza and paramyxovirus bronchopneumonia of mice. Antiviral Res **23:**107-18.
42. **Rott, R., M. Orlich, H. D. Klenk, M. L. Wang, J. J. Skehel, and D. C. Wiley.** 1984. Studies on the adaptation of influenza viruses to MDCK cells. EMBO J **3:**3329-32.
43. **Rott, R., M. Reinacher, M. Orlich, and H. D. Klenk.** 1980. Cleavability of hemagglutinin determines spread of avian influenza viruses in the chorioallantoic membrane of chicken embryo. Arch Virol **65:**123-33.
44. **Steinhauer, D. A.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.
45. **Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J **11:**2407-14.
46. **Su, B., S. Wurtzer, M. A. Rameix-Welti, D. Dwyer, S. van der Werf, N. Naffakh, F. Clavel, and B. Labrosse.** 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One **4:**e8495.
47. **Suarez, D. L.** 2000. Evolution of avian influenza viruses. Vet Microbiol **74:**15-27.
48. **Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol **72:**6678-88.
49. **Subbarao, K., and M. W. Shaw.** 2000. Molecular aspects of avian influenza (H5N1) viruses isolated from humans. Rev Med Virol **10:**337-48.
50. **Suzuki, Y., T. Ito, T. Suzuki, R. E. Holland, Jr., T. M. Chambers, M. Kiso, H. Ishida, and Y. Kawaoka.** 2000. Sialic acid species as a determinant of the host range of influenza A viruses. J Virol **74:**11825-31.
51. **Tashiro, M., P. Ciborowski, M. Reinacher, G. Pulverer, H. D. Klenk, and R. Rott.** 1987. Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. Virology **157:**421-30.
52. **Tashiro, M., H. D. Klenk, and R. Rott.** 1987. Inhibitory effect of a protease inhibitor, leupeptin, on the development of influenza pneumonia, mediated by concomitant bacteria. J Gen Virol **68 (Pt 7):**2039-41.
53. **Taubenberger, J. K.** 1998. Influenza virus hemagglutinin cleavage into HA1, HA2: no laughing matter. Proc Natl Acad Sci U S A **95:**9713-5.
54. **Tumpey, T. M., A. Garcia-Sastre, J. K. Taubenberger, P. Palese, D. E. Swayne, and C. F. Basler.** 2004. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci U S A **101:**3166-71.
55. **Tumpey, T. M., A. Garcia-Sastre, J. K. Taubenberger, P. Palese, D. E. Swayne, M. J. Pantin-Jackwood, S. Schultz-Cherry, A. Solorzano, N. Van Rooijen, J. M. Katz, and C. F. Basler.** 2005. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar

macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol **79:**14933-44.

56. **Walker, J. A., and Y. Kawaoka.** 1993. Importance of conserved amino acids at the cleavage site of the haemagglutinin of a virulent avian influenza A virus. J Gen Virol **74 (Pt 2):**311-4.
57. **Walker, J. A., S. S. Molloy, G. Thomas, T. Sakaguchi, T. Yoshida, T. M. Chambers, and Y. Kawaoka.** 1994. Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus. J Virol **68:**1213-8.
58. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-79.
59. **Webster, R. G., and R. Rott.** 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell **50:**665-6.
60. **WHO.** 2011. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011.
61. **Yates, P. J., J. S. Bootman, and J. S. Robertson.** 1990. The antigenic structure of a human influenza A (H1N1) virus isolate grown exclusively in MDCK cells. J Gen Virol **71 (Pt 8):**1683-8.
62. **Zhirnov, O. P., M. R. Ikizler, and P. F. Wright.** 2002. Cleavage of influenza a virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. J Virol **76:**8682-9.
63. **Zhirnov, O. P., T. Y. Matrosovich, M. N. Matrosovich, and H. D. Klenk.** 2011. Aprotinin, a protease inhibitor, suppresses proteolytic activation of pandemic H1N1v influenza virus. Antivir Chem Chemother **21:**169-74.

A.



B.

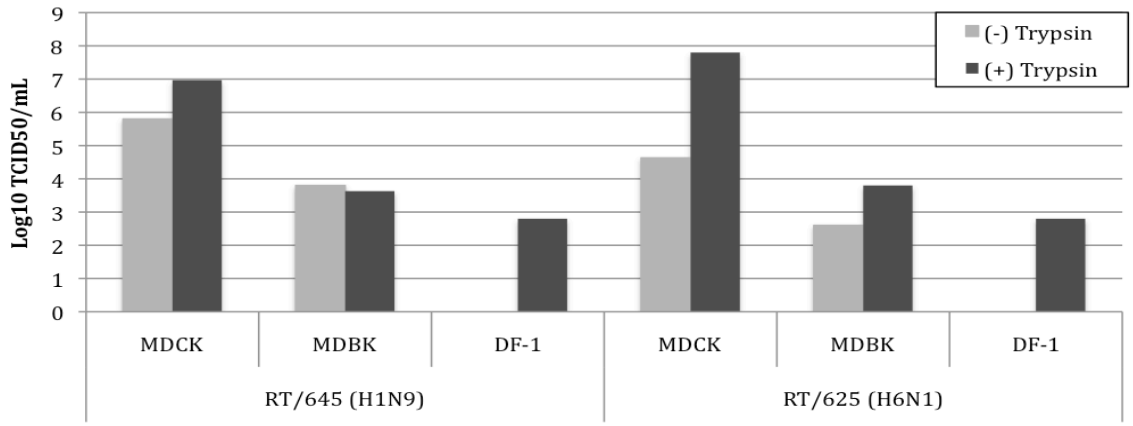
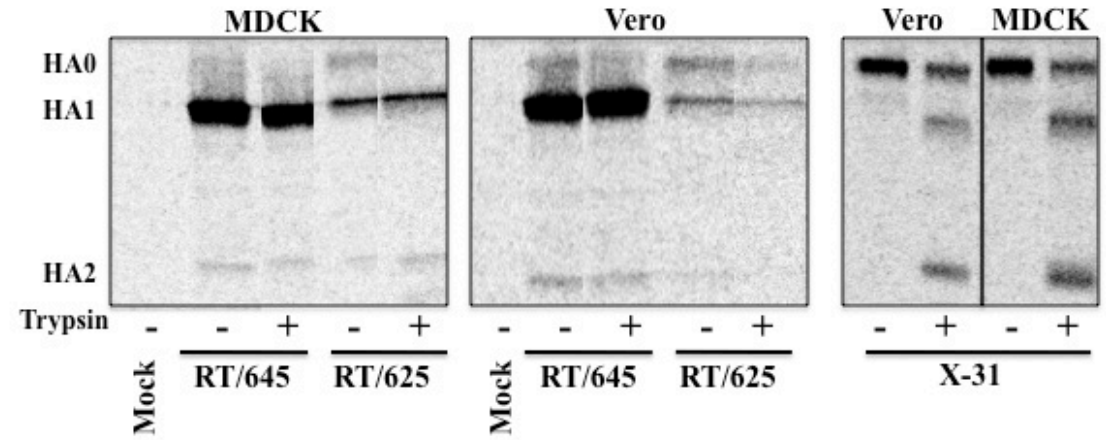


Figure 4.1. Trypsin independent replication observed in MDCK, MDBK and DF-1 cell lines. (A) Viral titers were determined by plaque assay on MDCK cells under (+) trypsin conditions, where the Avicel overlay supplemented with 1μg/mLTPCK trypsin and the (-) trypsin samples received Avicel only overlay. (B) MDCK, MDBK, and DF-1 cells were infected with RT/645 and RT/625 at a MOI 0.001 in MEM supplemented with 1μg/mLof TPCK-trypsin for 2 hours at 37°C. The infection medium was washed off before the addition of MEM only for the (-) trypsin samples, where the (+) trypsin samples received MEM supplemented with 1μg/mLof TPCK-trypsin. The cells incubated for 48 hours and time points were collected at 4,24, and 48 hours, where the titers shown represent the highest titers present during the course of the assay. All samples were titrated in MDCK cells by $TCID_{50}$ assay.

A.



B.

