EVALUATING THE PANDEMIC POTENTIAL OF INFLUENZA A VIRUSES IN PRIMARY AIRWAY MODELS

By

MADELYN CATHERINE KRUNKOSKY

(Under the Direction of Mark Tompkins)

ABSTRACT

Influenza A viruses (IAVs) are single stranded negative sense RNA viruses containing eight gene segments. IAVs have two genetic traits: (a) drift, resulting from mutations, usually in gene segments expressing the surface glycoproteins hemagglutinin and neuraminidase and (b) shift, or reassortment, which occurs when two viruses co-infect the same host cell and exchange gene segments. Migratory waterfowl are reservoirs of IAVs, while susceptible species include poultry, horses, humans, swine, and other mammals. IAVs are among the most important viral pathogens for animal health, causing outbreaks of respiratory disease in swine and poultry and occasional zoonotic transmission to humans. Swine are proposed as a "mixing" vessel since they are susceptible to avian and human IAVs and due to the distribution of avian- and human-like sialic acid receptors within their respiratory tract. H1N1 viruses have been circulating in pigs for over a century and, in the past decade, the predominant matrix gene has become the pdmM gene. The pdmM gene has been implicated as a determinant of improved respiratory transmission, which suggests that it might play an important role in the ability of these pdm viruses to spread in humans. Experiments performed in this dissertation will evaluate the pandemic potential and tropism of swine and avian influenza viruses as well as the effects of the matrix gene.

INDEX WORDS: swine influenza, pandemic potential, primary cells, matrix gene

EVALUATING THE PANDEMIC POTENTIAL OF INFLUENZA A VIRUSES IN PRIMARY AIRWAY MODELS

By

MADELYN CATHERINE KRUNKOSKY

BS, University of Georgia, Athens, GA, 2014
MS, University of Georgia, Athens, GA, 2017

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, GA, 2021

EVALUATING THE PANDEMIC POTENTIAL OF INFLUENZA A VIRUSES IN PRIMARY AIRWAY MODELS

By

MADELYN CATHERINE KRUNKOSKY

Major Professors: Mark Tompkins

Committee: Jeff Hogan

Balazs Rada

Daniela de Souza Rajao

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
August 2021

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major professor, Dr. Mark Tompkins not only for the research opportunity, continuous support, and encouragement, but also for his contributions and guidance throughout this entire research project.

I also would like to express my sincere appreciation to each professor that served on my advisory committee, Dr. Jeff Hogan, Dr. Balazs Rada and Dr. Daniela Rajao, for their expert input and all the time they have spent reviewing this work.

I would also like to thank Dr. Tom Krunkosky for helping develop the primary swine cells used in these experiments and his countless hours he spent with me acquiring images.

Finally, many thanks to my family and my fiancé for their confidence in my ambitions and their continuous support throughout this journey. This experience has been both challenging and immensely rewarding both personally and professionally. To all who have contributed to its completion in so many ways, I am and will always be especially grateful.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF TABLES vi

LIST OF FIGURES vii

1. LITERATURE REVIEW 1

2. ESTABLISHMENT OF PRIMARY PORCINE NASAL (PNE), TRACHEAL (PTE) AND BRONCHIAL EPITHELIAL (PBE) CELLS FOR THE STUDY OF SWINE RESPIRATORY VIRUSES 24

3. PRIMARY PORCINE NASAL (PNE), TRACHEAL (PTE) AND BRONCHIAL EPITHELIAL (PBE) CELLS ARE A FEASIBLE MODEL FOR INFLUENZA PANDEMIC RISK ASSESSMENT 45

4. THE MATRIX GENE ORIGIN OF SWINE INFLUENZA H1N1 AND H1N2 ISOLATES AFFECTS VIRAL TROPISM, REPLICATION KINETICS, AND PANDEMIC POTENTIAL IN PRIAMRY AIRWAY MODELS 63

5. DISCUSSION 106

LIST OF TABLES

Page

TABLE 4.1.1 GENETIC SIMILARITY OF CA/07/09 TO VARIOUS SWINE ISOLATES....76

TABLE 4.1.2 GENETIC SIMILARITY OF SW/IL/201/13 TO VARIOUS SWINE ISOLATES....77

TABLE 4.1.3 GENETIC SIMILARITY OF SW/NC/702/15 TO VARIOUS SWINE ISOLATES....78

TABLE 4.1.4 GENETIC SIMILARITY OF SW/NC/076/15 TO VARIOUS SWINE ISOLATES....79

TABLE 4.1.5 GENETIC SIMILARITY OF SW/MO/664/13 TO VARIOUS SWINE ISOLATES....79

TABLE 4.1.6 GENETIC SIMILARITY OF SW/IN/351/11 TO VARIOUS SWINE ISOLATES....80

SUPPLEMENTAL TABLE 4.1 MDCK KINETICS 2-WAY ANOVA MULTIPLE COMPARISONS....93

SUPPLEMENTAL TABLE 4.2 CALU-3 KINETICS 2-WAY ANOVA MULTIPLE COMPARISONS....94

SUPPLEMENTAL TABLE 4.3 NHBE KINETICS MIXED-EFFECT ANALYSIS MULTIPLE COMPARISONS....95

SUPPLEMENTAL TABLE 4.4 PNE KINETICS MIXED-EFFECT ANALYSIS MULTIPLE COMPARISONS....96

SUPPLEMENTAL TABLE 4.5 PTE KINETICS MIXED-EFFECT ANALYSIS MULTIPLE COMPARISONS....97

SUPPLEMENTAL TABLE 4.6 PBE KINETICS MIXED-EFFECT ANALYSIS MULTIPLE COMPARISONS....98

LIST OF FIGURES

Page

FIGURE 2.1 HARVESTING AND CULTURING OF PRIMARY BRONCHIAL AND TRACHEAL EPITHELIAL CELLS

.....36

FIGURE 2.2 PRIMARY PNE, PTE, AND PBE TRANS-EPITHELIAL ELECTRICAL RESISTANCE

.....37

FIGURE 2.3 PRIMARY PNE, PTE, AND PBE IMMUNOHISTOCHEMISTRY OF TIGHT JUNCTIONS

.....38

FIGURE 2.4 2,6-SIALIC ACID LECTIN IMMUNOHISTOCHEMISTRY OF PNE, PTE, AND PBE CELLS

.....39

FIGURE 2.5 2,3-SIALIC ACID LECTIN IMMUNOHISTOCHEMISTRY OF PNE, PTE, AND PBE CELLS
.....40

FIGURE 3.1. REPLICATION KINETICS OF H3N2, H3N1 SWINE ISOLATES IN PNE, PTE, PBE, CALU-3, AND NHBE CELLS 54

FIGURE 3.2. REPLICATION KINETICS OF H1N1, H1N2 SWINE ISOLATES IN PNE, PTE, PBE, CALU-3, AND NHBE CELLS 55

FIGUE 3.3. REPLICATION KINETICS OF H7 AVIAN ISOLATES IN PNE, PTE, PBE, CALU-3, AND NHBE CELLS.....
.....56

FIGUE 4.1. REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOLATES IN MDCK CELLS .....
.....81

FIGUE 4.2. REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOLATES IN CALU-3 CELLS .....
.....82

FIGUE 4.3.1. REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOLATES IN NHBE CELLS 83

FIGUE 4.3.2. IMMUNOHISTOCHEMISTRY OF NHBE CELLS 84

FIGUE 4.4.1. REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOLATES IN PNE CELLS 85

FIGUE 4.4.2. IMMUNOHISTOCHEMISTRY OF PNE CELLS 86

FIGUE 4.5.1. REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOLATES IN PTE CELLS 87

FIGUE 4.5.2. IMMUNOHISTOCHEMISTRY OF PTE CELLS 88

FIGUE 4.6.1. REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOLATES IN PBE CELLS 89

FIGUE 4.6.2. IMMUNOHISTOCHEMISTRY OF PBE CELLS 90

FIGUE 4.7. TEMPERATURE REPLICATION KINETICS OF H1N1 AND H1N2 SWINE ISOALTES IN PNE CELLS 91-92

CHAPTER 1
LITERATURE REVIEW

Introduction to Influenza A Viruses

Influenza viruses belong to the family *Orthomyxoviridae* and are divided into 4 genera, A, B, C, and D. Influenza A and B viruses are single stranded negative sense RNA viruses containing eight gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS). Influenza C and D viruses are single stranded negative sense RNA viruses that only contain seven gene segments since the hemagglutinin-esterase fusion protein vRNA replaces the HA and NA. Influenza B and C have sporadically been isolated from other mammals, but are largely restricted to humans. Influenza D viruses have been identified in cattle and swine. The focus of this dissertation will be pandemic influenza A viruses (IAVs) as they pose the greatest risk to human health (1-6).

IAVs have two genetic traits: (a) drift, resulting from mutations, usually in gene segments expressing the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), and (b) shift, or reassortment, which occurs when two viruses co-infect the same host cell and exchange gene segments (7). Migratory waterfowl are reservoirs of IAVs, while susceptible species include poultry, horses, humans, swine, and other mammals (7-10). IAVs are subtyped based on their surface antigens HA and NA, which mediate viral entry and release. To date there are a total of 18 HAs and 11 NAs that have been identified. H1-H16 and N1-N9 have been found in aquatic birds, while H17N10 and H18N11 have been identified in bats (8, 11-14). Swine IAVs are among the most important viral pathogens for animal health, causing outbreaks of respiratory disease in swine and occasional zoonotic transmission to humans.

The Porcine Respiratory Disease Complex and Swine Influenza Across the Globe

Swine are susceptible to multiple viral and bacterial respiratory pathogens. These pathogens are commonly found in co-infections that result in respiratory disease, referred to as the Porcine Respiratory Disease Complex (PRDC). Pathogens that cause PRDC include but are not limited to, swine influenza virus (swIAV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, and *Bordetella bronchiseptica* (15-20)**.** These viral and bacterial pathogens of swine are known to infect the respiratory epithelium. Respiratory diseases in swine are an important health concern for swine producers, since they cause tremendous economic loss due to poor growth performance, increased morbidity and mortality, and increased expenditures in control measures, thus greatly affecting the farmer's bottom line (15-20)**.**

The first significant outbreak of IAV in swine occurred in 1918 in the U.S., around the time of the 1918 pandemics in humans, caused by an H1N1 virus that was later shown to be similar to the virus causing the outbreak in humans. This virus, termed classical H1N1, remained genetically stable for almost 80 years. In 1979, an avian H1N1 virus jumped from wild ducks to pigs in Germany and Belgium. During the mid-1980s, H3N2 strains that were a descendant of the 1968 Hong Kong pandemic became prevalent in Europe. In the 1990s the H3N2 viruses that were circulating at the time, mixed with the seasonal H1N1 virus in circulation and created the H1N2 lineage (21-26). Then in 2009 the swine influenza pandemic occurred. In North America, a triple reassortant (TRIG) H3N2 virus was introduced in the late 1990s, which contained classical H1N1 NP, M, and NS, human seasonal H3N2 PB1, HA, and NA, and avian PB2 and PA genes (46-50). This virus reassorted with the H1N1 viruses that were circulating in pigs since 1918, resulting in the circulation of reassortant H1N1, H1N2, and H3N2 viruses. In the early 2000's, novel human-origin H1 viruses were detected

in pigs. Following the introduction of the 2009 pandemic H1N1 virus in humans, the virus was quickly reintroduced to pigs and became endemic. In the 2010-2011 season, a novel H3 virus lineage of human-origin was detected in swine in North America, which was genetically and antigenically distinct from viruses circulating at the time (27, 28). In Brazil, Chile, and Argentina, most of the H1N1, H1N2, and H3N2 viruses that have been detected contain the internal genes of the 2009 pandemic lineage (29-33). In China and in Southeast Asia, H1N1 viruses were endemic until the 1990s when the H1N1 and H3N2 triple reassortant viruses were introduced into the population (34, 35).

History of Past Pandemics of Avian and Swine Origin

The past four influenza pandemics were caused by viruses containing gene segments of either avian- or swine-origin. The 1918 pandemic was caused by an H1N1 virus with genes of avian origin. It was first identified in US military personnel in spring 1918. 500 million people, approximately 33% of the world's population, became infected. There was a total of 50 million deaths worldwide (36, 37). Next was the Asian pandemic in 1957. This virus was comprised of three different genes from an H2N2 virus, including the HA, NA and PB1, that originated from an avian influenza A virus. This pandemic led to 1.2 million deaths worldwide and 116,000 deaths in the United States (36-42). In 1968 the Hong Kong pandemic occurred, which was comprised of two genes from an avian influenza A virus, including the H3 and PB1, in addition to the N2 from 1957 pandemic. This pandemic lead to 1 million deaths worldwide and 100,000 deaths in the United States. Finally, we had the pandemic in 2009 (43-45). This pandemic virus was originated from the reassortment between two endemic swine viruses, the triple reassortment virus (PB2, PB1, PA, HA, NP, M, NS) and the M and NA genes derived from the Eurasian H1N1 virus, which then spread rapidly in both swine and human populations (51-55). During the 2009 H1N1 pandemic, it is

estimated that within the United States there were 60.8 million cases, 274,304 hospitalizations, and 12,469 deaths and an estimated range of 151,700-575,400 deaths worldwide (56).

Matrix Gene as a Determinate for Transmission

The matrix gene segment of influenza, consisting of 1027 base pairs, encodes two viral proteins, M1 (nucleotide position 26 to 784) and M2 (nucleotide position 26-51 and 740-1007). The M1 protein evolves slowly in human, avian, and swine IAV lineages. The M2 protein however has seen significant evolution in human and swine, but not as much in avian viruses. M1 forms a bridge between the inner core components of the virion and the membrane proteins. M1 promotes the formation of the RNP complex and causes it to dissociate from the nuclear matrix. M1 also plays a vital role in recruiting viral components and in the budding process (57-71). The M2 protein comprises 97 amino acids and its extracellular domain is recognized by the host's immune system. The M2 transmembrane domain forms an ion channel that participates in the uncoating of the virion once it is in the host cell (72-82).

H1N1 viruses have been circulating in pigs for over a century but, since the introduction of the H1N1pdm virus in pigs, the pdmM gene has become the predominant matrix gene in swine populations (46, 48, 50). During the 2009 H1N1 pandemic, the pdmM gene was implicated as a key determinant of increased respiratory transmission. This suggests that it might play an important role in the ability of viruses to be spread in humans (51-55). Swine-origin H3N2 viruses containing the pdmM gene from the 2009 pandemic have been isolated from the human population since 2011 and are referred to as "variant" viruses (83). Although the matrix gene has been implicated in the increased ability of swine influenza to transmit to humans, other genes may play a role. The viral polymerase, comprised of the PB1,

PB2, and PA gene segments has been shown to be a major host determinant. A single residue of the PB2 gene, E627K, has been correlated with increased viral replication and transmission in mammals. Other studies have also shown that other residues, A271 and D701N, are able to compensate for the absence of the E627K amino acid change (79, 84-90). The influenza viral NP gene segment has been shown to play a key role in MX sensitivity allowing for evasion of the host cell's Type-1 interferon response (91-95). Specifically, Y52, I100, P283, and Y313 have been shown to be essential to reduced MX sensitivity in cell culture and *in vivo* (96-98). Lastly the HA gene segment, which is essential for viral attachment as well as entry, also plays a role in host specificity. It has been found that viruses which contain a leucine at position 226 in the HA have a higher affinity for α2,6-sialic acid, and hence, have a preference to bind human-like sialic acid receptors (99, 100).

Swine as a "Mixing Vessel" and Primary Cell Culture

Pigs, proposed as a "mixing vessel" of IAVs, are linked to the generation of novel IAVs that may infect humans. Pigs are susceptible to swine, avian, and human influenza. This is thought to be linked to the distribution of sialic acids within their respiratory tract. The HA protein of IAVs binds to glycans on epithelial cells that have a terminal sialic acid (SA), triggering endocytosis of the viral particle. These sialic acids are either α2,3-linked or α2,6-linked. Avian IAVs preferentially infect cells expressing α2,3-SA, while mammalian IAVs prefer cells expressing the α2-6 SA, therefore avian IAV replication is restricted in humans, which contain a majority of α2-6 SA in the upper respiratory tract (101). Waterfowl, particularly ducks and geese, express mainly α2,3-SA in the respiratory tract, while white leghorn chickens express more α2,6-SA in their respiratory tract. Pheasants, turkeys, guinea fowl, and quail express both α2,3-SA and α2,6-SA in their respiratory tract (102, 103). The

upper respiratory tract of swine predominately contains cells expressing α2,6-SA, while cells expressing α2,3-SA dominate in the lower airways (104-106). The swine airway epithelium also has less sialylated glycans than humans and swine have a greater proportion of α2,6-SA than the human respiratory tract (107). In human bronchial and tracheal cells, it has been reported that α2,3-SA are expressed in ciliated cells, while α2,6 SA are primarily expressed in non-ciliated cells (108-110).