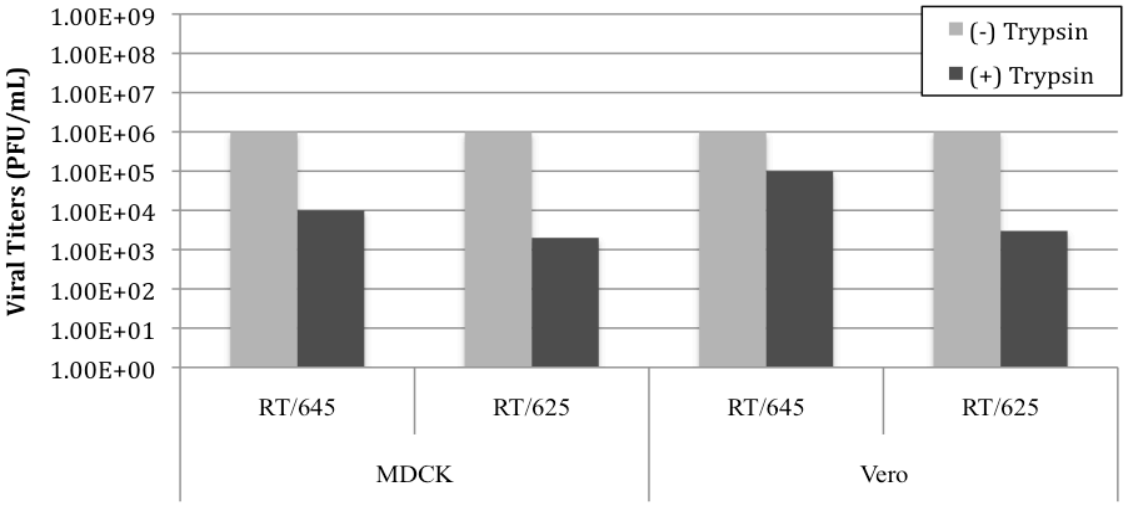
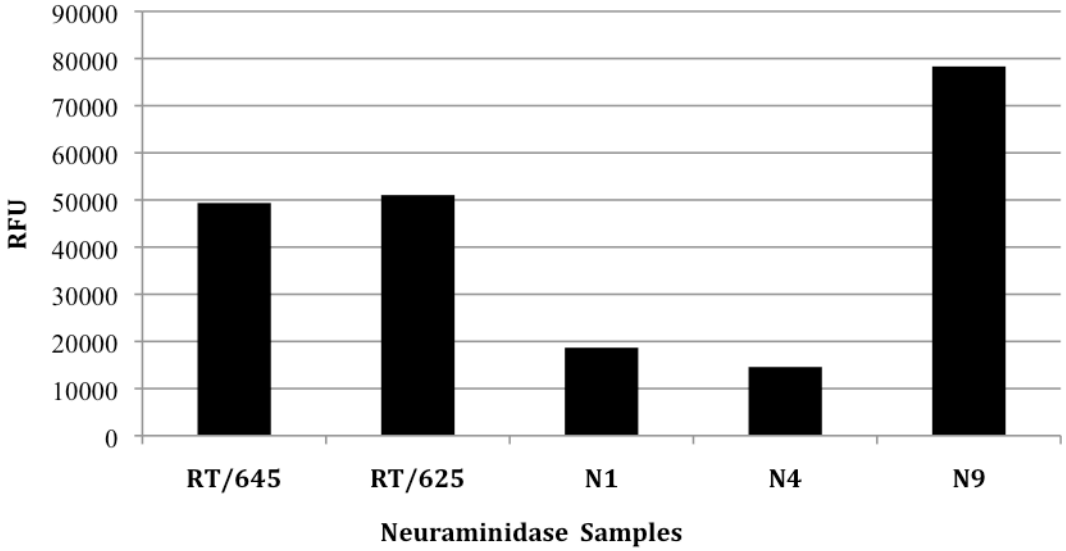


Figure 4.2 Trypsin independent hemagglutinin cleavage and replication in LPAI infected MDCK and Vero cells. (A). MDCK and Vero cells were infected with RT/645, RT/625, and X-31 at an MOI of 0.1 for 37°C for 2 hours. The mock-infected wells received DMEM only. The infected cells were washed prior to the addition of DMEM/10% FBS, which was allowed to incubate at 37°C for 4 hours before being radiolabeled and trypsin treated. The (-) trypsin cells received DMEM only, while the (+) trypsin cells were trypsin treated with 5μg/mLof TPCK-trypsin for 15 minutes. All samples were immunoprecipitated using HA subtype specific polyclonal antibodies and analyzed by SDS-PAGE. (B) Viral titers were examined in MDCK and Vero cells infected with RT/645 and RT/625 at an MOI of 0.1 for 2 hours before receiving MEM supplemented with 1μg/mLof TPCK-trypsin or MEM only. At 24 hours post infection, viral titers were determined by plaque assay on MDCK cells.

A.



B.

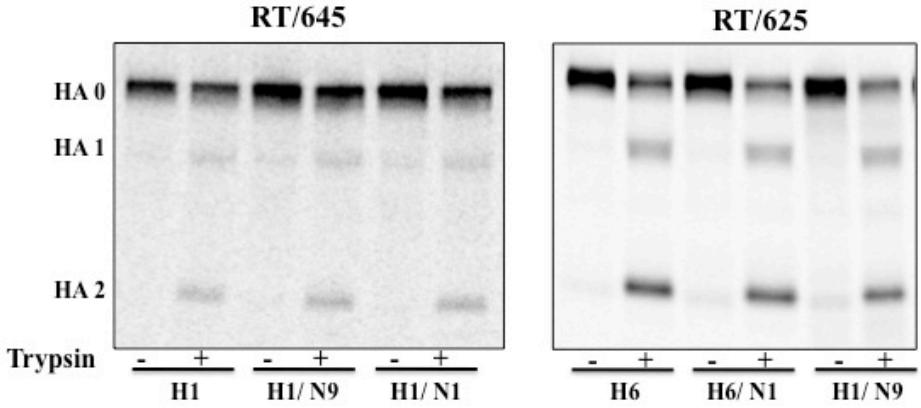


Figure 4.3. Trypsin independent hemagglutinin cleavage is not mediated by functionally active neuraminidase. (A) Vero cells were transfected with 1μg of RT/645 or RT/625 pCAGGS-NA plasmid and neuraminidase activity was measured by a fluorescent MUNANA substrate assay. The N1, N4, and N9 were previously identified as exhibiting neuraminidase activity and were used as positive controls. RFU: relative fluorescence units. (B) Vero cells were transfected with either HA alone, corresponding NA, or HA/mismatched NA from the RT/645 (H1N9) and RT/625 (H6N1) generated plasmids. Samples were radiolabeled and treated with (-) or (+) 5μg/mL of TPCK-trypsin before immunoprecipitated using HA subtype specific antibodies. HA cleavage was examined by SDS-PAGE.

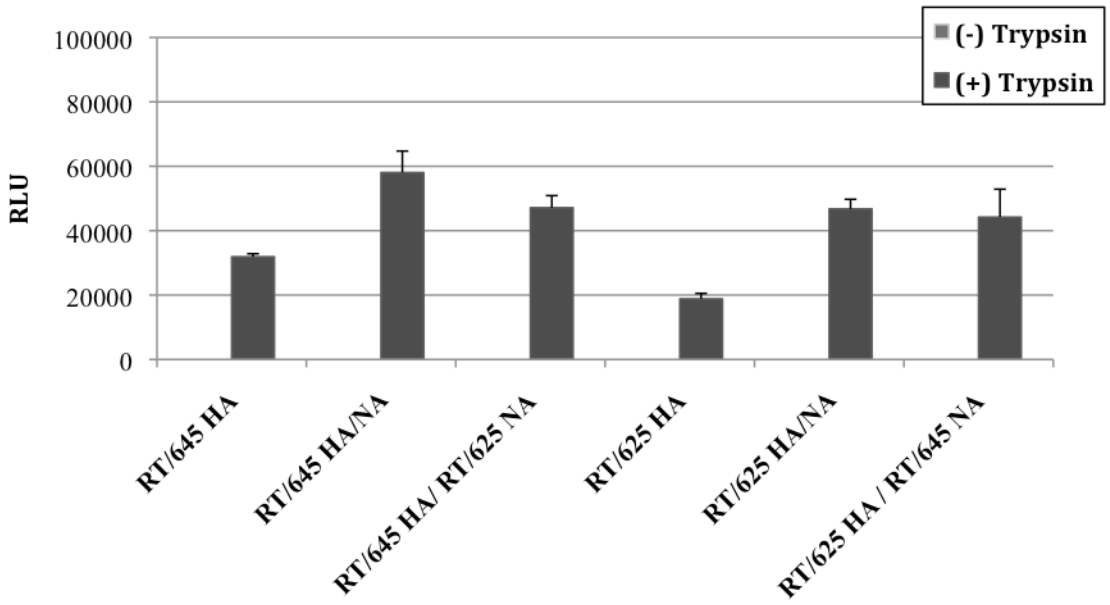
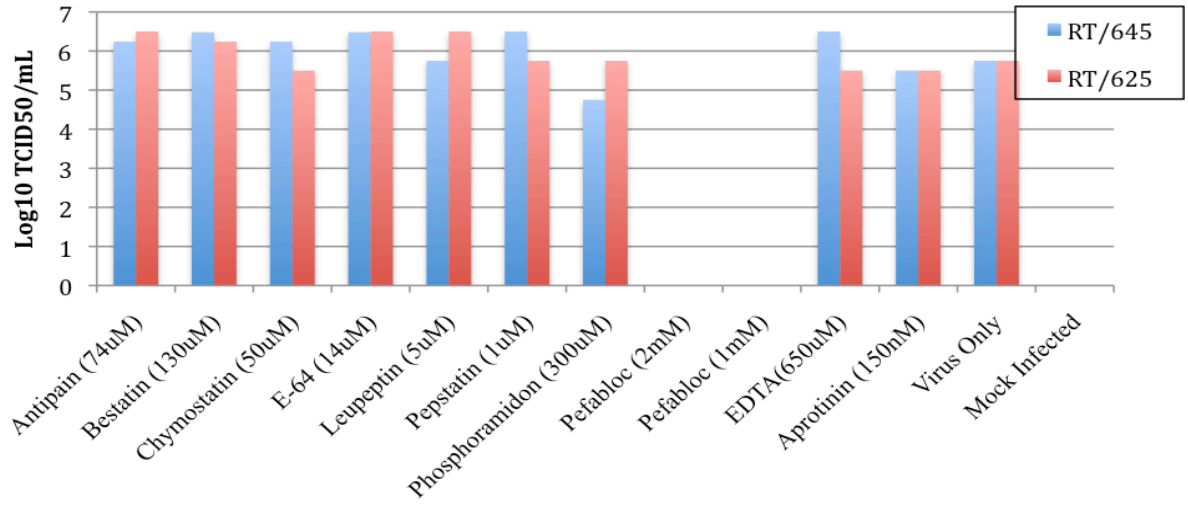


Figure 4.4. Trypsin is required for hemagglutinin mediated fusion in Vero cells. Using a T7 luciferase fusion assay to examine virus fusion in the absence (-) or presence (+) trypsin. Vero cells were transfected with pCAGGS-HA alone or cotransfected with congruent pCAGGS-NA plasmid and 24 hours post transfection the (-) cells received DMEM only, while the (+) cells were trypsin treated with 5µg/mL of TPCK-trypsin. The BSR-T7 effector cells overlayed the transfected Vero cells and pH pulsed with pH-adjusted PBS at pH5 or pH7. Luciferase activities of cell lysates were determined. The results are representative of experiment performed in triplicate; error bars indicate standard deviations. RFU: relative fluorescence units

A.



B.

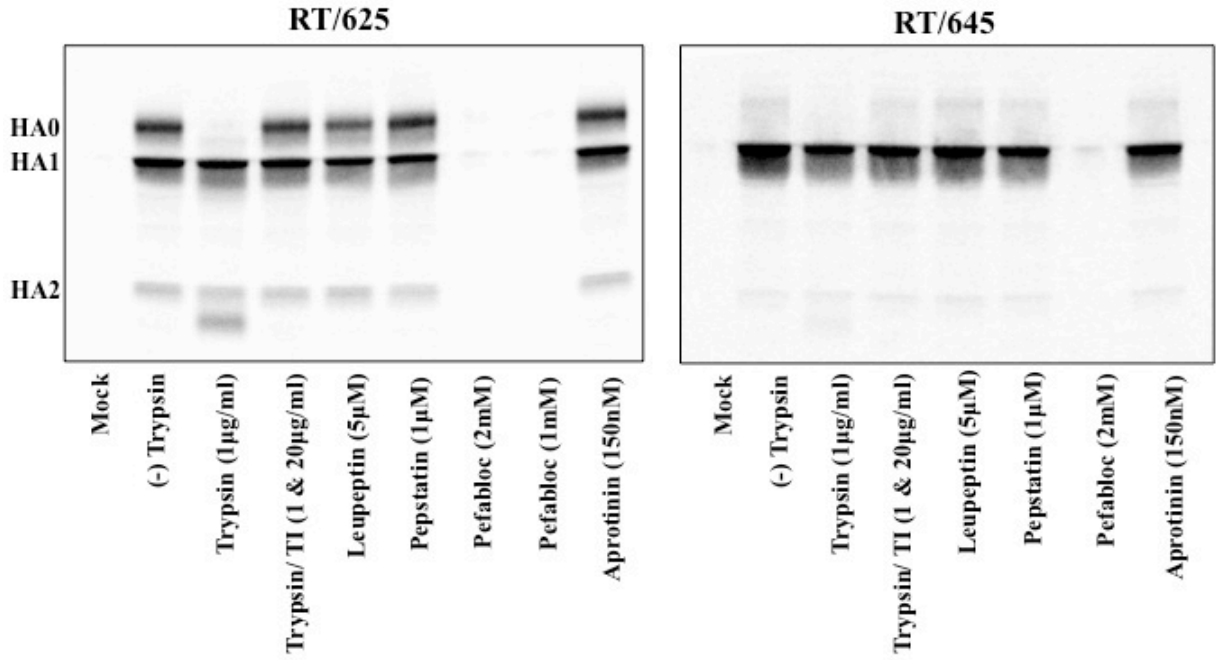
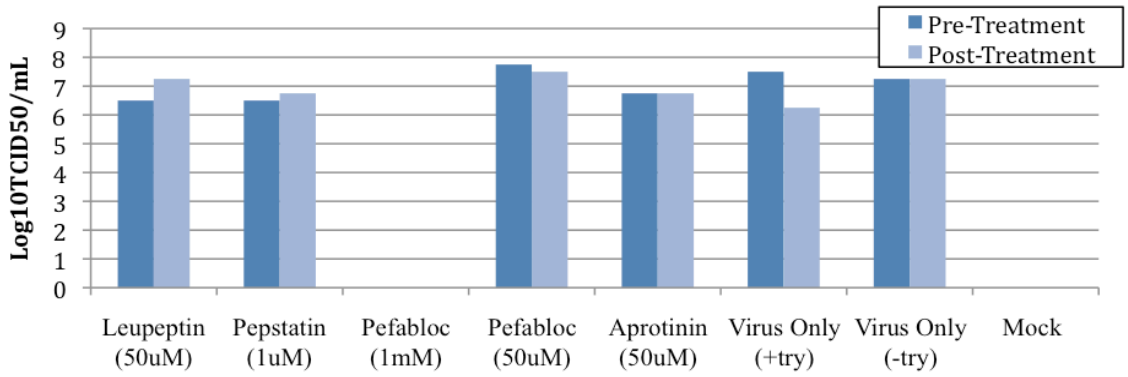


Figure 4.5. Initial protease inhibitor screen of LPAI infected MDCK cells. A protease inhibitor screen was completed to identify proteases involved in the RT/645 and RT/625 trypsin independent replication. (A) A screen of 10 protease inhibitors, within the manufacturer's suggested concentration (shown), was screened in MDCK cells pretreated with each protease inhibitor for 20 minutes prior to infection. The cells were infected with RT/645 and RT/625 at a MOI of 0.1 for 2 hours in the presence of the protease inhibitors. The infected MDCK cells were washed and cultured in MEM only for 24 hours. Viral titers were determined of the supernatant by $TCID_{50}$ assay. (B) The proteolytic activation of HA was examined in the presence of protease inhibitors, where MDCK cells were pretreated with protease inhibitors for 20 minutes prior to infection with RT/645 and RT/625 at an MOI of 0.1 for 5 hours. Protease inhibitors were present throughout the entire experiment. Samples were collected 5 hours post infection and radiolabeled before immunoprecipitated using HA subtype specific antibodies.

A.



B.

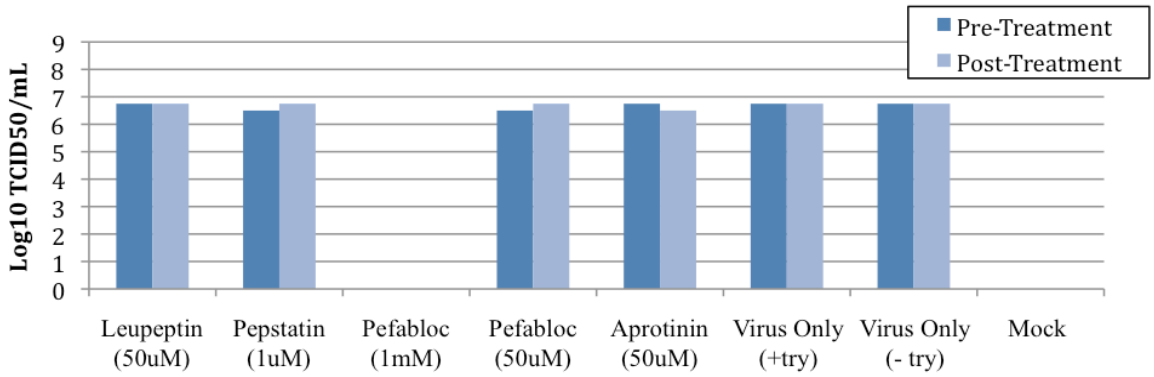
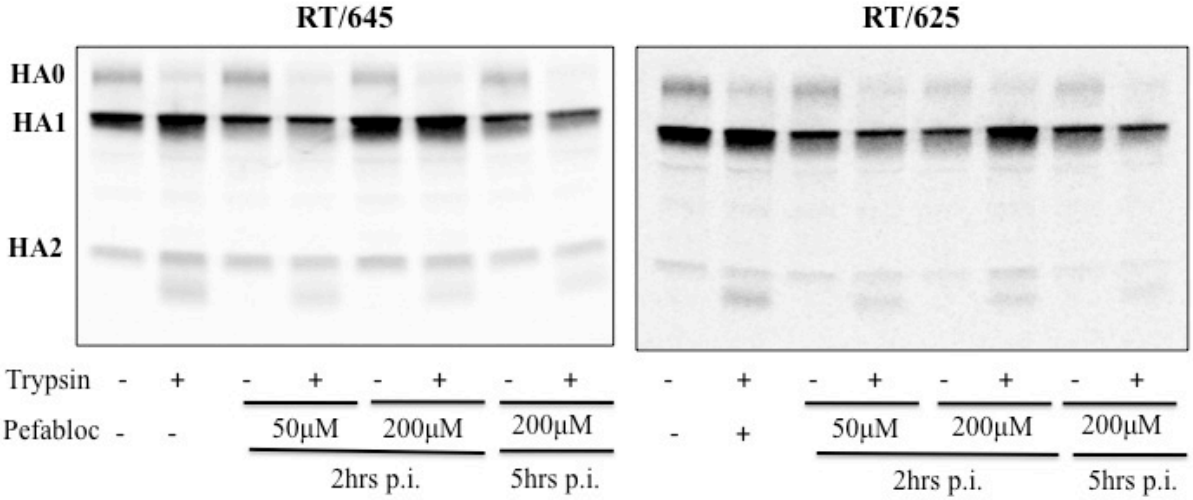


Figure 4.6. Serine protease inhibitors at lower concentrations exhibit negligible effects on avian influenza replication in MDCK cells. Examine viral replication in the presence of serine protease inhibitors (pefabloc, aprotinin, and leupeptin) at various concentrations. The MDCK cells were infected (A) RT/645 and (B) RT/625 at an MOI of 0.1 for 2 hours. The pretreated cells were treated with protease inhibitor at the stated concentrations during the 2 hour infection, while the post-treated samples were cultured in the protease inhibitor following a 2 hour infection. Supernatants were collected at 24 hours and viral titers were determined by $TCID_{50}$ assay

A.