Multiple different types of human and animal cultures have been developed for studying influenza (111-116). Previous work concerning the role of the swine epithelium during influenza infection has been performed in ex vivo models using swine bronchial and tracheal explants (104-108, 117-119). These models allow the determination of binding of influenza to the respiratory epithelium. In these types of respiratory explant models, it is incredibly difficult to evaluate the innate immune response. Furthermore, in many models that try and re-create the swine respiratory epithelium from harvesting from pig donors, the cells remain in submerged culture and never fully differentiate (120-122). Often these cells are transformed as well to make an immortal cell line. These cell lines can differentiate and are easily passaged, but viral replication can be affected (122-124). Few studies have developed air liquid interface (ALI) primary swine cell systems, but they are limited to the tracheal and bronchial epithelium (124-128). Therefore, the aims of this dissertation are as follows:

Aim 1: Establish primary porcine nasal (PNE), tracheal (PTE), and bronchial epithelial (PBE) cells for the study of swine respiratory viruses in multiple donors

Aim 2: Determine if primary porcine nasal (PNE), tracheal (PTE), and bronchial epithelial (PBE) cells are a feasible model for influenza pandemic risk assessment

Aim 3: Determine if the origin of the matrix gene influences the tropism, replication kinetics,

and pandemic potential of swine H1N1 and H1N2 influenza viruses

References

1. Dou D, Revol R, Östbye H, Wang H, Daniels R. 2018. Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. Frontiers in Immunology 9.

2. Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, Wang D, Nelson EA, Li F. 2014. Characterization of a novel influenza virus in cattle and swine: proposal for a new genus in the Orthomyxoviridae family. MBio 5:e00031-14.

3. Shaw M, Palese P. 2013. Fields virology, p 1151–1185. Fields virology, 6th ed Lippincott Williams & Wilkins, Philadelphia, PA.

4. Palese P, Schulman JL. 1976. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. Proceedings of the National Academy of Sciences 73:2142-2146.

5. McGeoch D, Fellner P, Newton C. 1976. Influenza virus genome consists of eight distinct RNA species. Proceedings of the National Academy of Sciences 73:3045-3049.

6. Houser K, Subbarao K. 2015. Influenza vaccines: challenges and solutions. Cell host & microbe 17:295-300.

7. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiological reviews 56:152-179.

8. Yoon S-W, Webby RJ, Webster RG. 2014. Evolution and ecology of influenza A viruses. Influenza Pathogenesis and Control-Volume I:359-375.

9. Hurt AC, Vijaykrishna D, Butler J, Baas C, Maurer-Stroh S, Silva-de-la-Fuente MC, Medina-Vogel G, Olsen B, Kelso A, Barr IG. 2014. Detection of evolutionarily distinct avian influenza A viruses in Antarctica. MBio 5:e01098-14.

10. Hurt AC, Su YC, Aban M, Peck H, Lau H, Baas C, Deng Y-M, Spirason N, Ellström P, Hernandez J. 2016. Evidence for the introduction, reassortment, and persistence of diverse influenza A viruses in Antarctica. Journal of Virology 90:9674-9682.

11. Katz G, Benkarroum Y, Wei H, Rice WJ, Bucher D, Alimova A, Katz A, Klukowska J, Herman GT, Gottlieb P. 2014. Morphology of influenza B/Lee/40 determined by cryo-electron microscopy. PloS one 9:e88288.

12. Gamblin SJ, Skehel JJ. 2010. Influenza hemagglutinin and neuraminidase membrane glycoproteins. Journal of Biological Chemistry 285:28403-28409.

13. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DAA, Chen L-M, Recuenco S, Ellison JA, Davis CT, York IA. 2012. A distinct lineage of influenza A virus from bats. Proceedings of the National Academy of Sciences 109:4269-4274.

14. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J. 2013. New world bats harbor diverse influenza A viruses. PLoS pathog 9:e1003657.

15. Qin S, Ruan W, Yue H, Tang C, Zhou K, Zhang B. 2018. Viral communities associated with porcine respiratory disease complex in intensive commercial farms in Sichuan province, China. Scientific Reports 8:13341.

16. Van Reeth K, Vincent AL. Diseases of Swine 11th Edition doi:https://doi.org/10.1002/9781119350927.ch36. John Wiley & Sons, Inc.

17. Opriessnig T, Giménez-Lirola L, Halbur P. 2011. Polymicrobial respiratory disease in pigs. Animal Health Research Reviews 12:133.

18. Fablet C, Marois-Créhan C, Simon G, Grasland B, Jestin A, Kobisch M, Madec F, Rose N. 2012. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. Veterinary microbiology 157:152-163.

19. Schmidt C, Cibulski S, Andrade C, Teixeira T, Varela A, Scheffer C, Franco A, de Almeida L, Roehe P. 2016. Swine influenza virus and association with the porcine respiratory disease complex in pig farms in southern Brazil. Zoonoses and public health 63:234-240.

20. Galán-Relaño A, Luque I, Gómez-Gascón L, Morales-Partera A, Tarradas C, Carrasco L, Gómez-Laguna J. 2015. Seroprevalence against selected pathogens involved in porcine respiratory disease complex in free-range fattening pigs in Spain. Veterinary Record:vetrec-2015-103432.

21. Mancera Gracia JC, Pearce DS, Masic A, Balasch M. 2020. Influenza A Virus in Swine: Epidemiology, Challenges and Vaccination Strategies. Frontiers in Veterinary Science 7.

22. Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA. 1981. Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man. Bulletin of the World Health Organization 59:75-78.

23. Simon G, Larsen LE, Dürrwald R, Foni E, Harder T, Van Reeth K, Markowska-Daniel I, Reid SM, Dan A, Maldonado J, Huovilainen A, Billinis C, Davidson I, Agüero M, Vila T, Hervé S, Breum SØ, Chiapponi C, Urbaniak K, Kyriakis CS, consortium E, Brown IH, Loeffen W. 2014. European Surveillance Network for Influenza in Pigs: Surveillance Programs, Diagnostic Tools and Swine Influenza Virus Subtypes Identified in 14 European Countries from 2010 to 2013. PLOS ONE 9:e115815.

24. Hervé S, Garin E, Calavas D, Lecarpentier L, Ngwa-Mbot D, Poliak S, Wendling S, Rose N, Simon G. 2019. Virological and epidemiological patterns of swine influenza A virus infections in France: Cumulative data from the RESAVIP surveillance network, 2011–2018. Veterinary Microbiology 239:108477.

25. Brown IH, Harris PA, McCauley JW, Alexander DJ. 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. Journal of General Virology 79:2947-2955.

26. Watson SJ, Langat P, Reid SM, Lam TT-Y, Cotten M, Kelly M, Reeth KV, Qiu Y, Simon G, Bonin E, Foni E, Chiapponi C, Larsen L, Hjulsager C, Markowska-Daniel I, Urbaniak K, Dürrwald R, Schlegel M, Huovilainen A, Davidson I, Dán Á, Loeffen W, Edwards S, Bublot M, Vila T, Maldonado J, Valls L, Brown IH, Pybus OG, Kellam P, García-Sastre A. 2015. Molecular Epidemiology and Evolution of Influenza Viruses Circulating within European Swine between 2009 and 2013. Journal of Virology 89:9920-9931.

27. Zeller MA, Anderson TK, Walia RW, Vincent AL, Gauger PC. 2018. ISU FLUture: a veterinary diagnostic laboratory web-based platform to monitor the temporal genetic patterns of Influenza A virus in swine. BMC Bioinformatics 19:397.

28. Rajão DS, Gauger PC, Anderson TK, Lewis NS, Abente EJ, Killian ML, Perez DR, Sutton TC, Zhang J, Vincent AL, García-Sastre A. 2015. Novel Reassortant Human-Like H3N2 and H3N1 Influenza A Viruses Detected in Pigs Are Virulent and Antigenically Distinct from Swine Viruses Endemic to the United States. Journal of Virology 89:11213-11222.

29. Rajão DS, Costa ATR, Brasil BSAF, Del Puerto HL, Oliveira FG, Alves F, Braz GF, Reis JKP, Guedes RMC, Lobato ZIP, Leite RC. 2013. Genetic characterization of influenza virus circulating in Brazilian pigs during 2009 and 2010 reveals a high prevalence of the pandemic H1N1 subtype. Influenza and Other Respiratory Viruses 7:783-790.

30. Nelson MI, Schaefer R, Gava D, Cantão ME, Ciacci-Zanella JR. 2015. Influenza A Viruses of Human Origin in Swine, Brazil. Emerging infectious diseases 21:1339-1347.

31. Nelson M, Culhane MR, Rovira A, Torremorell M, Guerrero P, Norambuena J. 2015. Novel Human-like Influenza A Viruses Circulate in Swine in Mexico and Chile. PLoS currents 7:ecurrents.outbreaks.c8b3207c9bad98474eca3013fa933ca6.

32. Cappuccio JA, Pena L, Dibárbora M, Rimondi A, Piñeyro P, Insarralde L, Quiroga MA, Machuca M, Craig MI, Olivera V, Chockalingam A, Perfumo CJ, Perez DR, Pereda A. 2011. Outbreak of swine influenza in Argentina reveals a non-contemporary human H3N2 virus highly transmissible among pigs. The Journal of general virology 92:2871-2878.

33. Pereda A, Cappuccio J, Quiroga MA, Baumeister E, Insarralde L, Ibar M, Sanguinetti R, Cannilla ML, Franzese D, Escobar Cabrera OE, Craig MI, Rimondi A, Machuca M, Debenedetti RT, Zenobi C, Barral L, Balzano R, Capalbo S, Risso A, Perfumo CJ. 2010. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerging infectious diseases 16:304-307.

34. Yu H, Zhang P-C, Zhou Y-J, Li G-X, Pan J, Yan L-P, Shi X-X, Liu H-L, Tong G-Z. 2009. Isolation and genetic characterization of avian-like H1N1 and novel ressortant H1N2 influenza viruses from pigs in China. Biochemical and Biophysical Research Communications 386:278-283.

35. Ngo LT, Hiromoto Y, Pham VP, Le HTH, Nguyen HT, Le VT, Takemae N, Saito T. 2012. Isolation of novel triple-reassortant swine H3N2 influenza viruses possessing the hemagglutinin and neuraminidase genes of a seasonal influenza virus in Vietnam in 2010. Influenza and Other Respiratory Viruses 6:6-10.

36. CDC. Past Pandemics.

37. Kilbourne ED. 2006. Influenza pandemics of the 20th century. Emerging infectious diseases 12:9-14.

38. Kilbourne ED. 1959. Studies on influenza in the pandemic of 1957-1958. III. Isolation of influenza A (Asian strain) viruses from influenza patients with pulmonary complications; details of virus isolation and characterization of isolates, with quantitative comparison of isolation methods. The Journal of clinical investigation 38:266-274.

39. Rogers DE, Louria DB, Kilbourne ED. 1958. The syndrome of fatal influenza virus pneumonia. Trans Assoc Am Physicians 71:260-73.

40. Smith W, Andrewes CH, Laidlaw PP. 1933. A VIRUS OBTAINED FROM INFLUENZA PATIENTS. The Lancet 222:66-68.

41. Langmuir AD. 1961. Epidemiology of Asian influenza. With special emphasis on the United States. Am Rev Respir Dis 83(2)Pt 2:2-14.

42. Choppin PW, Tamm I. 1960. Studies of two kinds of virus particles which comprise influenza A2 virus strains. II. Reactivity with virus inhibitors in normal sera. The Journal of experimental medicine 112:921-944.

43. Schulman JL, Kilbourne ED. 1969. Independent variation in nature of hemagglutinin and neuraminidase antigens of influenza virus: distinctiveness of hemagglutinin antigen of Hong Kong-68 virus. Proc Natl Acad Sci U S A 63:326-33.

44. Monto AS, Kendal AP. 1973. Effect of neuraminidase antibody on Hong Kong influenza. Lancet 1:623-5.

45. Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L. 2005. Multinational impact of the 1968 Hong Kong influenza pandemic: evidence for a smoldering pandemic. J Infect Dis 192:233-48.

46. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. 2000. Evolution of Swine H3N2 Influenza Viruses in the United States. Journal of Virology 74:8243.

47. Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Reeth KV, Brown IH, Swenson SL, Simon G, Saito T, Berhane Y, Ciacci-Zanella J, Pereda A, Davis CT, Donis RO, Webby RJ, Vincent AL, Lowen AC. 2016. A Phylogeny-Based Global Nomenclature System and Automated Annotation Tool for H1 Hemagglutinin Genes from Swine Influenza A Viruses. mSphere 1:e00275-16.

48. Brockwell-Staats C, Webster RG, Webby RJ. 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza and other respiratory viruses 3:207-213.

49. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73:8851-6.

50. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton J-C, Lowe J, Gramer M, Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerging infectious diseases 17:1624-1629.

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

52. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125.

53. Mena I, Nelson MI, Quezada-Monroy F, Dutta J, Cortes-Fernández R, Lara-Puente JH, Castro-Peralta F, Cunha LF, Trovão NS, Lozano-Dubernard B. 2016. Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. Elife 5:e16777.

54. Campbell PJ, Danzy S, Kyriakis CS, Deymier MJ, Lowen AC, Steel J. 2014. The M Segment of the 2009 Pandemic Influenza Virus Confers Increased Neuraminidase Activity, Filamentous Morphology, and Efficient Contact Transmissibility to A/Puerto Rico/8/1934-Based Reassortant Viruses. Journal of Virology 88:3802.

55. Chou Y-y, Albrecht RA, Pica N, Lowen AC, Richt JA, García-Sastre A, Palese P, Hai R. 2011. The M Segment of the 2009 New Pandemic H1N1 Influenza Virus Is Critical for Its High Transmission Efficiency in the Guinea Pig Model. Journal of Virology 85:11235.

56. CDC. 2019. 2009 H1N1 Pandemic (H1N1 pdm09 virus). https://wwwcdcgov/flu/pandemic-resources/2009-h1n1-pandemichtml.

57. Furuse Y, Suzuki A, Kamigaki T, Oshitani H. 2009. Evolution of the M gene of the influenza A virus in different host species: large-scale sequence analysis. Virology Journal 6:67.

58. Ito T, Gorman OT, Kawaoka Y, Bean WJ, Webster RG. 1991. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol 65:5491-8.

59. Nayak DP, Hui EK-W, Barman S. 2004. Assembly and budding of influenza virus. Virus research 106:147-165.

60. Schmitt AP, Lamb RA. 2005. Influenza virus assembly and budding at the viral budozone. Advances in virus research 64:383-416.

61. Cros JF, Palese P. 2003. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses. Virus research 95:3-12.

62. Garcia-Robles I, Akarsu H, Müller CW, Ruigrok RW, Baudin F. 2005. Interaction of influenza virus proteins with nucleosomes. Virology 332:329-336.

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

64. Elster C, Larsen K, Gagnon J, Ruigrok R, Baudin F. 1997. Influenza virus M1 protein binds to RNA through its nuclear localization signal. Journal of general virology 78:1589-1596.

65. Elster C, Fourest E, Baudin F, Larsen K, Cusack S, Ruigrok RW. 1994. A small percentage of influenza virus M1 protein contains zinc but zinc does not influence in vitro M1-RNA interaction. Journal of general virology 75:37-42.

66. Wakefield L, Brownlee GG. 1989. RNA-binding properties of influenza A virus matrix protein M1. Nucleic acids research 17:8569-8580.

67. Ye Z, Liu T, Offringa DP, McInnis J, Levandowski RA. 1999. Association of influenza virus matrix protein with ribonucleoproteins. Journal of virology 73:7467-7473.

68. Gómez-Puertas P, Albo C, Pérez-Pastrana E, Vivo A, Portela An. 2000. Influenza virus matrix protein is the major driving force in virus budding. Journal of virology 74:11538-11547.

69. Latham T, Galarza JM. 2001. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. Journal of virology 75:6154-6165.

70. Lamb RA. 1989. The genes and proteins of infleunza viruses. The influenza viruses:1-87.

71. Holsinger LJ, Alams R. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. Virology 183:32-43.

72. Gerhard W, Mozdzanowska K, Furchner M, Washko G, Maiese K. 1997. Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunological reviews 159:95-103.

73. Potter C, Oxford J. 1979. Determinants of immunity to influenza infection in man. British medical bulletin 35:69-75.

74. Treanor JJ, Tierney EL, Zebedee SL, Lamb RA, Murphy BR. 1990. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. Journal of virology 64:1375-1377.

75. Pinto LH, Holsinger LJ, Lamb RA. 1992. Influenza virus M2 protein has ion channel activity. cell 69:517-528.

76. Zebedee SL, Lamb RA. 1989. Growth restriction of influenza A virus by M2 protein antibody is genetically linked to the M1 protein. Proceedings of the National Academy of Sciences 86:1061-1065.

77. Hughey PG, Roberts PC, Holsinger LJ, Zebedee SL, Lamb RA, Compans RW. 1995. Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology 212:411-421.

78. Schroeder C, Heider H, Möncke-Buchner E, Lin T-I. 2005. The influenza virus ion channel and maturation cofactor M2 is a cholesterol-binding protein. European Biophysics Journal 34:52-66.

79. Chen R, Holmes EC. 2006. Avian Influenza Virus Exhibits Rapid Evolutionary Dynamics. Molecular Biology and Evolution 23:2336-2341.

80. Castrucci MR, Kawaoka Y. 1995. Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein. Journal of virology 69:2725-2728.

81. McCown MF, Pekosz A. 2006. Distinct domains of the influenza a virus M2 protein cytoplasmic tail mediate binding to the M1 protein and facilitate infectious virus production. Journal of virology 80:8178-8189.

82. Ozawa M, Maeda J, Iwatsuki-Horimoto K, Watanabe S, Goto H, Horimoto T, Kawaoka Y. 2009. Nucleotide sequence requirements at the 5′ end of the influenza A virus M RNA segment for efficient virus replication. Journal of virology 83:3384-3388.