B.

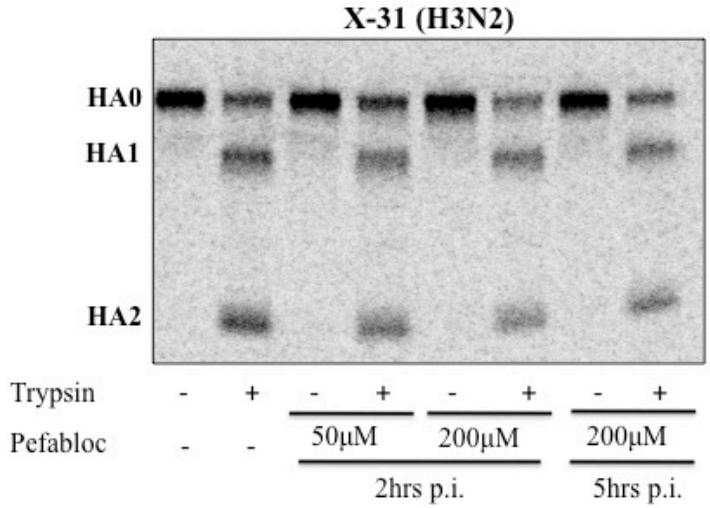


Figure 4.7. Trypsin and serine independent HA cleavage present during LPAI infection of MDCK cells. The proteolytic activation of HA was examined in the presence of protease inhibitors, where MDCK cells were infected with RT/645 and RT/625 at an MOI of 0.1 for 2 hours before pefabloc treatment at 50μM and 200μM at 2 and 5 hours post infection. Samples were collected 5 hours post infection and trypsin treated prior to being radiolabeled. The (-) trypsin cells received DMEM only, while the (+) trypsin cells were trypsin treated with 5μg/mLof TPCK-trypsin for 15 minutes. All samples were immunoprecipitated using HA subtype specific polyclonal antibodies and were analyzed by SDS-PAGE.

Table 4.1 Comparison of HA cleavage sites from LPAI isolates. All isolates contains the prototypical monobasic HA cleavage site, where a lysine (**K**) or an arginine (**R**) was present.

Strain ID	SCWDS ID#	AIV Isolates	Subtype	HA Cleavage Site
RT/645	1171	A/Ruddy Turnstone/NJ/650645/02	H1N9	LRNVPSIPS**R**GLF
RT/625	892	A/Ruddy Turnstone/NJ/650625/02	H6N1	LRNVPQIET**R**GLF

CHAPTER 5

SUMMARY AND CONCLUSIONS

Transmission of avian influenza viruses was originally thought to be restricted to the natural host reservoir of wild aquatic birds, but the direct transmission of highly pathogenic avian H5N1 in humans has highlighted the need to understand the pandemic potential these viruses pose. As most of the research efforts have concentrated on the highly pathogenic H5 and H7 subtypes, little is known about the pandemic potential of the low pathogenic avian influenza (LPAI) viruses found in nature. Several viral features have been identified that influence pathogenicity and tropism of avian viruses, including receptor specificity, the viral polymerase, cellular proteases responsible for hemagglutinin activation, and fusion. The objective of this study has been to examine the genetic features that mediate tropism and replication capacity, as well as the host features that may contribute to a trypsin independent replication phenotype and the transmission observed in mammals. We hypothesized that the hemagglutinin of avian influenza A virus contains features that affect tissue tropism, species tropism, and transmission. The specific aims examined the pandemic potential of North American LPAI isolates that exhibit a trypsin independent phenotype with a complete characterization of viral and host factors that influence receptor specificity, proteolytic activation of HA and fusion.

In our preliminary experiments, over 400 North American LPAI isolates were screened for their ability to replicate in the presence or absence of exogenous trypsin. Approximately 10% of the LPAI isolates replicated to high titers independent of trypsin

in Madin-Darby canine kidney (MDCK) cells and no correlation between subtype and avian species was evident (Appendix). To confirm the trypsin-independent phenotype, 23 isolates were selected and their trypsin-independent replication was evaluated in Madin-Darby bovine kidney (MDBK) and chicken embryo fibroblast (DF-1) cells. As expected, all the isolates replicated in the presence of trypsin, while a large subset were capable of replications in absence of trypsin in MDBK cells. One H6N1 isolate and three H3 isolates exhibited trypsin-independent replication in the DF-1 cells, while no virus was detected in DF-1 in the absence of trypsin for the remaining isolates (Appendix). Sequencing analysis revealed no mutations or insertions at the HA cleavage site in the trypsin-independent isolates, where either a single arginine (R) or lysine (K) was maintained. Finally, erythrocyte binding assays confirmed that all the LPAI isolates in this screen exhibited the predicted α2,3-linked sialic acid recognition. The general permissiveness of the cells and viral features influenced the trypsin-independent phenotype and enhanced viral tropism.

Several of the LPAI isolates examined in the original screen were used in parallel studies, where they were shown to infect and replicate in mammalian systems (Chapter 3). In a related study, a subset of H5 LPAI isolates were shown to infect and replicate in fully differentiated, primary normal human bronchial epithelial (NHBE) cells in the absence of α2,3-linked sialic acid receptors, where neuraminidase-treated NHBE were readily infected despite removal of 95% of α2,3-linked sialic acids. Moreover, several LPAI isolates were shown to infect and replicate in mammals (mice and ferrets) without prior adaptation; one isolate in particular (H1N9, RT/645) was able to transmit via direct contact in the ferret, which was surprising considering ferrets, like humans, express

predominantly α2,6-linked sialic acid receptors in their upper respiratory tract and a variety of other AIVs tested for transmission failed to transmit without adaptation or experimental mutation (1, 2). Several HA amino acid residues have been established and are known to influence receptor specificity and ferret infection or transmission (i.e. 190, 225, 226, and 228). Sequence analysis of several of the LPAI viruses confirmed that they all maintained the α2,3-linked sialic acid binding (avian) genotype (E190, G225, Q226, and G228). To functionally assess binding profiles of the LPAI isolates that were able to infect and replicate in mammalian model systems, several isolates were screened via glycan microarrays and all maintained a classical avian binding profile of α2,3-linked sialic acid recognition with little or no α2,6-linked sialic acid binding. These LPAI isolates established infections in mammalian model systems despite detectable α2,3-linked sialic acid receptors, which would suggest that there are other mechanisms that viruses can use to broaden their tropism, whether it be utilizing other receptors or mediating infection through derivatives of known sialic acids. While we have not identified the precise genotype or mechanism, we have shown that several LPAI isolates are able to infect and transmit in the ferret model without adaptation and that the species-specific barriers (i.e. receptor distribution and specificity) thought to reduce zoonotic transmission of low pathogenic viruses may be surpassed.

Finally, the proteolytic HA activation and fusion capacity of the trypsin-independent H1N9 (RT/645) and H6N1 (RT/625) isolates were evaluated (Chapter 4). In virus infected MDCK and African green monkey kidney (Vero) cells, the proteolytic cleavage of HA was observed regardless of trypsin treatment for both viruses. We tested the hypothesis that the HA cleavage observed in the absence of trypsin was mediated by

the neuraminidase (NA) glycoprotein. The HA and NA proteins expressed from plasmids were assayed for the trypsin-independent HA cleavage observed in the parental virus. There was no evidence that the NA enhanced HA cleavage in the presence or absence of exogenous trypsin and no distinguishable difference in the level of cleaved HA was observed with the addition of enzymatically active NA. The minor amount of cleaved HA was not sufficient to mediate viral fusion. However, the addition of the NA did increase the level of viral fusion in the presence of trypsin for both isolates. Since the trypsin-independent phenotype did not seem to be exclusively a viral mediated mechanism, we tested an alternative hypothesis in which viral infection induces expression of cellular proteases capable of cleaving viral HA. Using a protease inhibitor screen, it appeared that the RT/645 and RT/625 LPAI isolates were capable of inducing alternative proteases to facilitate HA cleavage and enhance infectivity. While this did not seem to be a common trait of LPAI isolates, the induction of cellular proteases by select avian strains demonstrated that viruses might exploit alternative methods for establishing infections.

Collectively, these results demonstrate that some LPAI viruses, unconstrained by subtype or avian species of origin, are capable of overcoming defined host range restrictions and establishing infections in mammals without undergoing adaptation. Despite classical avian receptor specificity and genotype, select LPAI isolates have mechanisms that enable infection and replication in mammals, which could allow for the introduction of novel potentially pandemic influenza viruses or provide opportunity for co-infection and reassortment with mammalian (i.e. swine or human) viruses. These

results highlight that LPAI viruses from the natural reservoir should not be overlooked, as they may be source for a future pandemic.

References:

1. **Maines, T. R., L. M. Chen, Y. Matsuoka, H. Chen, T. Rowe, J. Ortin, A. Falcon, T. H. Nguyen, Q. Mai le, E. R. Sedyaningsih, S. Harun, T. M. Tumpey, R. O. Donis, N. J. Cox, K. Subbarao, and J. M. Katz.** 2006. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A **103:**12121-6.
2. **Van Hoeven, N., C. Pappas, J. A. Belser, T. R. Maines, H. Zeng, A. Garcia-Sastre, R. Sasisekharan, J. M. Katz, and T. M. Tumpey.** 2009. Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A **106:**3366-71.

APPENDIX

CHARACTERIZATION OF TRYPSIN INDEPENDENT PHENOTYPE OBSERVED IN LOW PATHOGENIC AVIAN INFLUENZA STRAINS

Abstract

Little is known about the pandemic potential of low pathogenic avian influenza (LPAI) viruses circulating in North American wild bird populations. We screened over 400 North American LPAI isolates for their ability to replicate independent of exogenous trypsin, where most low pathogenic strains require trypsin to replicate in cell culture. Interestingly, we identified a subset of 23 LPAI isolates from various subtypes (H1-11) that exhibited a trypsin independent phenotype in Madin Darby canine kidney (MDCK) cells. This phenotype was further confirmed in other mammalian cell lines (Madin Darby bovine kidney (MDBK) and chicken embryo fibroblast (DF-1)). A subset were able to replicate independent of trypsin both the MDCK and MDBK cells but only the H3 subtyped viruses were able to replicate in all 3 cell lines regardless of trypsin. The permissiveness of the cells appeared to influence viral titers. Upon further characterization, these LPAI isolates expressed the classic monobasic HA cleavage site and exhibited α2,3-linked sialic acid recognition commonly observed among low pathogenic viruses. Much of our understanding of LPAI phenotypes remains to be elucidated and we have shown here that the LPAI trypsin independent phenotype is much more complex than originally suggested.

Introduction

The emergence of high pathogenic avian influenza (HPAI) virus in 1997 and subsequent human infections and deaths marked the beginning of intense investigation into the transmission of avian influenza viruses (AIV) into humans and other mammalian species. These "novel" AIVs are of concern not only because of the severity of disease observed but also because they have pandemic potential. All pandemic influenza viruses since 1918, including the 2009 H1N1 virus possess an avian genetic component (16, 31, 53, 54). There are at least 105 species of wild birds that serve as hosts for avian influenza viruses and all 16 hemagglutinin (HA) and 9 neuraminidases (NA) subtypes have been identified in the wild aquatic bird reservoir (37, 54). AIV isolates are characterized as either low pathogenic (LPAI) or high pathogenic (HPAI), while HPAI are any H5 or H7 subtypes that contain a polybasic cleavage site and result in greater than 75% mortality in intravenously inoculated chickens (38, 52). Those that do not fall under the high pathogenic criteria are considered LPAI and they posses a monobasic, single arginine (R) or lysine (K), HA cleavage site generating asymptomatic infections in birds. Currently, only HPAI H5 and H7 subtypes strains have been shown to directly infect humans causing substantial morbidity and mortality (2, 4, 10, 13, 15, 22, 48, 50, 55, 56).

The mechanisms by which avian viruses are capable of directly infecting humans and other mammals is multifactorial, including differences in hemagglutinin receptor specificity, protease requirements, and polymerase activity, and much still remains undefined. Avian viruses preferentially recognize α2,3-linked sialic acid receptors of the gastrointestinal and respiratory tracts of birds, while human viruses bind to α2,6-linked sialic acid receptors of the human upper respiratory tract (6, 8, 21, 25, 27, 30, 34, 39, 44,

51, 57). Proteolytic cleavage of the hemagglutinin (HA) precursor protein into HA1 and HA2 subunits is a prerequisite for the virus to become infectious. Distinct cellular proteases are known facilitate HA cleavage of LPAI and HPAI isolates. For LPAI viruses, localized, trypsin-like proteases of respiratory or intestinal tracts are responsible for proteolytic activation of HA; in contrast, HPAI viruses contain a series of basic amino acids at the HA cleavage site that are targeted by ubiquitously expressed intracellular subtilisin-like proteases that allow for systemic dissemination.

Most studies have focused on a select H5 and H7 HPAI viruses and there is minimal information available on the other abundant avian viruses that exist in nature. More importantly, there is little known about the risk posed by AIVs circulating in North American aquatic bird populations. To assess the potential for LPAI from the wild to infect humans or other mammals, we screened more than 400 North American avian influenza virus field isolates (including H1 through H11 subtypes) for trypsin independent replication in cell culture. Most LPAI isolates require exogenously added trypsin to replicate in cell culture and is often a phenotype observed in highly pathogenic influenza strains. Surprisingly, approximately 10% of the isolates were able to replicate to high titers independent of trypsin in MDCK cells. This phenotype was further confirmed for several individual viruses at low multiplicities of infection (MOI) in multiple mammalian culture systems, including bovine and avian cells. Furthermore, sequence analysis and erythrocyte binding assays confirm that these LPAI isolates maintain the classical low pathogenic HA cleavage site and α2,3-linked sialic acid recognition. We have demonstrated in this study the capacity of avian viruses to infect

and replicate in a variety of mammalian culture systems and better address the zoonotic potential of avian influenza viruses from the natural reservoir.

Material and Methods

Viruses and cells

The Southeastern Cooperative Wildlife Disease Study generously provided all avian influenza isolates, where cloacal swabs were collected as part of a North American wild aquatic bird surveillance initiative. All AIV isolates were minimally passaged (> 3 total) in specific pathogen free 9 day old embryonated chicken eggs (ECE) at 37°C for 72 hours. Virus stocks were generated in ECE at and stored at -80°C. Viral titers (PFU/mL) were determined via plaque assays or tissue culture infectious dose 50% ($TCID_{50}$) on Madin-Darby canine kidney cells (MDCK) (18).

The Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), and chicken embryo fibroblast (DF-1) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C with 5% CO_2 until confluent.

Trypsin independent plaque assay

All AIV isolates were screened by plaque assays in the presence or absence of 1μg/mL of TPCK-trypsin in the Avicel overlay as discussed previously (33). Briefly, AIV isolates were titrated on MDCK cells for 2 hours in MEM only. Following infection, Avicel overlay was added to the virus infected cells, where the (+) trypsin plates contained 1.2% Avicel supplemented with 1μg/mL of TPCK-trypsin, while the (-) trypsin

plates received Avicel overlay only. All plates were incubated at 37°C with 5% CO_2 for 48 to 72 hours. Cells were fixed with 60% acetone/40% methanol before stained with crystal violet.

Two-step infection assay

The MDCK, MDBK, and DF-1 cells were infected with each AIV isolate at a MOI 0.001 in MEM supplemented with 1μg/mL of TPCK-trypsin for 2 hours at 37°C. The infection medium was washed off before the addition of MEM only for the (-) trypsin samples, where the (+) trypsin samples received MEM supplemented with 1μg/mL of TPCK-trypsin. The cells incubated for 48 hours and supernatant samples were collected 4, 24, and 48 hours. All samples were titrated in MDCK cells by $TCID_{50}$ assay.

Sequencing of LPAI HA cleavage site

Each AIV HA cleavage site was sequenced from total viral RNA extracted from supernatant collected from virus infected MDCK cells in the presence or absence of exogenous trypsin using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. One-step RT-PCR (Qiagen, Valencia, CA) was performed on viral RNA using a set of HA0 primers as previously described by Gall et al (14).

HA0 Primer Name	**5' – 3' Sequence**
HA-1057.1-F	GGR GAA TGC CCC AAA TAY GT
HA-1057.2-F	GGR ARA TGC CCC AGR TAT GT
HA-1057.3-F	GGR GAA TGC CCC AAR TAY AT
HA-1232.1(555)-R	*CTG AGT CCG AAC ATT GAG* TTG CTA TGV TGR TAW CCA TAC CA
HA-1232.2(555)-R	*CTG AGT CCG AAC ATT GAG* TTY TGA TGY CTG AAD CCR TAC CA

The HA product was excised and gel purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). All sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA) using the primers listed above.

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. Each AIV strain was serially diluted in PBS and standard hemagglutination assay was performed, where 50 µL of 1% erythrocytes were added to each 50 µL of diluted virus and incubated at 4°C for 1 hour. Each plate was scored for the appearance of agglutination.

Results

Trypsin independent replication of low pathogenic avian influenza virus

A total of 419 low pathogenic avian influenza (LPAI) isolates (H1 through 11 subtypes) were provided from the Southeastern Cooperative Wildlife Disease Study surveillance initiative and represented 14 different species of waterfowl and shorebirds collected throughout North American from 1998 to 2006. It is well established that exogenously added trypsin is required for influenza replication in cell culture (3, 17, 28, 29). We were interested in examining the trypsin requirement for replication for these wild bird isolates, where they were screened by plaque assay for their ability to replicate in the absence of exogenous trypsin. Interestingly, we found that approximately 10% of the LPAI isolates were able to replicate to high viral titers independent of trypsin regardless of subtype and avian species. Of the LPAI isolates that exhibited the trypsin

independent phenotype, 23 of the isolates were selected for further examination (Figure A.1).