83. Prevention CfDCa. 2020. Reported Infections with Variant Influenza Viruses in the United States.

84. Neumann G, Kawaoka Y. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerging infectious diseases 12:881.

85. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67:1761-4.

86. Massin P, Werf Svd, Naffakh N. 2001. Residue 627 of PB2 Is a Determinant of Cold Sensitivity in RNA Replication of Avian Influenza Viruses. Journal of Virology 75:5398-5404.

87. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. 2007. Growth of H5N1 Influenza A Viruses in the Upper Respiratory Tracts of Mice. PLOS Pathogens 3:e133.

88. Labadie K, Dos Santos Afonso E, Rameix-Welti MA, van der Werf S, Naffakh N. 2007. Host-range determinants on the PB2 protein of influenza A viruses control the

interaction between the viral polymerase and nucleoprotein in human cells. Virology 362:271-82.

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

90. Manzoor R, Sakoda Y, Nomura N, Tsuda Y, Ozaki H, Okamatsu M, Kida H. 2009. PB2 protein of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) determines its replication potential in pigs. J Virol 83:1572-8.

91. La Gruta NL, Kedzierska K, Stambas J, Doherty PC. 2007. A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol 85:85-92.

92. Randall RE, Goodbourn S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. Journal of General Virology 89:1-47.

93. Killip MJ, Fodor E, Randall RE. 2015. Influenza virus activation of the interferon system. Virus Res 209:11-22.

94. Xiao H, Killip MJ, Staeheli P, Randall RE, Jackson D. 2013. The human interferon-induced MxA protein inhibits early stages of influenza A virus infection by retaining the incoming viral genome in the cytoplasm. J Virol 87:13053-8.

95. Ciancanelli MJ, Abel L, Zhang SY, Casanova JL. 2016. Host genetics of severe influenza: from mouse Mx1 to human IRF7. Curr Opin Immunol 38:109-20.

96. Riegger D, Hai R, Dornfeld D, Mänz B, Leyva-Grado V, Sánchez-Aparicio MT, Albrecht RA, Palese P, Haller O, Schwemmle M, García-Sastre A, Kochs G, Schmolke M, Lyles DS. 2015. The Nucleoprotein of Newly Emerged H7N9 Influenza A Virus Harbors a

Unique Motif Conferring Resistance to Antiviral Human MxA. Journal of Virology 89:2241-2252.

97. Mänz B, Dornfeld D, Götz V, Zell R, Zimmermann P, Haller O, Kochs G, Schwemmle M. 2013. Pandemic Influenza A Viruses Escape from Restriction by Human MxA through Adaptive Mutations in the Nucleoprotein. PLOS Pathogens 9:e1003279.

98. Zimmermann P, Mänz B, Haller O, Schwemmle M, Kochs G. 2011. The Viral Nucleoprotein Determines Mx Sensitivity of Influenza A Viruses. Journal of Virology 85:8133-8140.

99. Bateman Allen C, Busch Marc G, Karasin Alexander I, Bovin N, Olsen Christopher W. 2008. Amino Acid 226 in the Hemagglutinin of H4N6 Influenza Virus Determines Binding Affinity for α2,6-Linked Sialic Acid and Infectivity Levels in Primary Swine and Human Respiratory Epithelial Cells. Journal of Virology 82:8204-8209.

100. Obadan AO, Santos J, Ferreri L, Thompson AJ, Carnaccini S, Geiger G, Gonzalez Reiche AS, Rajão DS, Paulson JC, Perez DR. 2019. Flexibility In Vitro of Amino Acid 226 in the Receptor-Binding Site of an H9 Subtype Influenza A Virus and Its Effect In Vivo on Virus Replication, Tropism, and Transmission. J Virol 93.

101. Byrd-Leotis L, Cummings RD, Steinhauer DA. 2017. The Interplay between the Host Receptor and Influenza Virus Hemagglutinin and Neuraminidase. International journal of molecular sciences 18:1541.

102. Kimble B, Nieto GR, Perez DR. 2010. Characterization of influenza virus sialic acid receptors in minor poultry species. Virology Journal 7:365.

103. Pillai SPS, Lee CW. 2010. Species and age related differences in the type and distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. Virology Journal 7:5.

104. Punyadarsaniya D, Liang C-H, Winter C, Petersen H, Rautenschlein S, Hennig-Pauka I, Schwegmann-Wessels C, Wu C-Y, Wong C-H, Herrler G. 2011. Infection of differentiated porcine airway epithelial cells by influenza virus: differential susceptibility to infection by porcine and avian viruses. PloS one 6:e28429-e28429.

105. Trebbien R, Larsen LE, Viuff BM. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virology Journal 8:434.

106. Van Poucke SG, Nicholls JM, Nauwynck HJ, Van Reeth K. 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7:38.

107. Chan RW, Karamanska R, Van Poucke S, Van Reeth K, Chan IW, Chan MC, Dell A, Peiris JS, Haslam SM, Guan Y, Nicholls JM. 2013. Infection of swine ex vivo tissues with avian viruses including H7N9 and correlation with glycomic analysis. Influenza Other Respir Viruses 7:1269-82.

108. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang K-C. 2010. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Veterinary Research 6:4.

109. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. 2006. Avian flu: influenza virus receptors in the human airway. Nature 440:435-6.

110. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JSM. 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.

111. Travanty E, Zhou B, Zhang H, Di YP, Alcorn JF, Wentworth DE, Mason R, Wang J. 2015. Differential Susceptibilities of Human Lung Primary Cells to H1N1 Influenza Viruses. Journal of virology 89:11935-11944.

112. Zhou J, Li C, Sachs N, Chiu MC, Wong BH-Y, Chu H, Poon VK-M, Wang D, Zhao X, Wen L, Song W, Yuan S, Wong KK-Y, Chan JF-W, To KK-W, Chen H, Clevers H, Yuen K-Y. 2018. Differentiated human airway organoids to assess infectivity of emerging influenza virus. Proceedings of the National Academy of Sciences 115:6822-6827.

113. Kroeker AL, Ezzati P, Halayko AJ, Coombs KM. 2012. Response of Primary Human Airway Epithelial Cells to Influenza Infection: A Quantitative Proteomic Study. Journal of Proteome Research 11:4132-4146.

114. Newby CM, Rowe RK, Pekosz A. 2006. Influenza A virus infection of primary differentiated airway epithelial cell cultures derived from Syrian golden hamsters. Virology 354:80-90.

115. Crotta S, Davidson S, Mahlakoiv T, Desmet CJ, Buckwalter MR, Albert ML, Staeheli P, Wack A. 2013. Type I and Type III Interferons Drive Redundant Amplification Loops to Induce a Transcriptional Signature in Influenza-Infected Airway Epithelia. PLOS Pathogens 9:e1003773.

116. Kondo M, Tamaoki J, Takeyama K, Nakata J, Nagai A. 2002. Interleukin-13 Induces Goblet Cell Differentiation in Primary Cell Culture from Guinea Pig Tracheal Epithelium. American Journal of Respiratory Cell and Molecular Biology 27:536-541.

117. Shin D-L, Yang W, Peng J-Y, Sawatsky B, von Messling Vv, Herrler G, Wu N-H. 2020. Avian Influenza A Virus Infects Swine Airway Epithelial Cells without Prior Adaptation. Viruses 12:589.

118. Glorieux S, Van den Broeck W, van der Meulen KM, Van Reeth K, Favoreel HW, Nauwynck HJ. 2007. In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. Journal of Virological Methods 142:105-112.

119. Meng F, Wu NH, Nerlich A, Herrler G, Valentin-Weigand P, Seitz M, Camilli A. 2015. Dynamic Virus-Bacterium Interactions in a Porcine Precision-Cut Lung Slice Coinfection Model: Swine Influenza Virus Paves the Way for Streptococcus suis Infection in a Two-Step Process. Infection and Immunity 83:2806-2815.

120. Sreenivasan CC, Thomas M, Antony L, Wormstadt T, Hildreth MB, Wang D, Hause B, Francis DH, Li F, Kaushik RS. 2019. Development and characterization of swine primary respiratory epithelial cells and their susceptibility to infection by four influenza virus types. Virology 528:152-163.

121. Peitsch C, Klenk H-D, Garten W, Böttcher-Friebertshäuser E. 2014. Activation of Influenza A Viruses by Host Proteases from Swine Airway Epithelium. Journal of Virology 88:282-291.

122. Xie X, Gan Y, Pang M, Shao G, Zhang L, Liu B, Xu Q, Wang H, Feng Y, Yu Y, Chen R, Wu M, Zhang Z, Hua L, Xiong Q, Liu M, Feng Z. 2018. Establishment and characterization of a telomerase-immortalized porcine bronchial epithelial cell line. Journal of Cellular Physiology 233:9763-9776.

123. Meliopoulos V, Cherry S, Wohlgemuth N, Honce R, Barnard K, Gauger P, Davis T, Shult P, Parrish C, Schultz-Cherry S. 2020. Primary swine respiratory epithelial cell lines for the efficient isolation and propagation of influenza A viruses. Journal of Virology doi:10.1128/JVI.01091-20:JVI.01091-20.

124. Wang H, He L, Liu B, Feng Y, Zhou H, Zhang Z, Wu Y, Wang J, Gan Y, Yuan T, Wu M, Xie X, Feng Z. 2018. Establishment and comparison of air-liquid interface culture systems for primary and immortalized swine tracheal epithelial cells. BMC Cell Biology 19:10.

125. Khoufache K, Cabaret O, Farrugia C, Rivollet D, Alliot A, Allaire E, Cordonnier C, Bretagne S, Botterel F. 2010. Primary in vitro culture of porcine tracheal epithelial cells in an air-liquid interface as a model to study airway epithelium and Aspergillus fumigatus interactions. Medical Mycology 48:1049-1055.

126. Young TF, Thacker EL, Erickson BZ, Ross RF. 2000. A tissue culture system to study respiratory ciliary epithelial adherence of selected swine mycoplasmas. Veterinary Microbiology 71:269-279.

127. Lam E, Ramke M, Groos S, Warnecke G, Heim A. 2011. A differentiated porcine bronchial epithelial cell culture model for studying human adenovirus tropism and virulence. Journal of Virological Methods 178:117-123.

128. Peng J-Y, Shin D-L, Li G, Wu N-H, Herrler G. 2021. Time-dependent viral interference between influenza virus and coronavirus in the infection of differentiated porcine airway epithelial cells. Virulence 12:1111-1121.

CHAPTER 2

ESTABLISHMENT OF PRIMARY PORCINE NASAL (PNE), TRACHEAL (PTE), AND BRONCHIAL (PBE) CELLS FOR THE STUDY OF SWINE RESPIRATORY VIRUSES[1]

[1] Krunkosky M[1], Krunkosky T, Meliopoulos V, Kyriakis C, Schultz-Cherry S, and Tompkins M. To be submitted MDPI Pathogens

KEY WORDS swine epithelium, differentiation, sialic acid, respiratory viruses

ABSTRACT

In this study we have established primary porcine nasal (PNE), tracheal (PTE), and bronchial (PBE) epithelial cells that recapitulate the physical and functional properties of the respiratory tract and have the ability to fully differentiate. This is indicated functionally by increasing TEERs and histologically by the presence of tight junctions. Primary PNE, PTE, and PBE cells developed within our lab are fully differentiate indicated by the presence of cilia and mucus secretion and also express sialic acid receptors, which are necessary receptors for influenza. Swine are susceptible to multiple viral and bacterial pathogens that affect the respiratory tract. This is commonly referred to as the Porcine Respiratory Disease Complex (PRDC). Swine are also proposed as a "mixing vessel" of influenza A viruses (IAVs) since they are susceptible to viruses of avian and human origin based on the distribution of sialic acid receptors within their respiratory tract, with the potential to generate novel IAVs that may infect humans. Adding primary PNE, PTE, and PBE cells to surveillance studies can help assess the pandemic potential of emerging influenza viruses for humans as well as help understand the dynamics of polymicrobial infections in swine.

INTRODUCTION

Swine are susceptible to multiple viral and bacterial pathogens. These viral and bacterial pathogens, commonly referred to as the Porcine Respiratory Disease Complex (PRDC), primarily effect the respiratory tract of swine. These viral and bacterial pathogens are known to infect alveolar macrophages as well as the respiratory epithelium. Some of these viral and bacterial infections include, but are not limited to, swine influenza virus (swIAV), porcine circovirus 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, and *Bordetella bronchiseptica*. The PRDC is an important health concern for swine producers, since it causes tremendous economic loss to the industry (1, 2). Previous literature concerning the role of the swine epithelium during influenza infection has come from ex vivo models using swine bronchial and tracheal explants (3-10). These models allow the determination of binding of influenza to the respiratory epithelium, but these models don't last long in culture outside the host and it is incredibly difficult to evaluate the innate immune responses occurring within these explants. Also, in many other models that try and re-create the swine respiratory epithelium from harvesting from pig donors, the cells remain in submerged culture and never fully differentiate (11-13) or they are transformed to make an immortal cell line that fully differentiates, but can affect viral replication (13-15). Few studies have developed an air liquid interface (ALI) primary swine cells system that fully differentiates and retains most characteristics of the swine respiratory tract, but they are limited to the tracheal and bronchial epithelium (15-19) The goal of this study was to generate reliable harvest and culture methods for the creation of primary porcine nasal (PNE), tracheal (PTE), and bronchial (PBE) epithelial cells that will fully differentiate and allow us to evaluate the pandemic potential, viral tropism, and the immune response against influenza on the

different regions of the airway.

MATERIALS AND METHODS

Porcine Primary Nasal Epithelial Cells (PNE) Isolation and Propagation

Swine 4-6 weeks of age from the University of Georgia swine farm negative for influenza were humanely euthanized. A total of 3 donor pigs were used to culture and propagate porcine primary nasal epithelial (PNE) cells. Individual donors were kept separate. Transport media (DMEM F-12 (Corning 10-090-CV) + GA-1000 (Corning CC-4083, final concentration 30mg/mL Gentamicin and 15 ng/mL Amphotericin) + Penicillin/streptomycin, Pen/Strep, (Gibco 15140-148, final concentration 100mg/mL) was prepared and aliquoted in 15 milliliter conical tubes containing 12 milliliters of transport media. The snout of the pig was sprayed down with 70% ethanol and a Kim-wipe was used to remove excess feed and dirt from the inside of the nares. A sterile nylon bristle cytology brush (Medical Packaging 987406) was used to enter the nostril and swirled within the nasal cavity to obtain epithelial cells. Individual cytology swabs from the left and right nostril from each pig were kept separate to decrease chances of contamination. Two cytology swabs per nostril were obtained, which equals 4 cytology swabs total per donor pig. The cytology brushes were then swirled and scraped together to remove epithelial cells within the transport media. Primary PNE cells were brought back to the lab and spun down in a centrifuge at 300g for 5 minutes. The supernatant was then removed and primary PNE cells were resuspended in 5 milliliters of ACK lysis buffer (ThermoFisher A1049201), in order to remove red blood cells, and allowed to sit at room temperature for 2 minutes. 5 milliliters of DMEM F-12 media supplemented with 5% fetal bovine serum, FBS (Atlanta Biologicals), GA-1000, and Pen/Strep was added to the 5 milliliters of ACK lysis buffer for a total volume of 10 milliliters. Primary PNE cells

were spun down again at 300g for 5 minutes. The supernatant was removed and primary PNE cells were re-suspended in 12 milliliters of TEC Plus Media and added to collagen-coated T-75 flasks. TEC Plus Media formulation and final concentrations are as follows: DMEM F-12 (Corning 10-090-CV), Pen/Strep (Gibco 15140-148, final concentration 100mg/mL), GA-1000 (Corning CC-4083, final concentration 30mg/mL Gentamicin and 15 ng/mL Amphotericin), bovine insulin (Sigma, final concentration 10 mg/mL), human transferrin (Sigma T8158, final concentration 5 mg/mL), cholera toxin (Sigma C8052, final concentration 100ng/mL), human epidermal growth factor (ThermoFisher, final concentration 25ng/mL), bovine pituitary extract, BPE, (Corning 355100, final concentration 30 mg/mL), 5% FBS (Atlanta Biologicals), and retinoic acid (Sigma C8052, final concentration $1x10^{-10}$M). To coat flasks with collagen, diluted rat-tail collagen (Corning 354236) to a final concentration of 50 mg/mL in 0.02 N acetic acid. The flask was gently shaken to ensure the entire surface is covered. Leave flasks in laminar flow hood for one hour. After incubation period, remove excess collagen suspension and rinse the apical surface with Hank's Balanced Salt Solution, HBSS (Gibco 14025092). TEC Plus media was changed every 1-2 days depending on confluency. Once primary PNE cells reached 85-95% confluency, cells were washed with HBSS and Trypsin-EDTA Solution 1X 0.05% (Sigma 59417C) was added to release primary PNE cells from the collagen coated flasks. Cells began to release within 7-10 minutes after the addition of trypsin. Trypsin was neutralized using FBS for a final concentration of 10% FBS (v/v). Primary PNE cells were centrifuged again at 300g for 5 minutes. The supernatant was removed and primary PNE cells were re-suspended and counted on a hemacytometer. Cells were then re-suspended to contain 1.5-2 million cells per aliquot in a 50:50 ratio of TEC Plus Media and 2X Hyclone HyCryo Cryopreservation Media (FisherScientific SR3000102) and placed in liquid nitrogen.