Multiple mammalian cell types support LPAI replication independent of exogenous trypsin

The trypsin independent phenotype observed in a number of LPAI isolates from the initial screen led us to question if this phenotype was exclusive to MDCK cells or if it was present in other mammalian cell lines. We utilized a 2-step approach to examine the ability of these LPAI isolates to replicate independent of trypsin in Madin-Darby bovine kidney (MDBK) cells and chicken embryo fibroblast (DF-1) cells. First, the MDCK, MBDK, and DF-1 cells were infected with each isolate at an MOI 0.001 before receiving either MEM supplemented with 1μg/mL of TPCK-trypsin or MEM only and supernatant was collected at 4, 24, and 48 hours. The second step was to determine viral titers of collected supernatants by 50% tissue culture infectious dose ($TCID_{50}$). All isolates replicated in the presence of trypsin in all three cell lines. Most of the remaining isolates, excluding the H11 isolates, replicated well in both the MDCK and MDBK cells independent of trypsin. Interestingly, one H6N1 isolate and three H3 LPAI isolates were capable of replicating in all 3 cell lines (Figure A.2). All the LPAI viruses were able to replicate in a variety of cell types in the presence of trypsin, where the combination of general permissiveness of the cells influenced viral titers.

All the LPAI viruses exhibit monobasic HA cleavage site

Trypsin independent replication is a phenotype observed in high pathogenic avian influenza isolates that contain a polybasic amino acid (R-X-R/K-R consensus motif) HA cleavage site making them more accessible to a wider array of cellular proteases. Most HPAI strains undergo intracellular cleavage by ubiquitously expressed, subtilisin-like serine proteases, where progeny virions are infectious upon viral release from host cells (3, 7, 23, 24, 28, 45, 47, 58). We were interested in examining the HA cleavage site of these trypsin independent AIV isolates. Sequencing was completed for each isolate grown in the presence and absence of trypsin to see if there were modifications or mutations in the HA cleavage site. Sequencing analysis revealed that all isolates maintained a monobasic HA cleavage site and there was no amino acid changes observed in the virus grown in the absence of trypsin (Table A1). Interestingly, all of the H3 subtyped viruses contained the less common lysine (K), while the remaining isolates exhibited the typical arginine (R) at the cleavage site. There was no evident change in the HA cleavage site that might help explain the observed trypsin independent phenotype.

Erythrocyte agglutination suggests α2,3 specificity of LPAI viruses

To functionally assess the sialic acid receptor specificity of the LPAI isolates in this screen, we examined the erythrocyte binding of the 23 avian influenza strains. It is well established that avian viruses preferentially bind α2,3-linked sialic acid receptors, while human strains predominantly recognize α2,6-linked sialic acids (1, 5, 8, 9, 32, 35, 40-43, 46). Erythrocytes from most species, such as those from turkey, chicken, and guinea pig, express both α2,3- and α2,6-linked sialic acids, but equine erythrocytes are

unique in that they exhibit predominantly α2,3-linked sialic acids (26, 36). All LPAI isolates tested were able to agglutinate equine erythrocytes as high as 4096 HAU/mL titer (Table A2), while the control human influenza strains, A/Pennsylvania/08/008 generated no detectable agglutination of equine erythrocytes. All the LPAI isolates agglutinated both guinea pig and turkey erythrocytes to similar levels as compared to the human influenza strain, A/Pennsylvania/08/008. As expected, all the LPAI maintained the α2,3-linked sialic acid recognition. Furthermore, several isolates were examined by glycan array to confirm their receptor specificity and all preferentially bound α2,3-linked glycans (Chapter 3).

Discussion

The natural host for avian influenza viruses is wild aquatic birds and all 16 HA and 9 NA subtypes have been identified from this reservoir (12, 54). Avian influenza viruses are known to be well adapted in waterfowl and shorebirds and are considered to be in "evolutionary stasis" (19, 20, 54). Since the 1997 HPAI H5N1 outbreak that was particularly virulent in humans, most of the research has concentration only on the H5 and H7 subtypes with little focus on the LPAI isolates circulating in wild aquatic bird populations. Working along side the Southeastern Cooperative Wilderness Disease Study, we were interested in examining the zoonotic potential of North American LAPI isolates. This led us to do a complete *in vivo* and *in vitro* characterization of LAPI isolates collected throughout North American. In the initial screen, we examined trypsin independent replication for over 400 LPAI via plaque assay. The trypsin requirement for replication is often used as a pathogenicity determinant because most highly pathogenic

influenza strain can replicate independent of trypsin, while low pathogenic strains require exogenously added trypsin to replicate in cell culture. We used this criterion to select viruses and of the 400 LPAI isolates screened, greater than 10% of the isolates exhibited trypsin independent replication in MDCK cells (Figure A1). Various subtypes and species were included and there was no correlation between species, subtype, and trypsin independent replication. A select 28 LPAI isolates were examined *in vivo* and several isolates replicated in mice without prior adaptation and induced histopathologic lesions comparable to those observed during human influenza infections (11). A subset of these viruses was tested for trypsin independent replication in other mammalian and avian cell lines and several isolates maintained the trypsin-independent phenotype. Most of the viruses except for one H3N8 (RT/395) and all the H11 isolates, demonstrated trypsin independent replication in the MDCK and MDBK cell lines. One H6 and three H3 isolates replicated in all cell lines. The minimal medium conditions used in the 2-step experiments was not optimal for culturing the DF-1 cells, where by 48 hours most of the cells were detached from the plate, making it difficult to access trypsin-independent replication for the 48 hour time point. It appeared that under the experimental conditions tested, the DF-1 cells were less permissive than the mammalian cells. This may be due to viability of the DF-1 cells when they were cultured in sub-optimal medium (not supplemented with fetal bovine serum).

The viral feature that most clearly influences infectivity is the hemagglutinin glycoprotein, whose primary function is recognition and binding of cell surface receptors and facilitating membrane fusion. As mentioned previously, avian viruses predominantly recognize α2,3-linked sialic acid receptor and have to undergo HA cleavage by trypsin-

like protease to become infectious. Since a subset of these LPAI viruses were able to bypass the trypsin requirement for replication, we were interested in evaluating the HA cleavage site and receptor specificity. Sequencing analysis revealed that most of the LPAI isolates contained an arginine (R) while all the H3 subtypes exhibited the less common lysine (K) at the HA cleavage site (Table A1). The HA cleavage site sequence was maintained whether the LPAI isolate was grown in the presence or absence of trypsin, thus no mutations or basic amino acid insertions were present at the cleavage site when the LPAI isolates were grown in absence of exogenous trypsin. It did not appear that there were differences in the HA cleavage site when the viruses were grown in the absence of trypsin that could explain the trypsin independent phenotype.

Since, the trypsin independent phenotype was not the result of mutations within the HA cleavage site making it more accessible to cellular proteases. We questioned whether there was a change in receptor specificity using erythrocyte binding assays. It has been shown that LPAI viruses propagated in chicken eggs and MDCK cells can lose their ability to agglutinate chicken erythrocytes (26, 36, 49). Since all of the LPAI viral stocks were generated in chicken eggs, we employed turkey, guinea pig, and equine erythrocytes to examine the agglutination properties of each isolate. Equine erythrocytes express predominately α2,3-linked sialic acids and all the LPAI isolates exhibited up to 4096 HAU titers as compared to the human (A/Pennsylvania/08/2008) negative control (Table A2). All the LPAI isolates and the human control were able to agglutinate guinea pig and turkey erythrocytes because they express both α2,3- and α2,6-linked sialic acids. These results were confirmed for several of isolates by glycan array (Chapter 3) and preferentially recognized α2,3-linked glycans. There was no apparent shift in receptor

specificity of the LPAI isolates; they all maintained the classical avian α2,3-linked sialic acid recognition.

In this study, we have shown that LPAI isolates from the natural reservoir exhibit zoonotic potential, where a subset of the LPAI isolates we screen were able to replicate in multiple mammalian cell lines regardless of exogenous trypsin. The general permissiveness of the cells and viral features influenced the trypsin independent phenotype observed in theses wild bird influenza viruses. The LPAI isolates examined in this preliminary screen adhere to the low pathogenic avian influenza criteria of a monobasic HA cleavage site and α2,3-linked sialic acid receptor specificity with no obvious viral features that offer an explanation as to why some of these LPAI isolates exhibit a trypsin independent phenotype, while others require exogenous trypsin to replicate. This preliminary study illustrates that the mechanisms by which avian viruses are able broaden their tropism is more complex than originally suggested and a better understanding of how these viruses adapt needs to be further investigated.

References:

1. **Baum, L. G., and J. C. Paulson.** 1990. Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl **40:**35-8.
2. **Bender, C., H. Hall, J. Huang, A. Klimov, N. Cox, A. Hay, V. Gregory, K. Cameron, W. Lim, and K. Subbarao.** 1999. Characterization of the surface proteins of influenza A (H5N1) viruses isolated from humans in 1997-1998. Virology **254:**115-23.
3. **Bosch, F. X., W. Garten, H. D. Klenk, and R. Rott.** 1981. Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of Avian influenza viruses. Virology **113:**725-35.
4. **Capua, I., S. Marangon, P. Cordioli, L. Bonfanti, and U. Santucci.** 2002. H7N3 avian influenza in Italy. Vet Rec **151:**743-4.
5. **Carroll, S. M., H. H. Higa, and J. C. Paulson.** 1981. Different cell-surface receptor determinants of antigenically similar influenza virus hemagglutinins. J Biol Chem **256:**8357-63.
6. **Chan, R. W., K. M. Yuen, W. C. Yu, C. C. Ho, J. M. Nicholls, J. S. Peiris, and M. C. Chan.** 2010. Influenza H5N1 and H1N1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. PLoS One **5:**e8713.
7. **Chen, J., K. H. Lee, D. A. Steinhauer, D. J. Stevens, J. J. Skehel, and D. C. Wiley.** 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell **95:**409-17.
8. **Connor, R. J., Y. Kawaoka, R. G. Webster, and J. C. Paulson.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:**17-23.
9. **Couceiro, J. N., J. C. Paulson, and L. G. Baum.** 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res **29:**155-65.
10. **de Jong, J. C., E. C. Claas, and A. D. Osterhaus.** 1998. [Influenza A (H5N1) in Hong Kong: Forerunner of a pandemic or just a scientifically interesting phenomenon and a useful exercise in pandemiology?]. Ned Tijdschr Geneeskd **142:**1252-6.
11. **Driskell, E. A., C. A. Jones, D. E. Stallknecht, E. W. Howerth, and S. M. Tompkins.** 2010. Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. Virology **399:**280-9.
12. **Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus.** 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol **79:**2814-22.
13. **Fouchier, R. A. M., P. M. Schneeberger, F. W. Rozendaal, J. M. Broekman, S. A. G. Kemink, V. Munster, T. Kuiken, G. F. Rimmelzwaan, M. Schutten,**

G. J. J. van Doornum, G. Koch, A. Bosman, M. Koopmans, and A. D. M. E. Osterhaus. 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.

14. **Gall, A., B. Hoffmann, T. Harder, C. Grund, and M. Beer.** 2008. Universal primer set for amplification and sequencing of HA0 cleavage sites of all influenza A viruses. J Clin Microbiol **46:**2561-7.
15. **Gao, P., S. Watanabe, T. Ito, H. Goto, K. Wells, M. McGregor, A. J. Cooley, and Y. Kawaoka.** 1999. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol **73:**3184-9.
16. **Garten, R. J., C. T. Davis, C. A. Russell, B. Shu, S. Lindstrom, A. Balish, W. M. Sessions, X. Xu, E. Skepner, V. Deyde, M. Okomo-Adhiambo, L. Gubareva, J. Barnes, C. B. Smith, S. L. Emery, M. J. Hillman, P. Rivailler, J. Smagala, M. de Graaf, D. F. Burke, R. A. Fouchier, C. Pappas, C. M. Alpuche-Aranda, H. Lopez-Gatell, H. Olivera, I. Lopez, C. A. Myers, D. Faix, P. J. Blair, C. Yu, K. M. Keene, P. D. Dotson, Jr., D. Boxrud, A. R. Sambol, S. H. Abid, K. St George, T. Bannerman, A. L. Moore, D. J. Stringer, P. Blevins, G. J. Demmler-Harrison, M. Ginsberg, P. Kriner, S. Waterman, S. Smole, H. F. Guevara, E. A. Belongia, P. A. Clark, S. T. Beatrice, R. Donis, J. Katz, L. Finelli, C. B. Bridges, M. Shaw, D. B. Jernigan, T. M. Uyeki, D. J. Smith, A. I. Klimov, and N. J. Cox.** 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **325:**197-201.
17. **Garten, W., F. X. Bosch, D. Linder, R. Rott, and H. D. Klenk.** 1981. Proteolytic activation of the influenza virus hemagglutinin: The structure of the cleavage site and the enzymes involved in cleavage. Virology **115:**361-74.
18. **Gaush, C. R., and T. F. Smith.** 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl Microbiol **16:**588-94.
19. **Gorman, O. T., W. J. Bean, and R. G. Webster.** 1992. Evolutionary processes in influenza viruses: divergence, rapid evolution, and stasis. Curr Top Microbiol Immunol **176:**75-97.
20. **Gorman, O. T., R. O. Donis, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of influenza A virus PB2 genes: implications for evolution of the ribonucleoprotein complex and origin of human influenza A virus. J Virol **64:**4893-902.
21. **Hatakeyama, S., Y. Sakai-Tagawa, M. Kiso, H. Goto, C. Kawakami, K. Mitamura, N. Sugaya, Y. Suzuki, and Y. Kawaoka.** 2005. Enhanced expression of an alpha2,6-linked sialic acid on MDCK cells improves isolation of human influenza viruses and evaluation of their sensitivity to a neuraminidase inhibitor. J Clin Microbiol **43:**4139-46.
22. **Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840-2.

23. **Horimoto, T., T. Ito, D. J. Alexander, and Y. Kawaoka.** 1995. Cleavability of hemagglutinin from an extremely virulent strain of avian influenza virus containing a unique cleavage site sequence. J Vet Med Sci **57:**927-30.
24. **Horimoto, T., and Y. Kawaoka.** 1997. Biologic effects of introducing additional basic amino acid residues into the hemagglutinin cleavage site of a virulent avian influenza virus. Virus Res **50:**35-40.
25. **Ibricevic, A., A. Pekosz, M. J. Walter, C. Newby, J. T. Battaile, E. G. Brown, M. J. Holtzman, and S. L. Brody.** 2006. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. J Virol **80:**7469-80.
26. **Ito, T., Y. Suzuki, L. Mitnaul, A. Vines, H. Kida, and Y. Kawaoka.** 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology **227:**493-9.
27. **Ito, T., Y. Suzuki, T. Suzuki, A. Takada, T. Horimoto, K. Wells, H. Kida, K. Otsuki, M. Kiso, H. Ishida, and Y. Kawaoka.** 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. J Virol **74:**9300-5.
28. **Kawaoka, Y., and R. G. Webster.** 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A **85:**324-8.
29. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426-39.
30. **Kogure, T., T. Suzuki, T. Takahashi, D. Miyamoto, K. I. Hidari, C. T. Guo, T. Ito, Y. Kawaoka, and Y. Suzuki.** 2006. Human trachea primary epithelial cells express both sialyl(alpha2-3)Gal receptor for human parainfluenza virus type 1 and avian influenza viruses, and sialyl(alpha2-6)Gal receptor for human influenza viruses. Glycoconj J **23:**101-6.
31. **Lahariya, C., A. K. Sharma, and S. K. Pradhan.** 2006. Avian flu and possible human pandemic. Indian Pediatr **43:**317-25.
32. **Markwell, M. A., P. Fredman, and L. Svennerholm.** 1984. Receptor ganglioside content of three hosts for Sendai virus. MDBK, HeLa, and MDCK cells. Biochim Biophys Acta **775:**7-16.
33. **Matrosovich, M., T. Matrosovich, W. Garten, and H. D. Klenk.** 2006. New low-viscosity overlay medium for viral plaque assays. Virol J **3:**63.
34. **Matrosovich, M., A. Tuzikov, N. Bovin, A. Gambaryan, A. Klimov, M. R. Castrucci, I. Donatelli, and Y. Kawaoka.** 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol **74:**8502-12.
35. **Matrosovich, M. N., A. S. Gambaryan, S. Teneberg, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, and K. A. Karlsson.** 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:**224-34.
36. **Medeiros, R., N. Escriou, N. Naffakh, J. C. Manuguerra, and S. van der Werf.** 2001. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology **289:**74-85.

37. **Munster, V. J., C. Baas, P. Lexmond, J. Waldenstrom, A. Wallensten, T. Fransson, G. F. Rimmelzwaan, W. E. Beyer, M. Schutten, B. Olsen, A. D. Osterhaus, and R. A. Fouchier.** 2007. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog **3:**e61.
38. **OIE.** 2003. Manual of standards for diagnostic tests and vaccines. . Office of International des Epizooties.
39. **Oshansky, C. M., J. A. Pickens, K. C. Bradley, L. P. Jones, G. M. Saavedra-Ebner, J. P. Barber, J. M. Crabtree, D. A. Steinhauer, S. M. Tompkins, and R. A. Tripp.** 2011. Avian Influenza Viruses Infect Primary Human Bronchial Epithelial Cells Unconstrained by Sialic Acid alpha2,3 Residues. PLoS One **6:**e21183.
40. **Rogers, G. N., and B. L. D'Souza.** 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology **173:**317-22.
41. **Rogers, G. N., and J. C. Paulson.** 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:**361-73.
42. **Rogers, G. N., T. J. Pritchett, J. L. Lane, and J. C. Paulson.** 1983. Differential sensitivity of human, avian, and equine influenza a viruses to a glycoprotein inhibitor of infection: Selection of receptor specific variants. Virology **131:**394-408.
43. **Shelton, H., G. Ayora-Talavera, J. Ren, S. Loureiro, R. J. Pickles, W. S. Barclay, and I. M. Jones.** 2011. Receptor binding profiles of avian influenza virus hemagglutinin subtypes on human cells as a predictor of pandemic potential. J Virol **85:**1875-80.
44. **Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka.** 2006. Avian flu: influenza virus receptors in the human airway. Nature **440:**435-6.
45. **Steinhauer, D. A.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.
46. **Stevens, J., O. Blixt, L. Glaser, J. K. Taubenberger, P. Palese, J. C. Paulson, and I. A. Wilson.** 2006. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J Mol Biol **355:**1143-55.
47. **Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J **11:**2407-14.
48. **Suarez, D. L., M. L. Perdue, N. Cox, T. Rowe, C. Bender, J. Huang, and D. E. Swayne.** 1998. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol **72:**6678-88.
49. **Suarez, D. L., P. R. Woolcock, A. J. Bermudez, and D. A. Senne.** 2002. Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis **46:**111-21.
50. **Subbarao, K., and M. W. Shaw.** 2000. Molecular aspects of avian influenza (H5N1) viruses isolated from humans. Rev Med Virol **10:**337-48.