Porcine Primary Trachea (PTE) and Bronchial Epithelial (PBE) Cells Isolation and Propagation

The same pigs used to harvest the porcine primary nasal cells were used for harvesting primary trachea and bronchial epithelial cells. Donors were kept separate. Gallon zip-lock bags were used to collect the lungs and bronchi. Suture was used to tie off the upper part of the trachea by the larynx. The respiratory apparatus (trachea, bronchi, and lungs) was removed intact from the body cavity. The heart and the aorta were removed from the respiratory organs. Respiratory organs, having been tied off below the larynx, were brought back to the lab and placed in Pyrex dishes for further dissection. The outside of the lungs, trachea, and bronchi were sprayed down with 70% ethanol. The upper trachea was separated from the larynx and the lower portion of the trachea was cut before the bifurcation of the carina, where the primary bronchi enter into the lungs. As much connective tissue as possible was removed from the trachea and the whole intact trachea was placed in a 50-milliliter conical tube containing 25-30 milliliters of digestion buffer. Digestion buffer formulation are as follows: DMEM F-12 supplemented with GA-1000, 1X Pen/Strep, and 1.4mg/mL of Pronase (Sigma 10165921001). Connective tissue was removed from the primary bronchi and the lobes of the lungs were dissected away to reveal secondary and tertiary bronchi. The intact bronchi were placed in a 50-milliliter conical tube containing 25-30 milliliters of digestion buffer. The trachea and bronchi were allowed to digest overnight. Following the overnight digestion, both the trachea and bronchi samples along with the digestion buffer was poured into a petri dish. The trachea and bronchi were flushed with digestion buffer within the petri dish 8-10 times. The trachea and bronchi were cut longitudinally with a scalpel and the inside of the trachea and the larger bronchi were gently scraped. Leaving the digestion buffer within the petri dish, 10 milliliters of collection media was added (DMEM F-12 supplemented with 5% FBS, GA-1000, and 1X

Pen/Strep) to empty 50 milliliter conical tube and the digested trachea and bronchi were added to the collection media. The tubes containing the digested trachea or bronchi were inverted 20 times to collect any extra epithelial cells. 25-30 milliliters of digestion buffer were added back to the 50 milliliter conical tube which contained 10 milliliters of collection media. Each 50-milliliter conical tube contained a volume of 35-40 milliliters. Primary PTE and PBE cells were centrifuged at 300g for 5 minutes. At this point you can leave the primary PTE and PBE cells in 50 milliliter conical tubes or transfer them to a new set of 15 milliliter conical tubes. The supernatant was removed and primary PTE and PBE cells were re-suspended in 2 milliliters of ACK lysis buffer to remove red blood cells and were incubated at room temperature for 2 minutes. 10 milliliters of collection media was added to primary PTE and PBE tubes, for a total volume of 12 milliliters. Primary PTE and PBE cells were centrifuged again at 300xg for 5 minutes. The supernatant was removed and primary PTE and PBE cells were re-suspended in 2 milliliters of DNASE solution. DNASE solution formulation was as follows: DMEM F-12 supplemented with GA-1000, 1X Pen/Strep, and 100mg/mL of DNASE (Sigma 10104159001). Primary PTE and PBE cells were incubated at room temperature for 10 minutes. After the incubation period, 5 milliliters of collection media were added to primary PTE and PBE cells were centrifuged at 300g for 5 minutes. The supernatant was removed and primary PTE and PBE cells were re-suspended in 10 milliliters of TEC Plus Media and incubated in petri dishes at 37ºC and 5% CO_2 for two hours to allow for fibroblasts to attach. Non-adherent primary PTE and PBE cells were collected in a 15-milliliter conical tube and the petri dish was washed with 2 milliliters of TEC Plus Media twice, for a total volume of 12 milliliters. Primary PTE and PBE cells were added to collagen coated T-75 flasks. Two T-75 flasks per donor were used to decrease chances of contamination. To coat flasks with collagen, the same protocol for collagen coating in primary PNE was followed.

TEC Plus media was changed every 1-2 days depending on confluency. Once primary PTE and PBE cells reached 85-95% confluency, cells were washed with HBSS and Trypsin-EDTA Solution 1X 0.05% was added to release primary PTE and PBE cells from the collagen coated flasks. Cells began to release within 10-12 minutes after the addition of trypsin. Trypsin was neutralized using FBS for a final concentration of 10% FBS (w/v). Primary PTE and PBE cells were spun down again at 300g for 5 minutes. The supernatant was removed and primary PTE and PBE cells were re-suspended and counted on a hemocytometer. Cells were then re-suspended to contain 1.5-2 million cells per aliquot in a 50:50 ratio of TEC Plus Media and 2X Hyclone HyCryo Cryopreservation Media (FisherScientific SR3000102) and placed in liquid nitrogen.

Porcine Primary Nasal Epithelial Cells (PNE), Porcine Primary Trachea (PTE), and Bronchial Epithelial (PBE) Cell Air-liquid Interface Culture for Differentiation

Expanded primary PNE, PTE, and PBE cells were seeded onto 24 well, 12 trans-wells, 0.32cm^2 collagen-coated trans-well inserts (Corning Costar 3470). Cultures were maintained in submerged culture, fed apically and basally, at 37º Celsius 5% CO_2. TEC Plus apical and basal media was changed every 48 hours until the cells were 100% confluent, approximately 6-8 days. Once the primary PNE, PTE, and PBE cells reached confluency, cells were left in air-liquid interface, ALI, and cells were fed basolaterally with TEC ALI media (DMEM F-12 supplemented with 1X Pen/Strep, GA-1000, retinoic acid ($1x10^{-10}$M final concentration), and 2% Nuserum (Corning 355100). Primary PNE, PTE, and PBE cells were washed apically with HBSS and TEC ALI basal media was changed every 48 hours until ciliated cells and mucus were present, approximately 6-10 days in ALI.

Immunohistochemistry of primary PNE, PTE, and PBE Cells Cultured at Air-liquid Interface

Differentiated Primary PNE, PTE, and PBE cells in trans-wells were fixed in 2% paraformaldehyde (Electron Microscopy Sciences 16% Paraformaldehyde Aqueous Solution, EM Grade, Fischer Scientific 50-980-487) at room temperature for 15 minutes. Primary PNE, PTE, and PBE cells were permeabilized in 0.5% Triton X-100 (Sigma 9002-93-1) in phosphate buffered saline, PBS (Fischer Scientific MT21030CM) - PBST, for 30 minutes at room temperature. Primary PNE, PTE, and PBE cells were then washed 3 times with 0.1% PBST. Primary PNE, PTE, and PBE cells were blocked with 10% goat serum (Sigma G9023) for an hour at room temperature. Block was removed and primary antibodies for cilia (mouse IgG2b monoclonal anti-tubulin, acetylated antibody, Sigma T6793, final working concentration 4 mg/mL), mucus (Muc5AC monoclonal antibody (45M1)-Mouse IgG1, Thermo Fischer Scientific MA5-12178, final working concentration 2mg/mL), ZO-1 (Anti-ZO-1 tight junction protein antibody rabbit polycolonal, Abcam ab96587, final working concentration 1:1000), and Claudin 4 (Anti-Claudin 4 antibody rabbit polyclonal, Abcam ab53156, final working concentration 5mg/mL) were added along with directly conjugated lectins SNA-Cy3 (Glycomatrix 21761140-2, final working concentration of 30mg/mL) or MAL2-Cy5 (Glycomatrix 21511110-1, final working concentration of 30mg/mL). Primary antibodies and lectins were allowed to incubate overnight in the dark at 4 degrees Celsius on a plate rocker. Primary PNE, PTE, and PBE cells were then washed 3 times with 0.1% PBST and incubated with secondary antibody, Alexa Fluor 488 goat anti-mouse (Invitrogen A-32723), Alexa Fluor 488 goat anti-rabbit (Invitrogen A-11008), or Alexa Fluor 546 goat anti-mouse (Invitrogen A-11003) at a 1:400 dilution for 30 minutes at room temperature in the dark. Primary PNE, PTE, and PBE cells were washed 3 times again with 0.1% PBST and were incubated with Alexa Fluor 647 Phalloidin (ThermoFisher Scientific A22287) or Alexa Fluor 488 Phalloidin (ThermoFisher Scientific A12379) for 20 minutes at room temperature

in the dark. Primary PNE, PTE, and PBE cells were washed one more time with 0.1% PBST and wet mounted on slides with SlowFade Gold Antifade Mountant with Dapi (ThermoFisher Scientific, S36938). Images of primary porcine nasal, tracheal, and bronchial cells were acquired using a Nikon confocal microscope via cross-sectional z-plane imaging with a 60X objective and 2X optical zoom. Files were saved in the Nikon Nd2 file format and image analysis on 20-30 mm tissue optical sections was conducted with the NIS-Elements viewer version 4.2. Images were then exported in JPEG format.

Transepithelial Electrical Resistance (TEERs) of primary PNE, PTE, and PBE Cells Cultured at Air-liquid Interface

HBSS was added to the apical compartment of differentiated PNE, PTE, and PBE cells. TEER was measured in Ohms using an EVOM epithelial voltmeter (World Precision Instruments). Three readings per well was used to get an average TEER of an individual well, and a total of 6 wells were used per time-point. The same set of wells were used for all time-points. After TEER measurements were taken, HBSS was removed from apical surface and TEC ALI media was added to the basal chamber.

RESULTS

Development of Primary PNE, PTE, and PBE cells for the study of Swine Respiratory Diseases and Human Pandemic Risk Assessment

Primary PNE cells were collected via cytology bristle brush, refer back to materials and methods. Primary PTE and PBE cells were isolated from the respiratory system of 4–6-week-old pigs (Figure 2.1A). The pulmonary lung lobules were removed revealing an intact trachea and bronchi (Figure 2.1B). Swine tracheal and bronchial tissues were separated from each other and allowed to digest overnight, refer back to materials and methods for complete procedure (Figure 2.1C, 2.1D, 2.1E, and 2.1F). Primary PTE and Primary PBE

cells were placed on T-75 flasks and allowed to reach confluency (Figure 2.1G).

PNE, PTE, and PBE cells retain similar functional and physical properties as *in vivo*

Primary PNE, PTE, and PBE cells all established tight junctions as seen by an increase in TEERs (Figure 2.2) and seen by IHC staining with Claudin 4, Zo-1, and F-actin (Figure 2.3). Primary PNE, PTE, and PBE cells also fully differentiate as seen by staining of cilia and mucus granules (Figure 2.4 and 2.5). Furthermore, primary PNE, PTE, and PBE cells possess 2,3 and 2,6 sialic acid receptors (Figure 2.4 and 2.5), which makes swine a potential mixing vessel for avian and human influenza viruses.

DISCUSSION

In this study we have established primary PNE, PTE, and PBE cells that recapitulate the physical and functional properties of the respiratory tract, develop tight junctions and have TEERs (Figure 2.2, 2.3, and 2.4). Primary PNE, PTE, and PBE cells also have the ability to fully differentiate, indicated by the presence of cilia and mucus (Figures 2.3 and 2.4). Adding primary PNE, PTE, and PBE cells to surveillance studies can help assess the pandemic potential of emerging influenza viruses for humans as well as help understand the dynamics of polymicrobial infections in swine. Swine primary PNE, PTE, and PBE cells developed through the methodology of this paper, revealed the presence of α2,3-SA and α2,6-SA indicated by positive lectin staining with MAL2 or SNA respectively (Figure 2.3 and 2.4). Pigs, susceptible to avian and human IAVs, are proposed as a "mixing vessel" linked to the generation of novel IAVs that may infect humans based on the distribution of sialic acid receptors within their respiratory tract. The hemagglutinin (HA) protein of IAVs bind to glycans on epithelial cells that have a terminal sialic acid (SA) in order to trigger endocytosis of the viral particle. These sialic acids are either α2,3-linked or α2,6-linked. It is currently thought that avian IAV replication is restricted in humans due to differing

distribution of cellular receptors and differing affinity for these receptors (20). Avian IAVs preferentially infect cells expressing α2,3-SA, while mammalian IAVs prefer cells expressing the α2-6 SA (20). Water fowl, particularly ducks and geese express mainly α2,3-SA in the respiratory tract, while white leghorn chickens and Japanese quail express mainly α2,6-SA in their respiratory tract. Pheasants, turkeys, guinea fowl, and quail have been found to express both α2,3-SA and α2,6-SA in their respiratory tract (21, 22). The upper respiratory tract of swine predominately contains cells expressing α2,6-SA, while cells expressing α2,3-SA dominate in the lower airways (5-7). The swine airway epithelium also has less sialylated glycans that humans and swine have a greater proportion of α2,6-SA than the human respiratory tract (4). In human bronchial and tracheal cells, it has been reported that α2,3-SA are expressed in ciliated cells, while α2,6 SA are primarily expressed in non-ciliated cells (3, 23, 24). Future studies within our lab will evaluate the pandemic potential of swine influenza field virus isolates by kinetics studies and evaluating viral tropism by using multiple cellular substrates.

Figure 2.1: Harvesting and Culturing of Primary Porcine Bronchial and Tracheal Epithelial Cells A. Pluck harvested from a 4-6 week old pig **B.** Dissected out trachea and bronchi intact **C.** Dissected trachea **D.** Dissected bronchi and bronchioles **E.** Post digestion trachea **F.** Post digestion bronchi and bronchioles G. Single cell suspension with multiple ciliated epithelial rafts 20X objective phase contrast on inverted microscope

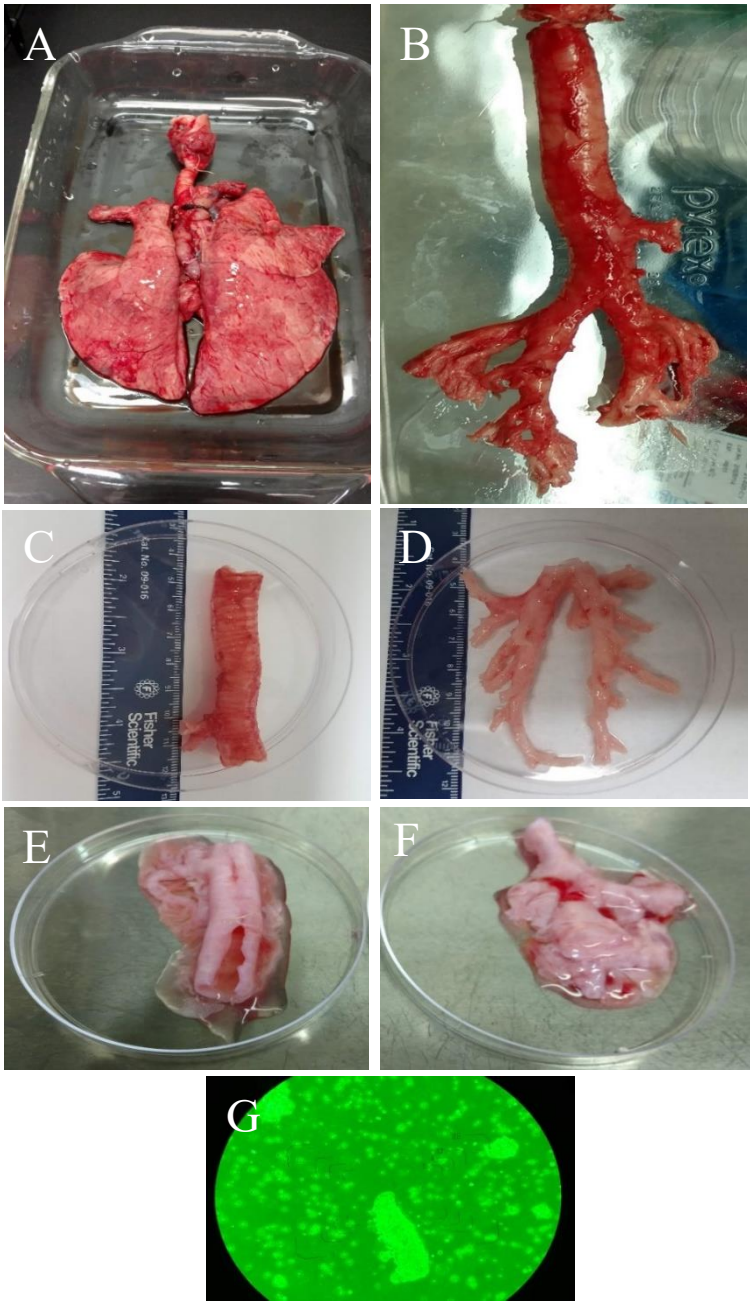


Figure 2.2: Primary PNE, PTE, and PBE Trans-epithelial Electrical Resistance. Trans-epithelial electrical resistance (TEERs) of differentiated swine cells cultured at air-liquid interface (ALI). Data represents one independent experiment. 3 measurements were taken from each well at each individual time point and averaged. n=6 wells per time-point +/- SEM.