51. **Suzuki, Y., T. Ito, T. Suzuki, R. E. Holland, Jr., T. M. Chambers, M. Kiso, H. Ishida, and Y. Kawaoka.** 2000. Sialic acid species as a determinant of the host range of influenza A viruses. J Virol **74:**11825-31.
52. **Swayne, D. E.** 2008. Avian influenza, 1st ed. Blackwell Pub., Ames, Iowa.
53. **Taubenberger, J. K., and D. M. Morens.** 2006. 1918 Influenza: the mother of all pandemics. Emerg Infect Dis **12:**15-22.
54. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-79.
55. **Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351:**467-71.
56. **Zanella, A., P. Dall'Ara, and P. A. Martino.** 2001. Avian influenza epidemic in Italy due to serovar H7N1. Avian Dis **45:**257-61.
57. **Zhirnov, O. P., M. R. Ikizler, and P. F. Wright.** 2002. Cleavage of influenza a virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. J Virol **76:**8682-9.
58. **Zhirnov, O. P., I. V. Vorobjeva, A. V. Ovcharenko, and H. D. Klenk.** 2003. Intracellular cleavage of human influenza a virus hemagglutinin and its inhibition. Biochemistry (Mosc) **68:**1020-6.

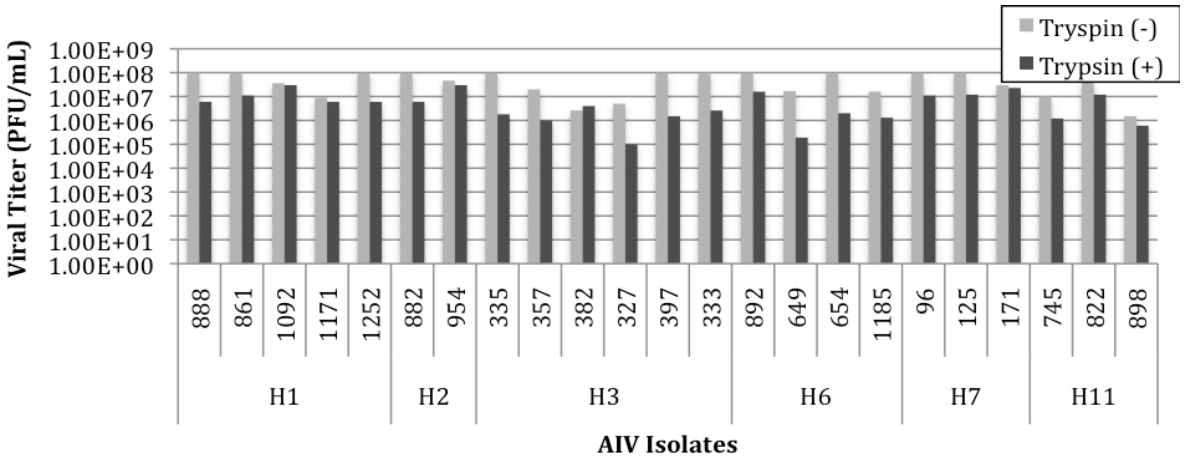


Figure A1. Trypsin independent replication of North American low pathogenic avian influenza (LPAI) isolates. Plaque assays were performed in the presence (1μg/mL of TPCK-trypsin) or in the absence of exogenous trypsin in the Avicel overlay. A total of 23 LPAI isolates of various subtypes were included in the screen.

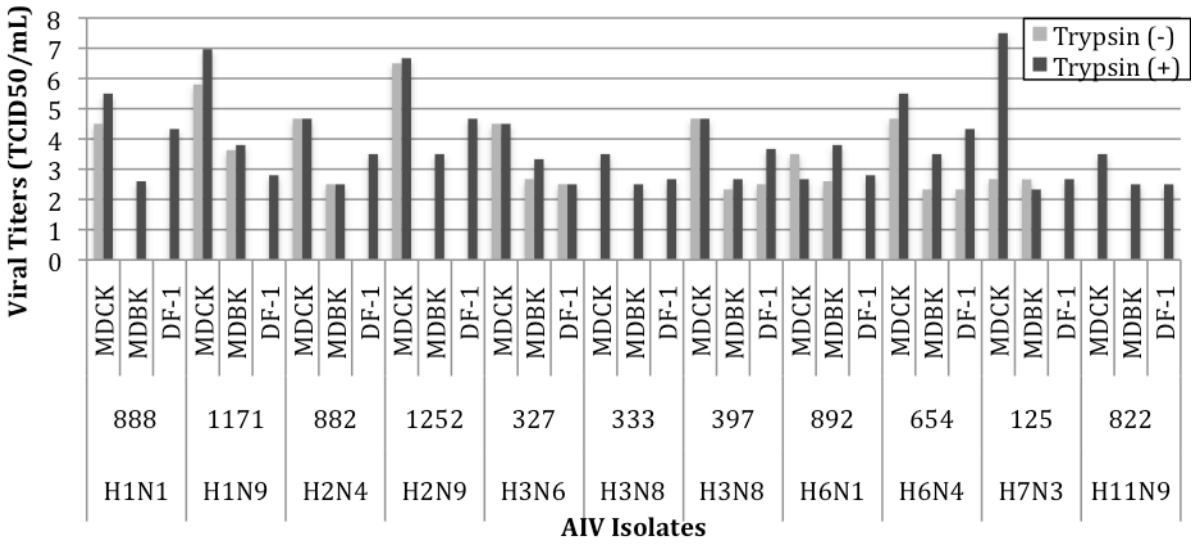


Figure A2. Trypsin independent replication observed in various cell types. All LPAI isolates were further screened using a 2-step assay, where Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), and chicken embryo fibroblast (DF-1) were infected with each isolate at an MOI of 0.001 for 2 hours before receiving MEM supplemented with 1μg/mL of TPCK-trypsin (+) or MEM only (-). Supernatant was taken at 4, 24, and 48 hour time points. Viral titers were determined by $TCID_{50}$ on MDCK cells. The data represents the highest viral titer observed for each distinct LPAI subtype over a 48 hour time period.

Table A1. All LPAI express the prototypic monobasic HA cleavage site.

AIV isolate	Subtype	HA Cleavage Site	Accession #
A/Ruddy Turnstone/NJ/650626/02	H1N9	LRNVPSIPS**R**GLF	
A/Ruddy Turnstone/DE/650621/02	H2N9	LRNVPSIQS**R**GLF	
A/Ruddy Turnstone/NJ/1321394/05	H3N6	RNVPEKQT**K**GLF	
A/Ruddy Turnstone/NJ/1321395/05	H3N8	RNVPEKQT**K**GLF	
A/Mute Swan/MI/451072-02/06	H5N1	RNVPQRET**R**GLF	ABV25968.1
A/Sanderling/DE/650680/02	H6N4	LRNVPQIET**R**GLF	
A/Sanderling/NJ/1523471/06	H7N3	NVPENPKT**R**GLF	

The amino acid, arginine (**R**) or a lysine (**K**), indicative of the HA cleavage site for each HA subtype included in the study. The HA cleavage site shown represents the sequence present under (+) and (-) trypsin conditions.

Table A2. Agglutination of erythrocytes from different animal species by LPAI isolates. Hemagglutination titers are provided as the reciprocal of the highest virus dilution generating agglutination. NB abbreviation for no binding.

AIV Virus Strain	ID#	Subtype	Turkey	Horse	Guinea Pig
A/Ruddy Turnstone/NJ/650624/02	888	H1N1	512	512	256
A/Ruddy Turnstone/DE/650621/02	861	H1N9	1024	1024	1024
A/Ruddy Turnstsone/DE/650580/02	1252	H1N9	1024	256	512
A/Ruddy Turnstsone/NJ/650638/02	1092	H1N9	512	128	512
A/Sanderling/DE/650623/02	882	H2N4	1024	128	128
A/Ruddy Turnstsone/NJ/650627/02	954	H2N9	512	256	512
A/Ruddy Turnstsone/DE/650645/02	1171	H1N9	1024	512	512
A/Ruddy Turnstone/NJ/1321397/05	357	H3N6	512	2048	512
A/Ruddy Turnstone/NJ/1321396/05	335	H3N6	512	2048	512
A /Ruddy Turnstone/NJ/1321398/05	382	H3N6	128	256	32
A/Ruddy Turnstone/NJ/1321395/05	333	H3N8	256	1024	256
A /Ruddy Turnstone/NJ/1321399/05	397	H3N8	512	2048	512
A/Ruddy Turnstone/NJ/1321394/05	327	H3N6	32	NB	256
A/Sanderling/DE/650680/02	654	H6N4	128	256	256
A/Ruddy Turnstone/NJ/650677/02	649	H6N4	128	128	256
A/Ruddy Turnstone/DE/650625/02	892	H6N1	1024	512	256
A/feces/DE/650574/02	1185	H6N8	256	128	128
A/Red Knot/NJ/1523470/06	096	H7N3	1024	4096	256
A/Sanderling/NJ/1523471/06	125	H7N3	512	4096	256
A/Ruddy Turnstone/NJ/1523477/06	171	H7N3	512	4096	512
A/Ruddy Turnstsone/NJ/650626/02	898	H11N9	256	1024	512
A/Ruddy Turnstone/NJ/650615/02	745	H11N2	512	512	512
A/feces/DE/650619/02	822	H11N9	256	512	512
Human Virus Strain					
A/Pennsylvania/08/2008	N/A	H1N1	128	NB	32