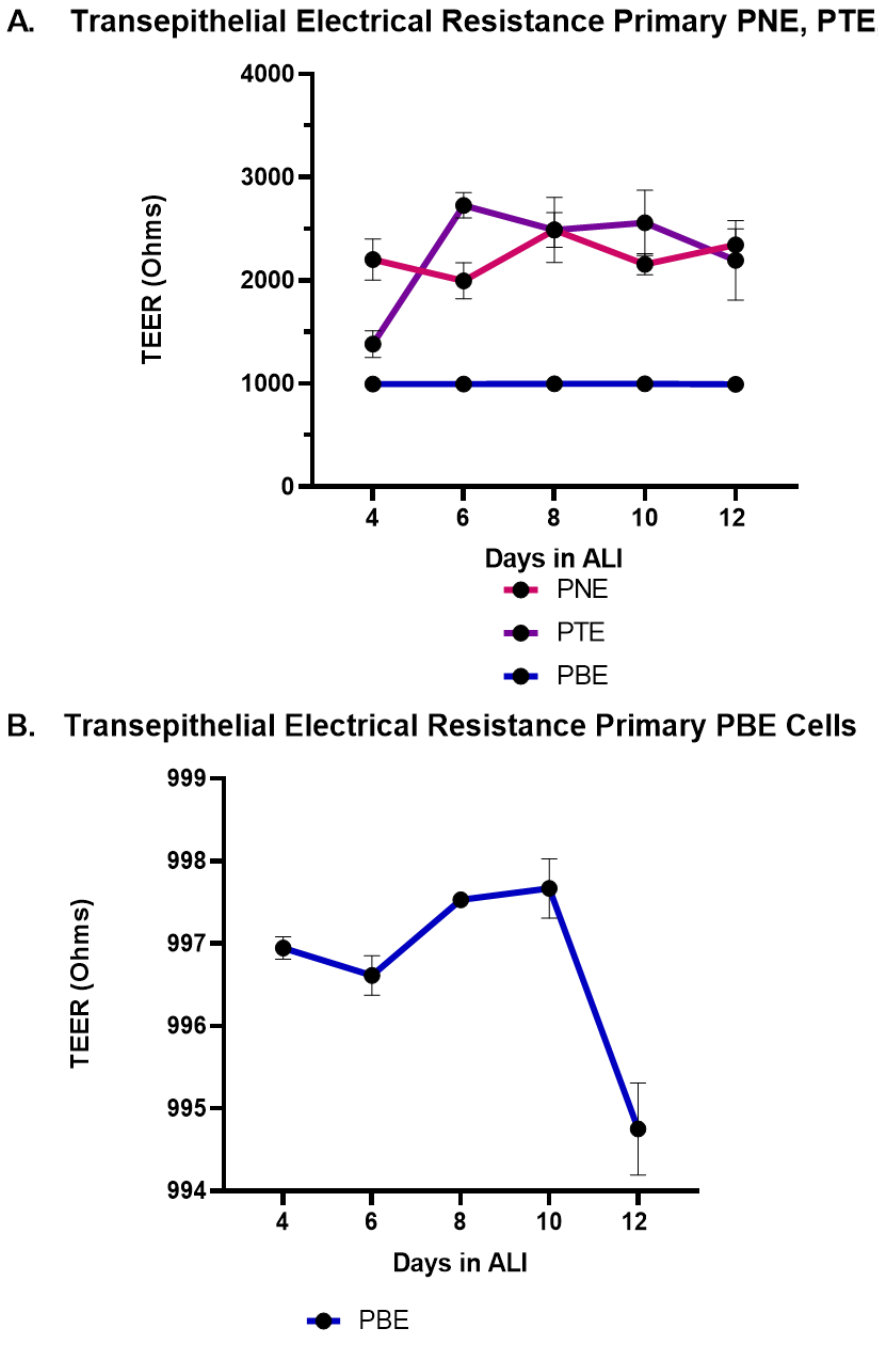


Figure 2.3: Primary PNE, PTE, and PBE Immunohistochemistry of Tight Junctions.

Claudin 4 and ZO-1 to detect presence of tight junctions on primary swine epithelial cells in air-liquid interface (ALI)=green, acetylated tubulin to detect the presence of cilia on primary swine epithelial cells in ALI=red, and Phallodin to detect colocalization of Claudin 4 and ZO-1 with F-actin=purple, white bar indicates a measurement of 25 microns **A.** PNE-Claudin 4 and cilia (top view) **B.** PTE-Claudin 4 and cilia (top view) **C.** PBE-Claudin 4 and cilia (top view) **D.** PNE-ZO-1 and cilia (lateral view)**E.** PTE-ZO-1 and cilia (lateral view), and **F.** PBE-ZO-1 and cilia (lateral view). Figures D-F green represents ZO-1 creating tight junctions on apical surface colocalizing with F-actin. Yellow represents colocalization indicating tight junction formation of F-actin, ZO-1, and cilia basal bodies, which is indicated by the white arrow.

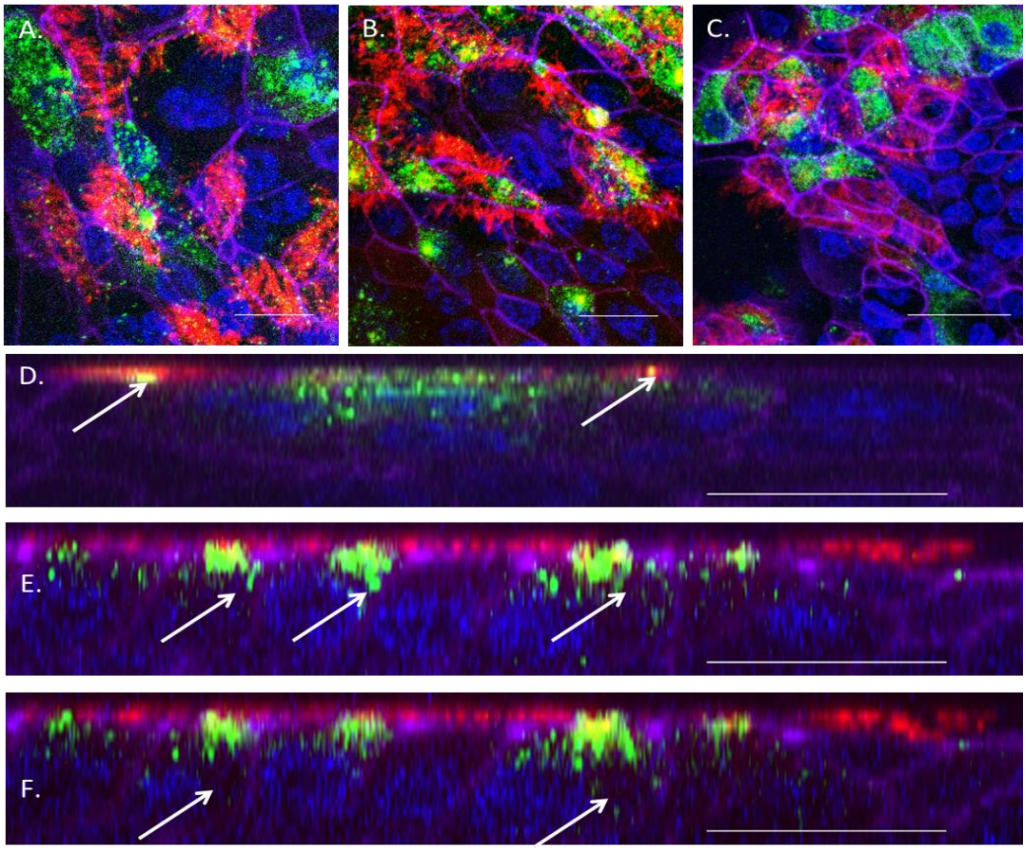


Figure 2.4: 2,6-Sialic Acid Lectin Immunohistochemistry of PNE, PTE, and PBE Cells. SNA lectin to detect presence of 2,6- sialic acid receptors on primary swine epithelial cells in air-liquid interface (ALI)=red, beta and acetylated tubulin to detect the presence of cilia on primary swine epithelial cells in ALI=green (A,B,C), Muc5AC to detect the presence of mucus and mucin granules in goblet cells in primary swine epithelial cells in ALI=green (D, E, F), dapi, nuclear stain=blue, and Phallodin to detect the presence of tight junctions, F-actin=purple, white bar indicates a measurement of 25 microns **A.** PNE-SNA and cilia **B.** PTE-SNA and cilia **C.** PBE-SNA and cilia **D.** PNE-SNA and mucus **E.** PTE-SNA and mucus, and **F.** PBE SNA and mucus.

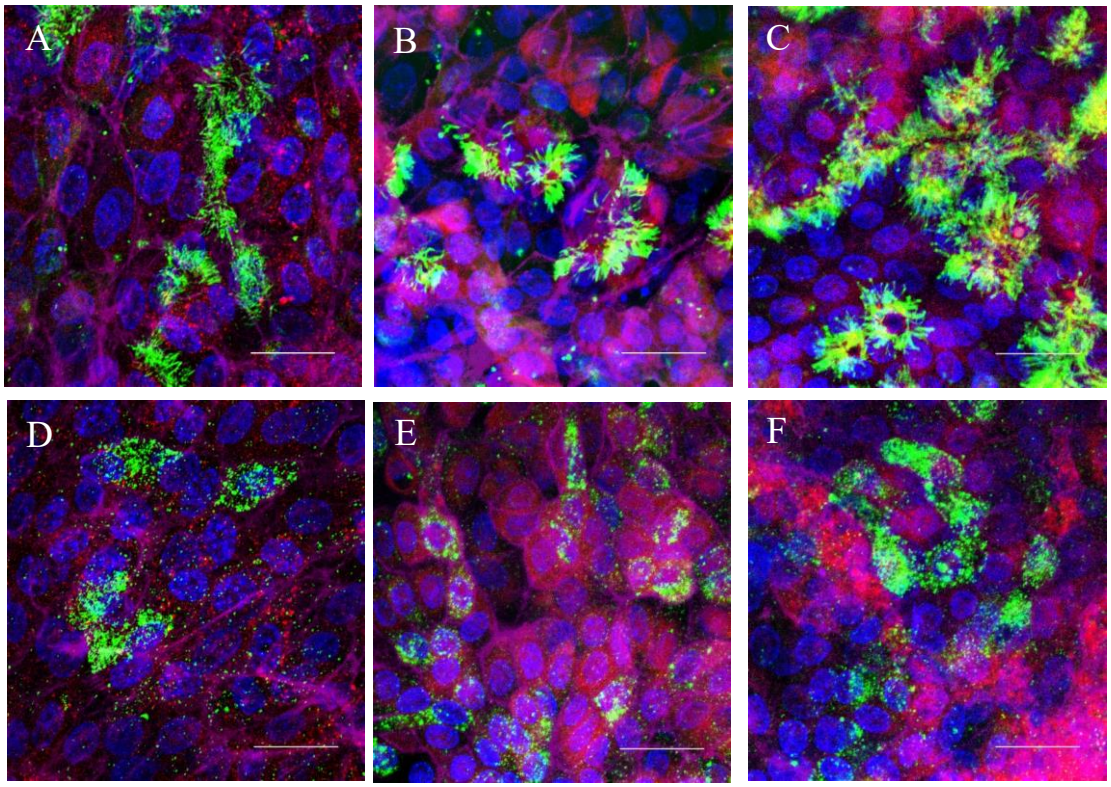
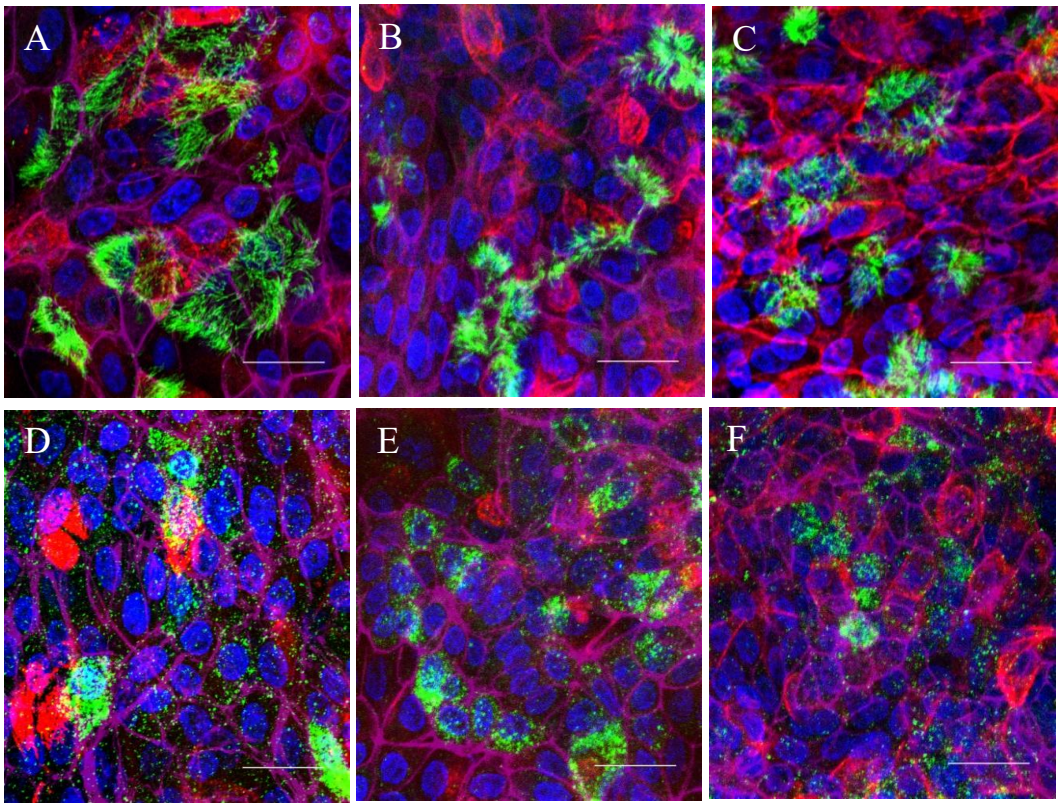


Figure 2.5: 2,3-Sialic Acid Lectin Immunohistochemistry of PNE, PTE, and PBE Cells. MAL-2 lectin to detect presence of 2,3- sialic acid receptors on primary swine epithelial cells in air-liquid interface (ALI)=red, beta and acetylated tubulin to detect the presence of cilia on primary swine epithelial cells in ALI=green (A,B,C), Muc5AC to detect the presence of mucus and mucin granules in goblet cells in primary swine epithelial cells in ALI=green (D, E, F), dapi, nuclear stain=blue, and Phallodin to detect the presence of tight junctions, F-actin=purple, white bar indicates a measurement of 25 microns **A.** PNE-MAL-2 and cilia **B.** PTE-MAL-2 and cilia **C.** PBE-MAL-2 and cilia **D.** PNE-MAL-2 and mucus **E.** PTE-MAL-2 and mucus, and **F**. PBE MAL-2 and mucus.



REFERENCES

1. Van Reeth K, Vincent AL. Diseases of Swine 11th Edition doi:https://doi.org/10.1002/9781119350927.ch36. John Wiley & Sons, Inc.

2. Brockmeier SL, Halbur P.G., Thacker E.L. 2002. Porcine Respiratory Disease Complex Chapter 13. Polymicrobial Diseases.

3. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang K-C. 2010. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Veterinary Research 6:4.

4. Chan RW, Karamanska R, Van Poucke S, Van Reeth K, Chan IW, Chan MC, Dell A, Peiris JS, Haslam SM, Guan Y, Nicholls JM. 2013. Infection of swine ex vivo tissues with avian viruses including H7N9 and correlation with glycomic analysis. Influenza Other Respir Viruses 7:1269-82.

5. Van Poucke SG, Nicholls JM, Nauwynck HJ, Van Reeth K. 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7:38.

6. Punyadarsaniya D, Liang C-H, Winter C, Petersen H, Rautenschlein S, Hennig-Pauka I, Schwegmann-Wessels C, Wu C-Y, Wong C-H, Herrler G. 2011. Infection of differentiated porcine airway epithelial cells by influenza virus: differential susceptibility to infection by porcine and avian viruses. PloS one 6:e28429-e28429.

7. Trebbien R, Larsen LE, Viuff BM. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virology Journal 8:434.

8. Shin D-L, Yang W, Peng J-Y, Sawatsky B, von Messling Vv, Herrler G, Wu N-H.

2020. Avian Influenza A Virus Infects Swine Airway Epithelial Cells without Prior Adaptation. Viruses 12:589.

9. Glorieux S, Van den Broeck W, van der Meulen KM, Van Reeth K, Favoreel HW, Nauwynck HJ. 2007. In vitro culture of porcine respiratory nasal mucosa explants for studying the interaction of porcine viruses with the respiratory tract. Journal of Virological Methods 142:105-112.

10. Meng F, Wu NH, Nerlich A, Herrler G, Valentin-Weigand P, Seitz M, Camilli A. 2015. Dynamic Virus-Bacterium Interactions in a Porcine Precision-Cut Lung Slice Coinfection Model: Swine Influenza Virus Paves the Way for Streptococcus suis Infection in a Two-Step Process. Infection and Immunity 83:2806-2815.

11. Sreenivasan CC, Thomas M, Antony L, Wormstadt T, Hildreth MB, Wang D, Hause B, Francis DH, Li F, Kaushik RS. 2019. Development and characterization of swine primary respiratory epithelial cells and their susceptibility to infection by four influenza virus types. Virology 528:152-163.

12. Peitsch C, Klenk H-D, Garten W, Böttcher-Friebertshäuser E. 2014. Activation of Influenza A Viruses by Host Proteases from Swine Airway Epithelium. Journal of Virology 88:282-291.

13. Xie X, Gan Y, Pang M, Shao G, Zhang L, Liu B, Xu Q, Wang H, Feng Y, Yu Y, Chen R, Wu M, Zhang Z, Hua L, Xiong Q, Liu M, Feng Z. 2018. Establishment and characterization of a telomerase-immortalized porcine bronchial epithelial cell line. Journal of Cellular Physiology 233:9763-9776.

14. Meliopoulos V, Cherry S, Wohlgemuth N, Honce R, Barnard K, Gauger P, Davis T, Shult P, Parrish C, Schultz-Cherry S. 2020. Primary swine respiratory epithelial cell lines for the efficient isolation and propagation of influenza A viruses. Journal of Virology

doi:10.1128/JVI.01091-20:JVI.01091-20.

15. Wang H, He L, Liu B, Feng Y, Zhou H, Zhang Z, Wu Y, Wang J, Gan Y, Yuan T, Wu M, Xie X, Feng Z. 2018. Establishment and comparison of air-liquid interface culture systems for primary and immortalized swine tracheal epithelial cells. BMC Cell Biology 19:10.

16. Khoufache K, Cabaret O, Farrugia C, Rivollet D, Alliot A, Allaire E, Cordonnier C, Bretagne S, Botterel F. 2010. Primary in vitro culture of porcine tracheal epithelial cells in an air-liquid interface as a model to study airway epithelium and Aspergillus fumigatus interactions. Medical Mycology 48:1049-1055.

17. Young TF, Thacker EL, Erickson BZ, Ross RF. 2000. A tissue culture system to study respiratory ciliary epithelial adherence of selected swine mycoplasmas. Veterinary Microbiology 71:269-279.

18. Lam E, Ramke M, Groos S, Warnecke G, Heim A. 2011. A differentiated porcine bronchial epithelial cell culture model for studying human adenovirus tropism and virulence. Journal of Virological Methods 178:117-123.

19. Peng J-Y, Shin D-L, Li G, Wu N-H, Herrler G. 2021. Time-dependent viral interference between influenza virus and coronavirus in the infection of differentiated porcine airway epithelial cells. Virulence 12:1111-1121.

20. Byrd-Leotis L, Cummings RD, Steinhauer DA. 2017. The Interplay between the Host Receptor and Influenza Virus Hemagglutinin and Neuraminidase. International journal of molecular sciences 18:1541.

21. Kimble B, Nieto GR, Perez DR. 2010. Characterization of influenza virus sialic acid receptors in minor poultry species. Virology Journal 7:365.

22. Pillai SPS, Lee CW. 2010. Species and age related differences in the type and

distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. Virology Journal 7:5.

23. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. 2006. Avian flu: influenza virus receptors in the human airway. Nature 440:435-6.

24. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JSM. 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.

CHAPTER 3

PRIMARY PORCINE NASAL (PNE), TRACHEAL (PTE), AND BRONCHIAL EPITHELIAL CELLS ARE A FEASIBLE MODEL FOR INFLUENZA PANDEMIC RISK ASSESSMENT[1]

[1] Krunkosky M[1], Krunkosky T, Kyriakis C, and Tompkins M. To be submitted to MDPI Viruses

KEY WORDS swine influenza, avian influenza, primary cells, pandemic assessment

ABSTRACT

Influenza A viruses (IAVs) are single stranded negative sense RNA viruses containing eight gene segments. IAVs have two genetic traits: (a) drift, resulting from mutations, usually in gene segments expressing the surface glycoproteins hemagglutinin and neuraminidase and (b) shift, reassortment, which occurs when two viruses co-infect the same host cell and exchange gene segments. Migratory waterfowl are reservoirs of IAVs, while susceptible species include poultry, horses, humans, swine, and other mammals. IAVs are among the most important viral pathogens for animal health, causing outbreaks of respiratory disease in swine and poultry and occasional zoonotic transmission to humans. We have developed primary swine nasal, tracheal, and bronchial cells that have the ability to differentiate and can support influenza virus replication. Utilizing these substrates as a tool for pandemic assessment along with increased surveillance will help detect possible variants that may pose a threat to human health. These cell substrates can also be utilized as a tool for the isolation of difficult viruses and to learn more about innate responses of the airway epithelium to influenza.

INTRODUCTION

Swine and avian species are susceptible to multiple viral and bacterial respiratory pathogens. These pathogens are commonly found in co-infections referred to as the Porcine Respiratory Disease Complex (PRDC) and the Avian Respiratory Disease Complex (ARDC). Diseases of the PRDC include but are not limited to, swine influenza virus (swIAV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, and *Bordetella* (1-6). Diseases of the ARDC include, but are not limited to avian influenza virus (avIAV), Infectious Bronchitis Virus (IBV), Infectious Laryngotracheitis Virus (ILTV), Avian pneumovirus, Newcastle Disease Virus (NDV), *Mycoplasma gallisepticum*, *Escheria Coli*, and *Staphylococcus* (7-12). These viral and bacterial pathogens of swine and avian are known to infect the respiratory epithelium. Respiratory diseases in pigs and avian species are an important health concern for swine and poultry producers, since this causes tremendous economic loss to the industry. The pathogens associated with the PRDC cause poor growth performance, increased morbidity and mortality, and increased expenditures in control measures, thus greatly affecting the farmer's bottom line (1-6). Clinical signs associated with pathogens of the ARDC include sneezing, head swelling, conjunctivitis, nasal exudates, and rattling noises. Postmortem lesions include tracheitis, airsacculitis, and pericarditis. Morbidity can be as high as 30% and mortality as high as 10%, and much higher mortality rates associated with highly pathogenic avian influenza (HPAI) (7-12).

One of the most important pathogens in the PRDC and ARDC is influenza. SwIAVs and AvIAVs also pose a threat to human health. The past four influenza pandemics were caused by viruses containing gene segments originated in either avian or swine. The 1918

pandemic was caused by an H1N1 virus with genes of avian origin. It was first identified in US military personnel in spring 1918. 500 million people, approximately 33% of the world's population, became infected. There was a total of 50 million deaths worldwide (13, 14). The Asian pandemic occurred in 1957, caused by a virus containing three different genes from an H2N2 virus that originated from an avian influenza A virus, including the H2 hemagglutinin and the N2 neuraminidase genes. This pandemic led to 1.2 million deaths worldwide and 116,000 deaths in the United States (13-19). Next, in 1968 the Hong Kong pandemic occurred, caused by an influenza A H3N2 virus comprised of two genes from an avian influenza A virus, including the H3 hemagglutinin, and all other genes including the N2 from the 1957 pandemic virus. This pandemic lead to 1 million deaths worldwide and 100,000 deaths in the United States. Lastly, the 2009 H1N1 pandemic (20-22) led to 60.8 million cases, 274,304 hospitalizations, and 12,469 deaths in the United States and an estimated range of 151,700-575,400 deaths worldwide (23). This pandemic virus originated from the reassortment between a swine H3N2 triple reassortment virus containing swine NP, M, and NS, human seasonal PB1, HA, and NA, and avian PB2 and PA genes (24-28) and the M and NA genes derived from the Eurasian avian-like H1N1. This virus spread rapidly in both swine and human populations (29-33).

Recently there has been a few human infections of low pathogenic avian influenza virus (LPAIV) H7 subtypes. AvIAVs can be divided into high and low pathogenic based on their pathogenicity in chickens. Furthermore, high pathogen avian influenza has multiple basic amino acids, either arginine or lysine, at the HA cleavage site. Only avian influenza of the H5 and H7 subtypes have been shown the ability to become highly pathogenic. Avian influenza H7 viruses are able to infect a broad range of species, including seals, pigs, horses, and humans. The ability of these H7 viruses to transmit between multiple species is

important in the evolution of H7 avian influenza viruses. Seasonal waves of LPAIV H7N9 infection in humans in China have been shown to be lethal but most cases were associated with contact with poultry and no humanto-human transmission has been reported (34-44). Therefore, the purpose of this study was to utilize the primary porcine nasal, tracheal, and bronchial epithelial cells that we developed in our lab to survey a panel of H3 and H1 swine viruses and H7 LPAI viruses. We also compared the replication kinetics of these viruses to replication kinetics in human cell substrates.

MATERIALS AND METHODS

Culturing of Swine and Avian Isolates

Viral stocks of Sw/MN/A01125993/2012, Sw/NC/KH1552516/2016, Sw/MO/A01410819/2014, CA/07/09, Sw/NC/154076/2015, and Sw/MO/A01444664/2013 were propagated in MDCK Atlanta cells. Viral stocks of A/CK/NY/131/94, A/CK/NJ/150/02, and A/NY/16 were propagated in embryonated eggs.

Culturing of Calu-3s and MDCKs for Viral Kinetics and Plaque Assays

MDCK cells were brought out of liquid nitrogen storage and seeded into T75 flasks with DMEM supplemented with 5% FBS and 1X antibiotic. Once MDCK cells were confluent, cells were trypsinized and plated on 12-well tissue culture treated plates for plaque assays to determine viral titer. Plaque assays were incubated for 72 hours at 37º C in 5% CO_2 with a 1.2% Avicel MEM+1X antibiotics overlay with 4X N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin.

Calu-3 cells were brought out of liquid nitrogen storage and seeded into T75 flasks with DMEM F-12 supplemented with 10% FBS and 1X antibiotics. Once Calu-3 cells were confluent, cells were trypsinized and plated on 12-well tissue culture treated plates for viral infection (250 PFU/well). For Calu-3 kinetics, 1 milliliter of DMEM F-12 1X antibiotic and

2% Nuserum was used to maintain cultures during the course of the experiment. Media was removed and replaced at each time-point.

Culturing of Differentiated Porcine Primary Nasal (PNE), Porcine Primary Trachea (PTE), and Bronchial Epithelial (PBE) Cells Maintained in Air-liquid Interface (ALI)

Expanded primary nasal, tracheal, and bronchial cells were brought out of liquid nitrogen storage, thawed for 3 minutes in a 37º Celsius water bath, and re-suspended in 10 milliliters of TECplus media (DMEM F-12 (Corning 10-090-CV), Pen/Strep (Gibco 15140-148, final concentration 100mg/mL), GA-1000 (Corning CC-4083, final concentration 30mg/mL Gentamicin and 15 ng/mL Amphotericin), bovine insulin (Sigma, final concentration 10 mg/mL), human transferrin (Sigma T8158, final concentration 5 mg/mL), cholera toxin (Sigma C8052, final concentration 100ng/mL), human epidermal growth factor (ThermoFisher, final concentration 25ng/mL), bovine pituitary extract, BPE, (Corning 355100, final concentration 30 mg/mL), 5% FBS (Atlanta Biologicals), and retinoic acid (Sigma C8052, final concentration $1x10^{-10}$M). Cells were washed and resuspended in TEC plus media and seeded onto 24 well, 12 trans-wells, 0.32cm^2 collagen-coated trans-well inserts (Corning Costar 3470). Cultures were maintained at 37º C in 5% CO_2 in submerged culture, until the cells were 100% confluent, 6-8 days. TEC Plus media was replaced every other day. Once the primary nasal, tracheal, and bronchial cells reached confluency, cells went to air-liquid interface, ALI, and maintained with TEC ALI media (DMEM F-12 supplemented with 1X Pen/Strep, GA-1000, retinoic acid ($1x10^{-10}$M final concentration), and 2% Nuserum (Corning 355100). Basal media was changed every 48 hours until ciliated cells and mucus were present, approximately 6-10 days ALI.

Culturing of Differentiated Normal Human Bronchial Epithelial Cells (NHBE) Maintained in Air-liquid Interface (ALI)

NHBE cells purchased from Lonza were brought out of liquid nitrogen storage, thawed for 3 minutes in a 37º Celsius water bath, and re-suspended in a 1:1 mixture of bronchial epithelial growth media (BEGM) (Clonetics): Dulbecco's modified Eagle's medium with high glucose (BEGM: DMEM-H) containing supplements provided in Lonza growth media kit, with the exception of EGF (0.5ng/mL). NHBE cells were seeded onto 24 well, 12 trans-wells, 0.32cm^2 collagen-coated trans-well inserts (Corning Costar 3470). Cultures were maintained at 37º C in 5% CO_2 in submerged culture, until the cells were 100% confluent, 7-10 days. Media was replaced every other day. Once the NHBEs reached confluency, cells went to air-liquid interface, ALI, and were maintained with PneumaCult-ALI Medium (Stem Cell Technologies).

Inoculation of CALU-3, PNE, PTE, and PBE Cultures

Calu-3 cells after reaching confluency on 12-well tissue culture plates, were washed twice with PBS and inoculated with 250 PFU/well of virus diluted in PBS. Calu-3 cells were incubated at 37º C in 5% CO2 for 2 hours. The inoculum was then removed, and cells were maintained with DMEM F-12, 1X antibiotic and 2% Nuserum. At the indicated time points, media was collected for plaque assays and replaced with fresh media.

PNE, PTE, PBE, and NHBE cells, after having fully differentiated on 24-well trans-well plates, were washed twice with HBSS. PNE, PTE, PBE, and NHBE cells were inoculated apically with 250 PFU/well of virus diluted in PBS and incubated at 37º C in 5% CO2 for 2 hours. The inoculum was then removed, and cells were maintained in air-liquid interface with TEC ALI media or PneumaCult-ALI Medium. At the indicated time points the apical surface was washed with HBSS and basal media was collected for plaque assays. TEC ALI media or PneumaCult-ALI Medium was replaced with fresh media at 48 hours.

RESULTS

H3 and H1 SwIAVs were able to efficiently replicate in PNE, PTE, PBE, Calu-3, and NHBE cells (Figure 3.1 and Figure 3.2), but showed different growth profiles. H7N2 avian IAVs were able to efficiently replicate in multiple different epithelial cell substrates, including NHBEs (Figure 3.3). NY/94 H7N2 avian IAV was able to replicate in all epithelial cell substrates, Calu-3, PNE, PTE, and PBE cells, although only to very low titers in PTE cells (Figure 3.3). NJ/02 H7N2 avian IAV was able to replicate efficiently in Calu-3 and PBE cells, but only replicated to low titers in PTE and NHBE cells and was unable to replicate in PNE cells (Figure 3.3). Interestingly, NY/16 H7N2 avian-origin IAV was unable to replicate in any of the porcine respiratory cells and only replicated in the human Calu-3 cell substrate (Figure 3.3). The ability of the avian IAVs to replicate in NHBEs and Calu-3s suggests they might be able to infect humans and may have pandemic potential and risk to human health.

DISCUSSION

H1 and H3 viruses of swine all replicated efficiently in porcine cell substrates as expected. Interestingly, these H1N1 and H3N2 viruses were also able to replicate in human cell substrates, indicating their possibility for human infection (Figure 3.1 and Figure 3.2). In addition to the pandemic of 2009, there have been multiple cases of human infections after exposure to infected pigs, but spread of these variant viruses from human-to-human is rare. In the United States alone there has been 9 cases of H1N1, 25 cases of H1N2, and 430 cases of H3N2 variant viruses since 2009/2010 (45, 46).

Avian H7N2 viruses, NY/94 and NJ/02, were able to replicate in at least one porcine substrate. However, all of the avian viruses were able to replicate in human substrates, indicating their potential to infect humans and possibility for pandemic potential (Figure 3.3). In addition to the pandemics of avian origin mentioned above, there have been

multiple reports of human infections with H7 LPAIV, such as seasonal waves of H7N9 in China since 2013 (34-44). There also have been reports of human infections with the H7N2 subtype, notably the A/NY/16 used in this study. Highly pathogenic H7 viruses have been known to emerge from ancestral low pathogenic avian influenza (47-49).

Pigs are proposed as a "mixing vessel" for the generation of variant viruses which may be able to infect and transmit amongst humans. This is partially due to the distribution of sialic acids within their respiratory tract. The upper respiratory tract of swine predominately contains cells expressing α2,6-SA, while cells expressing α2,3-SA dominate in the lower airways (50-52). Utilizing these porcine and human cell substrates as a tool for pandemic assessment along with increased surveillance will help detect possible variants that may pose a threat to human health. These cell substrates can also be utilized as a tool for the isolation of difficult viruses and to learn more about reassortment events and virus evolution. Furthermore, these cell substrates can be utilized to learn more about innate responses of the airway epithelium to influenza.

Figure 3.1: Replication Kinetics of H3N2, H3N1 Swine Isolates in PNE, PTE, PBE, Calu-3 and NHBE Cells. Calu-3 cells were grown in 12-well tissue culture plates. PNE, PTE, PBE, and NHBE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with **A.** Sw/MN/A01125993/2012 **B.** Sw/NC/KH1552516/2016, and **C.** Sw/MO/A01410819/2014 at 250 PFU/well. Data represents one independent experiment. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM.

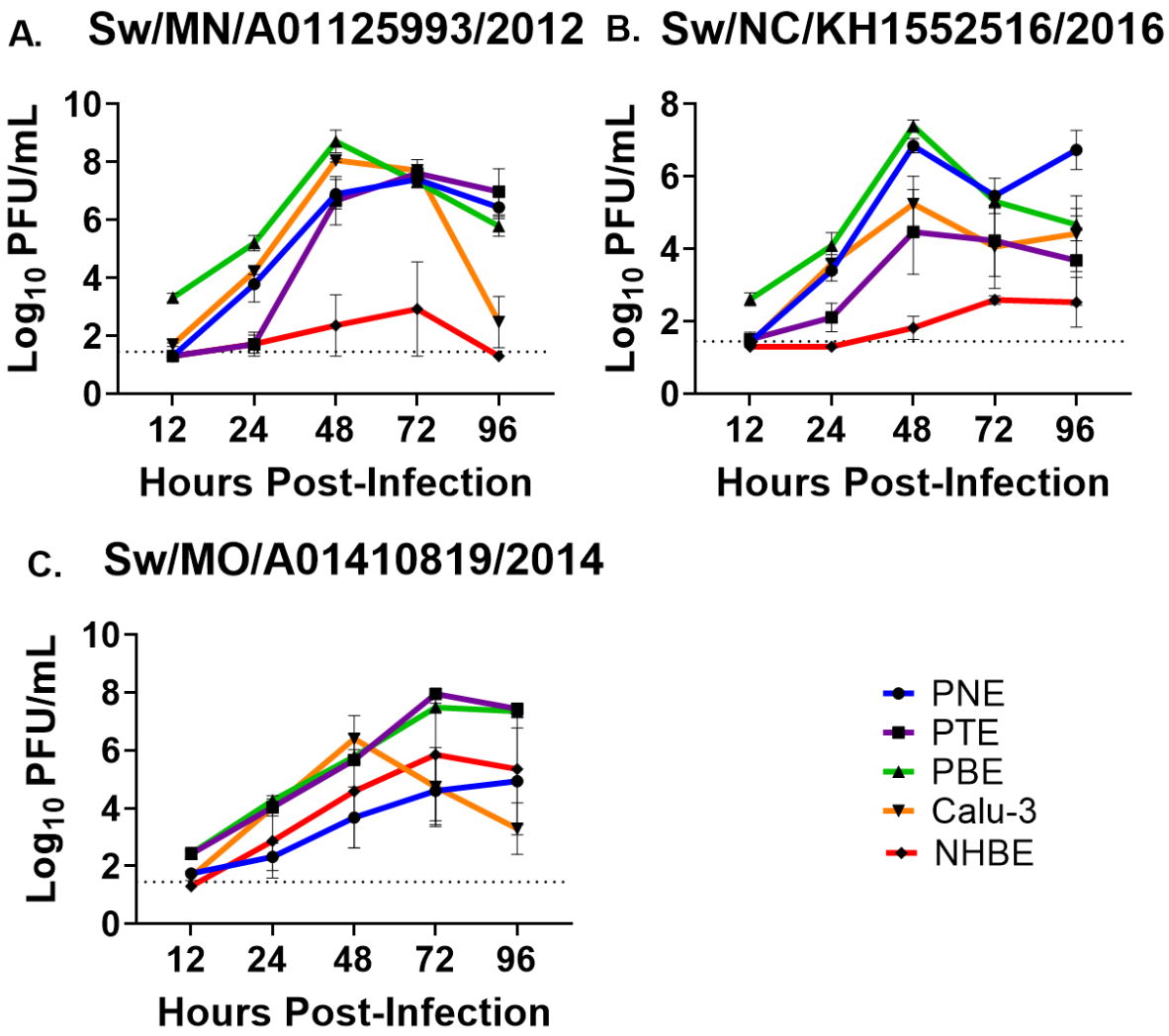


Figure 3.2: Replication Kinetics of H1N1, H1N2 Swine Isolates in PNE, PTE, PBE, Calu-3, and NHBE Cells. Calu-3 cells were grown in 12-well tissue culture plates. PNE, PTE, PBE, and NHBE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with **A.** CA/07/09 **B.** Sw/NC/154076/2015, and **C.** Sw/MO/A014444664/2013 at 250 PFU/well. Data represents one independent experiment. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM.

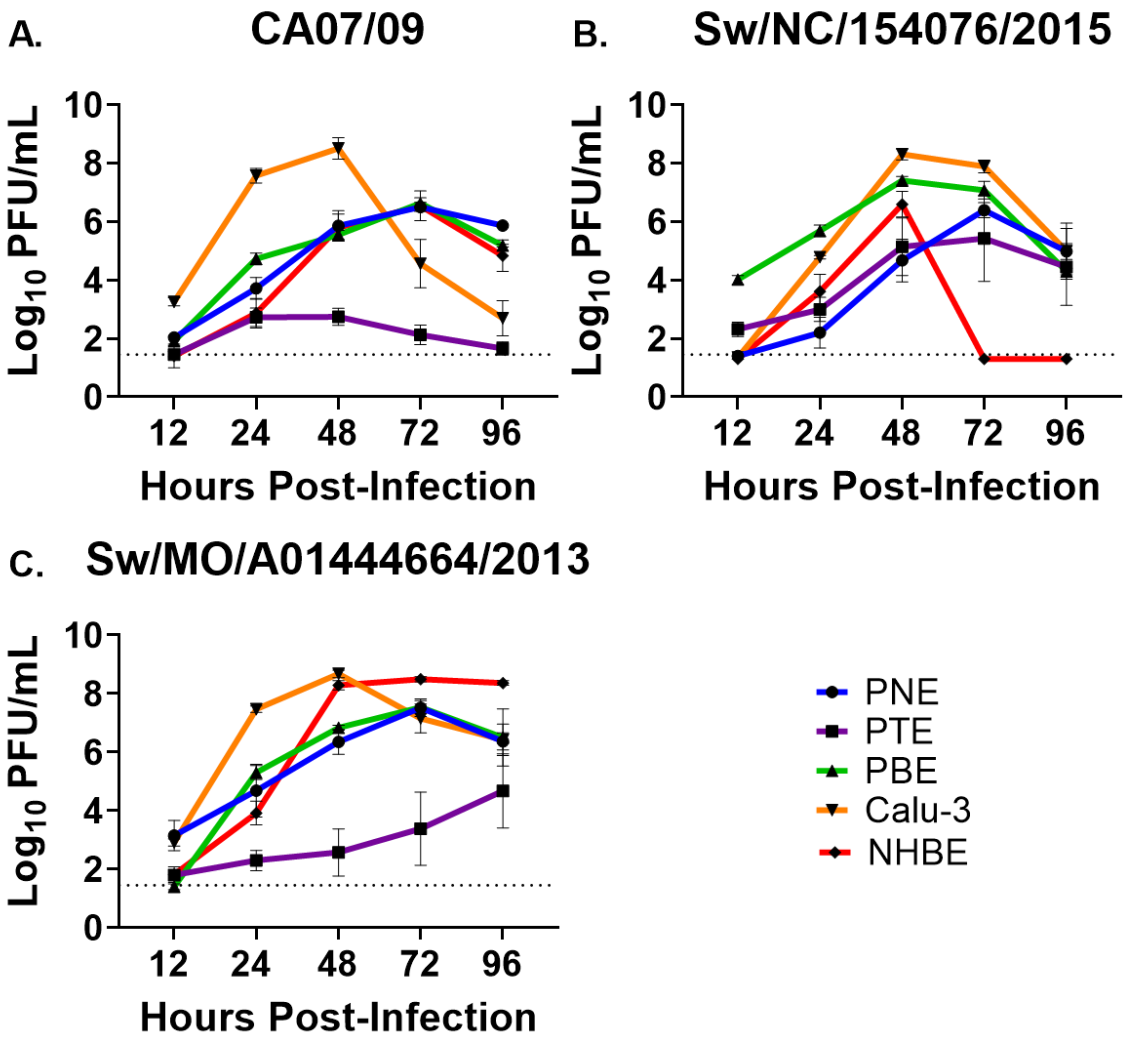
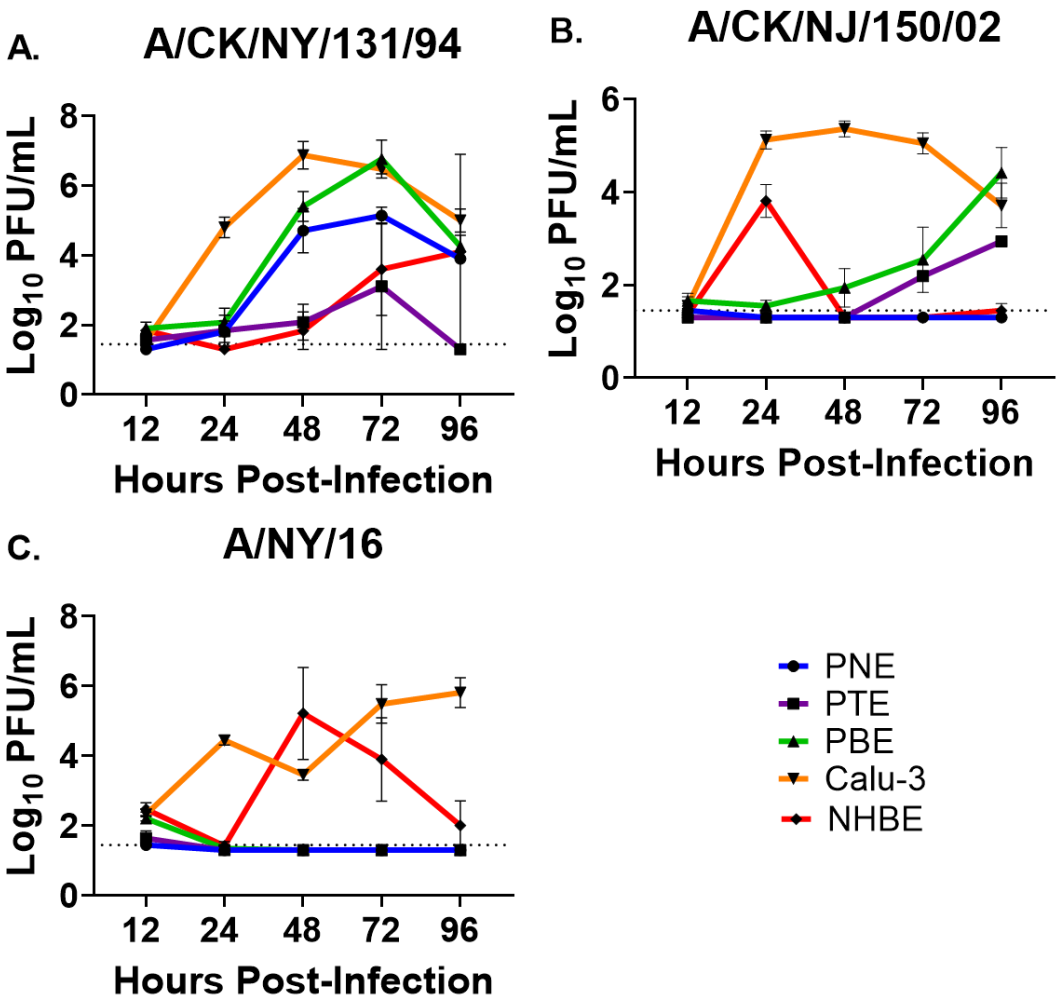


Figure 3.3: Replication Kinetics of H7 Avian Isolates in PNE, PTE, PBE, Calu-3, and NHBE Cells. Calu-3 cells were grown in 12-well tissue culture plates. PNE, PTE, PBE, and NHBE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with **A.** CK/NY/131/94 **B.** CK/NJ/150/02, and **C.** A/NY/16 at 250 PFU/well. Data represents one independent experiment. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM.



REFERENCES

1. Qin S, Ruan W, Yue H, Tang C, Zhou K, Zhang B. 2018. Viral communities associated with porcine respiratory disease complex in intensive commercial farms in Sichuan province, China. Scientific Reports 8:13341.

2. Van Reeth K, Vincent AL. Diseases of Swine 11th Edition doi:https://doi.org/10.1002/9781119350927.ch36. John Wiley & Sons, Inc.

3. Opriessnig T, Giménez-Lirola L, Halbur P. 2011. Polymicrobial respiratory disease in pigs. Animal Health Research Reviews 12:133.

4. Fablet C, Marois-Créhan C, Simon G, Grasland B, Jestin A, Kobisch M, Madec F, Rose N. 2012. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. Veterinary microbiology 157:152-163.

5. Schmidt C, Cibulski S, Andrade C, Teixeira T, Varela A, Scheffer C, Franco A, de Almeida L, Roehe P. 2016. Swine influenza virus and association with the porcine respiratory disease complex in pig farms in southern Brazil. Zoonoses and public health 63:234-240.

6. Galán-Relaño A, Luque I, Gómez-Gascón L, Morales-Partera A, Tarradas C, Carrasco L, Gómez-Laguna J. 2015. Seroprevalence against selected pathogens involved in porcine respiratory disease complex in free-range fattening pigs in Spain. Veterinary Record:vetrec-2015-103432.

7. Thomas JK, Noppenberger J. 2007. Avian influenza: a review. Am J Health Syst Pharm 64:149-65.

8. Stipkovits L, Egyed L, Palfi V, Beres A, Pitlik E, Somogyi M, Szathmary S, Denes B. 2012. Effect of low-pathogenicity influenza virus H3N8 infection on Mycoplasma gallisepticum infection of chickens. Avian Pathol 41:51-7.

9. Haghighat-Jahromi M, Asasi K, Nili H, Dadras H, Shooshtari AH. 2008. Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Arch Virol

153:651-5.

10. França M, Howerth EW, Carter D, Byas A, Poulson R, Afonso CL, Stallknecht DE. 2014. Co-infection of mallards with low-virulence Newcastle disease virus and low-pathogenic avian influenza virus. Avian Pathol 43:96-104.

11. Cavanagh D. 2007. Coronavirus avian infectious bronchitis virus. Vet Res 38:281-97.

12. Scheiblauer H, Reinacher M, Tashiro M, Rott R. 1992. Interactions between bacteria and influenza A virus in the development of influenza pneumonia. J Infect Dis 166:783-91.

13. CDC. Past Pandemics.

14. Kilbourne ED. 2006. Influenza pandemics of the 20th century. Emerging infectious diseases 12:9-14.

15. Kilbourne ED. 1959. Studies on influenza in the pandemic of 1957-1958. III. Isolation of influenza A (Asian strain) viruses from influenza patients with pulmonary complications; details of virus isolation and characterization of isolates, with quantitative comparison of isolation methods. The Journal of clinical investigation 38:266-274.

16. Rogers DE, Louria DB, Kilbourne ED. 1958. The syndrome of fatal influenza virus pneumonia. Trans Assoc Am Physicians 71:260-73.

17. Smith W, Andrewes CH, Laidlaw PP. 1933. A VIRUS OBTAINED FROM INFLUENZA PATIENTS. The Lancet 222:66-68.

18. Langmuir AD. 1961. Epidemiology of Asian influenza. With special emphasis on the United States. Am Rev Respir Dis 83(2)Pt 2:2-14.

19. Choppin PW, Tamm I. 1960. Studies of two kinds of virus particles which comprise influenza A2 virus strains. II. Reactivity with virus inhibitors in normal sera. The Journal of experimental medicine 112:921-944.

20. Schulman JL, Kilbourne ED. 1969. Independent variation in nature of hemagglutinin and neuraminidase antigens of influenza virus: distinctiveness of hemagglutinin antigen of Hong

Kong-68 virus. Proc Natl Acad Sci U S A 63:326-33.

21. Monto AS, Kendal AP. 1973. Effect of neuraminidase antibody on Hong Kong influenza. Lancet 1:623-5.

22. Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L. 2005. Multinational impact of the 1968 Hong Kong influenza pandemic: evidence for a smoldering pandemic. J Infect Dis 192:233-48.

23. CDC. 2019. 2009 H1N1 Pandemic (H1N1 pdm09 virus). https://wwwcdcgov/flu/pandemic-resources/2009-h1n1-pandemichtml.

24. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. 2000. Evolution of Swine H3N2 Influenza Viruses in the United States. Journal of Virology 74:8243.

25. Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Reeth KV, Brown IH, Swenson SL, Simon G, Saito T, Berhane Y, Ciacci-Zanella J, Pereda A, Davis CT, Donis RO, Webby RJ, Vincent AL, Lowen AC. 2016. A Phylogeny-Based Global Nomenclature System and Automated Annotation Tool for H1 Hemagglutinin Genes from Swine Influenza A Viruses. mSphere 1:e00275-16.

26. Brockwell-Staats C, Webster RG, Webby RJ. 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza and other respiratory viruses 3:207-213.

27. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73:8851-6.

28. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton J-C, Lowe J, Gramer M, Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerging infectious diseases 17:1624-1629.

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

30. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125.

31. Mena I, Nelson MI, Quezada-Monroy F, Dutta J, Cortes-Fernández R, Lara-Puente JH, Castro-Peralta F, Cunha LF, Trovão NS, Lozano-Dubernard B. 2016. Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. Elife 5:e16777.

32. Campbell PJ, Danzy S, Kyriakis CS, Deymier MJ, Lowen AC, Steel J. 2014. The M Segment of the 2009 Pandemic Influenza Virus Confers Increased Neuraminidase Activity, Filamentous Morphology, and Efficient Contact Transmissibility to A/Puerto Rico/8/1934-Based Reassortant Viruses. Journal of Virology 88:3802.

33. Chou Y-y, Albrecht RA, Pica N, Lowen AC, Richt JA, García-Sastre A, Palese P, Hai R. 2011. The M Segment of the 2009 New Pandemic H1N1 Influenza Virus Is Critical for Its High Transmission Efficiency in the Guinea Pig Model. Journal of Virology 85:11235.

34. Swayne DE. 2009. Avian influenza. John Wiley & Sons.

35. Capua I, Marangon S. 2000. The avian influenza epidemic in Italy, 1999—2000: a review. Avian Pathology 29:289-294.

36. Capua I, Alexander DJ. 2007. Avian influenza infections in birds–a moving target. Influenza and other respiratory viruses 1:11-18.

37. Capua I, Alexander DJ. 2004. Avian influenza: recent developments. Avian Pathology 33:393-404.

38. Belser JA, Pulit-Penaloza JA, Sun X, Brock N, Pappas C, Creager HM, Zeng H,

Tumpey TM, Maines TR. 2017. A novel A (H7N2) influenza virus isolated from a veterinarian caring for cats in a New York City animal shelter causes mild disease and transmits poorly in the ferret model. Journal of virology 91:e00672-17.

39. Naguib MM, Verhagen JH, Mostafa A, Wille M, Li R, Graaf A, Järhult JD, Ellström P, Zohari S, Lundkvist Å, Olsen B. 2019. Global patterns of avian influenza A (H7): virus evolution and zoonotic threats. FEMS Microbiology Reviews 43:608-621.

40. Mostafa A, Abdelwhab EM, Mettenleiter TC, Pleschka S. 2018. Zoonotic potential of influenza A viruses: a comprehensive overview. Viruses 10:497.

41. Bonfanti L, Monne I, Tamba M, Santucci U, Massi P, Patregnani T, Piccolomini LL, Natalini S, Ferri G, Cattoli G. 2014. Highly pathogenic H7N7 avian influenza in Italy. Veterinary Record.

42. Joseph U, Su YC, Vijaykrishna D, Smith GJ. 2017. The ecology and adaptive evolution of influenza A interspecies transmission. Influenza and other respiratory viruses 11:74-84.

43. Abdelwhab E, Veits J, Mettenleiter T. 2014. Prevalence and control of H7 avian influenza viruses in birds and humans. Epidemiology & Infection 142:896-920.

44. Abdelwhab E-SM, Veits J, Tauscher K, Ziller M, Teifke JP, Stech J, Mettenleiter TC. 2016. A unique multibasic proteolytic cleavage site and three mutations in the HA2 domain confer high virulence of H7N1 avian influenza virus in chickens. Journal of virology 90:400-411.

45. Prevention CfDCa. 2020. Reported Infections with Variant Influenza Viruses in the United States.

46. Control CfD, Prevention. 2017. Reported Infections with Variant Influenza Viruses in the United States.

47. Anonymous. 2007. Avian influenza A/(H7N2) outbreak in the United Kingdom. Euro Surveill 12:E070531.2.

48. Marinova-Petkova A, Laplante J, Jang Y, Lynch B, Zanders N, Rodriguez M, Jones J, Thor S, Hodges E, De La Cruz JA, Belser J, Yang H, Carney P, Shu B, Berman L, Stark T, Barnes J, Havers F, Yang P, Trock SC, Fry A, Gubareva L, Bresee JS, Stevens J, Daskalakis D, Liu D, Lee CT, Torchetti MK, Newbury S, Cigel F, Toohey-Kurth K, St George K, Wentworth DE, Lindstrom S, Davis CT. 2017. Avian Influenza A(H7N2) Virus in Human Exposed to Sick Cats, New York, USA, 2016. Emerging infectious diseases 23:2046-2049.

49. Terebuh P, Adija A, Edwards L, Rowe T, Jenkins S, Kleene J, Fukuda K, Katz JM, Bridges CB. 2018. Human infection with avian influenza A(H7N2) virus-Virginia, 2002. Influenza Other Respir Viruses 12:529-532.

50. Punyadarsaniya D, Liang C-H, Winter C, Petersen H, Rautenschlein S, Hennig-Pauka I, Schwegmann-Wessels C, Wu C-Y, Wong C-H, Herrler G. 2011. Infection of differentiated porcine airway epithelial cells by influenza virus: differential susceptibility to infection by porcine and avian viruses. PloS one 6:e28429-e28429.

51. Trebbien R, Larsen LE, Viuff BM. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virology Journal 8:434.

52. Van Poucke SG, Nicholls JM, Nauwynck HJ, Van Reeth K. 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7:38.

CHAPTER 4

THE MATRIX GENE ORIGIN OF SWINE INFLUENZA H1N1 AND H1N2 ISOLATES AFFECTS VIRAL TISSUE TROPISM, REPLICATION KINETICS, AND PANDEMIC POTENTIAL IN PRIMARY AIRWAY MODELS[1]

[1] Krunkosky M[1], Bryan K, Krunkosky T, Meliopoulos V, Kyriakis C, Schultz-Cherry S, and Tompkins M. To be submitted to Journal of Virology

KEY WORDS influenza, swine, pandemic potential

ABSTRACT

Pigs are proposed as a "mixing vessel" linked to the generation of novel reassortant IAVs that may infect humans since they are susceptible to infection with both avian and human IAVs. To investigate the zoonotic capacity of swIAVs, we compared the replication properties of human and swine H1N1 and H1N2 viruses in swine primary nasal, tracheal, and bronchial epithelial cells, Calu-3, and normal human bronchoepithelial (NHBE) cells. SwIAVs were sequenced to identify differences within the internal genes, and viruses were classified based upon the presence of a matrix gene of classical swIAV origin (swM) or pdmH1N1 origin (pdmM), which was established in swine populations after the 2009 H1N1 pandemic and is now the predominant M gene found in swIAVs. Notably, swIAVs containing the pdmM gene replicated more efficiently in Calu-3 and NHBE cells than swIAVs containing the swM gene, suggesting a greater pandemic potential. Interestingly, swIAVs that contained the swM gene replicated more efficiently in PTE and PBE cells. Interestingly, swIAVs which contained the pdmM gene replicated more efficiently in primary nasal swine cells than those containing the swM gene. Furthermore, at later time-points of infection, swIAVs which contained the pdmM gene replicated more efficiently in primary nasal swine cells at 32º C than at 37º C.

INTRODUCTION

During the 2018-2019 influenza season in the United States, it is estimated that there were 36 million cases, 490,000 hospitalizations, and 34,000 deaths related to seasonal influenza. These numbers remain consistent with estimates of the 2019-2020 influenza season in the United States where it is estimated that there will be 38,000,000 cases, 400,000 hospitalizations, and 22,000 deaths related to seasonal flu (1). Furthermore, during the 2009 H1N1 pandemic, it is estimated that there were 60.8 million cases, 274,304 hospitalizations, and 12,469 deaths in the United States and an estimated range of 151,700-575,400 deaths worldwide due to the pandemic (2). Influenza A viruses (IAVs) are single stranded negative sense RNA viruses containing eight gene segments. Pigs are susceptible to avian and human IAVs and are proposed as a "mixing vessel" capable of generating novel IAVs that may infect humans. This is thought to be partially attributed to the distribution of sialic acids found along the swine respiratory tract. The upper respiratory tract of swine predominately contains cells expressing a2,6-SA, while cells expressing a2,3-SA dominate in the lower airways (3-5). Swine IAVs (swIAVs) are among the most important viral pathogens for animal health, causing outbreaks of respiratory disease in pigs and occasional zoonotic transmission to humans (6-8). Zoonotic transmission of swine influenza is thought to be partially attributed to the distribution of sialic acids found distributed within the human respiratory tract. In the human airway it has been reported in tracheal and bronchial cells that a2,3-SA are expressed in ciliated cells, while a2,6 SA are primarily expressed in non-ciliated cells (9-11).

Three subtypes of swIAVs currently circulate in swine in North America and contain gene segments from swine, avian, or human origin: H1N1, H3N2 and H1N2. H1N1

subtypes of the classical swine lineage circulated stably from 1918-1998. However, in 1998 the influenza H3N2 triple reassortment viruses appeared. This virus contained classical H1N1 NP, M, and NS, human seasonal H3N2 PB1, HA, and NA, and avian PB2 and PA genes (12-16). Then in 2009 a reassortant virus (H1N1 pandemic virus) emerged in Mexico that contained the M and NA genes derived from the Eurasian avian-like H1N1 subtype which spread rapidly in both swine and human populations. The pdmM gene has been implicated as a key determinate of increased respiratory transmission during the 2009 H1N1 pandemic, which suggests that it might play an important role in the ability of viruses to be spread in humans (17-21). H1N1 viruses have been circulating in pigs for over a century. The predominant matrix gene in IAVs circulating in North American pigs has become the pdmM gene (12, 14, 16). Swine-origin viruses that contain the pdmM gene from the 2009 pandemic have been detected in the human population since 2011 and are referred to as "variant" viruses (22). Therefore, our hypothesis is that H1N1 and H1N2 swine field virus isolates that contain the pdmM gene will replicate more efficiently in MDCK ATL cells, NHBEs, Calu-3s, PBE, PTE, and PNE cell substrates than swine field virus isolates that contain the classical swine matrix gene. Furthermore, H1N1 and H1N2 swine field virus isolates that contain the pdmM gene will exhibit greater respiratory epithelial tissue tropism and greater pandemic potential.

MATERIALS AND METHODS

Culturing and Sequencing of Swine Field Isolates

Viral stocks of CA/07/09, Sw/IL/A01395201/2013, Sw/NC/154076/2015, Sw/NC/152702/2015 N2, Sw/MO/A01444664/2013 N2, Sw/IN/A00968351/2011, and Sw/IL/A00857300/2011 were propagated in MDCK Atlanta cells. Viral stocks were sent to collaborators at UGA and St. Jude for NGS sequencing.

Culturing of Calu-3s and MDCKs for Viral Kinetics and Plaque Assays

MDCK cells were brought out of liquid nitrogen storage, thawed for 3 minutes in a 37º Celsius water bath, washed, and then re-suspended in DMEM supplemented with 5% FBS and 1X antibiotics and seeded into T75 flasks. Once MDCK cells were confluent, cells were trypsinized and plated on 12-well tissue culture treated plates for viral infection (250 PFU/well) or for plaque assays to determine viral titer. For MDCK kinetics, 1 milliliter of MEM+1X antibiotic was used to maintain cultures during the course of the experiment. Media was removed and replaced at each time-point Plaque assays were incubated for 72 hours at 37º C in 5% CO_2 with a 1.2% Avicel MEM+1X antibiotics overlay with 4X TPCK trypsin.

Calu-3 cells were brought out of liquid nitrogen storage, thawed for 3 minutes in a 37º Celsius water bath, washed, and then re-suspended in DMEM F-12 supplemented with 10% FBS and 1X antibiotics and seeded into T75 flasks. Once Calu-3 cells were confluent, cells were trypsinized and plated on 12-well tissue culture treated plates for viral infection (250 PFU/well). For Calu-3 kinetics, 1 milliliter of DMEM F-12 1X antibiotic and 2% Nuserum was used to maintain cultures during the course of the experiment. Media was removed and replaced at each time-point.

Culturing of Differentiated Normal Human Bronchial Epithelial Cells (NHBE) Maintained in Air-liquid Interface (ALI)

Expanded NHBE cells were brought out of liquid nitrogen storage, thawed for 3 minutes in a 37º Celsius water bath, and re-suspended in a 1:1 mixture of bronchial epithelial growth media (BEGM) (Clonetics): Dulbecco's modified Eagle's medium with high glucose (BEGM: DMEM-H) containing supplements provided in Lonza growth media kit, with the exception of EGF (0.5ng/mL). NHBE cells were seeded onto 24 well, 12 trans-wells, 0.32cm^2 collagen-coated trans-well inserts (Corning Costar 3470). Cultures were maintained at 37º

Celsius 5% CO_2 in submerged culture, until the cells were 100% confluent, 7-10 days. Media was replaced every other day. Once the NHBEs reached confluency, cells went to air-liquid interface, ALI, and were maintained with PneumaCult-ALI Medium (Stem Cell Technologies).

Culturing of Differentiated Porcine Primary Nasal (PNE), Porcine Primary Trachea (PTE), and Bronchial Epithelial (PBE) Cells Maintained in Air-liquid Interface (ALI)

Expanded primary swine nasal, tracheal, and bronchial cells were brought out of liquid nitrogen storage, thawed for 3 minutes in a 37º C water bath, and re-suspended in 10 milliliters of TEC+ media (DMEM F-12 (Corning 10-090-CV), Pen/Strep (Gibco 15140-148, final concentration 100mg/mL), GA-1000 (Corning CC-4083, final concentration 30mg/mL Gentamicin and 15 ng/mL Amphotericin), bovine insulin (Sigma, final concentration 10 mg/mL), human transferrin (Sigma T8158, final concentration 5 mg/mL), cholera toxin (Sigma C8052, final concentration 100ng/mL), human epidermal growth factor (ThermoFisher, final concentration 25ng/mL), bovine pituitary extract, BPE, (Corning 355100, final concentration 30 mg/mL), 5% FBS (Atlanta Biologicals), and retinoic acid (Sigma C8052, final concentration $1x10^{-10}$M). Cells were spun down at 300g for 5 minutes. Supernatant was removed and primary cells were re-suspended in TEC plus media and were seeded onto 24 well, 12 trans-wells, 0.32cm^2 collagen-coated trans-well inserts (Corning Costar 3470). Cultures were maintained at 37º C 5% CO_2 in submerged culture, until the cells were 100% confluent, 6-8 days. TEC Plus media was replaced every other day. Once the primary nasal, tracheal, and bronchial cells reached confluency, cells went to air-liquid interface, ALI, and maintained with TEC ALI media (DMEM F-12 supplemented with 1X Pen/Strep, GA-1000, retinoic acid ($1x10^{-10}$M final concentration), and 2% Nuserum (Corning 355100). Basal media was changed every 48 hours until ciliated cells and mucus were present,

approximately 6-10 days ALI.

Inoculation of MDCK, CALU-3, NHBE, PNE, PTE, and PBE Cultures

MDCK and Calu-3 cells after reaching confluency on 12-well tissue culture plates, were washed twice with PBS. MDCK cells were inoculated with 250 PFU/well of virus diluted in PBS with trypsin. Calu-3 cells were not trypsin treated. MDCK and Calu-3 cells were incubated at 37° Celsius 5% CO2 for 2 hours. The inoculum was then removed, and cells were maintained with MEM with 1X antibiotics for MDCKs and DMEM F-12 1X antibiotic and 2% Nuserum for Calu-3s. At the indicated time points, media was collected for plaque assays and replaced with fresh media.

NHBE, PNE, PTE, and PBE cells after having fully differentiated on 24-well trans-well plates, were washed twice with HBSS. NHBE, PNE, PTE, and PBE cells were inoculated apically with 250 PFU/well of virus diluted in PBS and incubated at 37° Celsius 5% CO2 for 2 hours. The inoculum was then removed, and cells were maintained in air-liquid interface with TEC ALI media. At the indicated time points the apical surface was washed with HBSS and basal media was collected for plaque assays. TEC ALI media was replaced with fresh media at 48 hours.

Statistical Analysis

Statistical analysis was done using GraphPad Prism v9. Multiple comparison analysis was conducted via 2-way ANOVA or mixed-effects analysis as indicated in supplemental figure description and legend. $P<0.05$ was considered significant.

Immunohistochemistry of NHBE, PNE, PTE, and PBE Cells to Determine Viral Tropism and Pandemic Potential of H1N1 and H1N2 Viruses

Differentiated NHBEs and primary PNE, PTE, and PBE trans-wells were fixed in 2% paraformaldehyde (Electron Microscopy Sciences 16% Paraformaldehyde Aqueous Solution,

EM Grade, Fischer Scientific 50-980-487) at room temperature for 15 minutes. Differentiated NHBE and primary PNE, PTE, and PBE trans-wells were permeabilized in 0.5% Triton X-100 (Sigma 9002-93-1) in phosphate buffered saline, PBS (Fischer Scientific MT21030CM), for 30 minutes at room temperature on a plate rocker. NHBE, PNE, PTE, and PBE cells were then washed 3 times with 0.1% PBST and blocked with 10% goat serum (Sigma G9023) for an hour at room temperature on a plate rocker. Block was removed and primary antibodies for cilia (rabbit anti acetylated tubulin, Cell Signaling D20G3, final working dilution 1:800 and rabbit anti beta tubulin, Cell Signaling D2NG5, final working dilution 1:200) and influenza viral np (mouse anti influenza A nucleoprotein, Genetex 14213, final working dilution 1:500) were added.

Primary antibodies were allowed to incubate overnight in the dark at 4º Celsius on a plate rocker. NHBE, PNE, PTE, and PBE cells were then washed 3 times with 0.1% PBST and incubated with secondary antibodies, Alexa Fluor 488 goat anti-rabbit (Invitrogen A-11008) and Alexa Fluor 546 goat anti-mouse (Invitrogen A-11003) at a 1:400 dilution for 30 minutes at room temperature in the dark. NHBE, PNE, PTE, and PBE cells were washed 3 times again with 0.1% PBST and were incubated with Alexa Fluor 647 Phalloidin (ThermoFisher Scientific A22287) for 20 minutes at room temperature in the dark. NHBE, PNE, PTE, and PBE cells were washed one more time with 0.1% PBST and wet mounted on slides with SlowFade Gold Antifade Mountant with dapi (ThermoFisher Scientific, S36938). Images of NHBE, PNE, PTE, and PBE cells were acquired using a Nikon confocal microscope via cross-sectional z-plane imaging with a 60X objective and 2X optical zoom. Files were saved in the Nikon Nd2 file format and image analysis on 20-30 mm tissue optical sections was conducted with the NIS-Elements viewer version 4.2. Images were then exported in JPEG format.

RESULTS

NGS Sequencing Alignments

Viral stocks were sequenced in order to determine the degree of genetic similarity among the swine field virus isolates. The greatest difference can be seen within the matrix gene, when comparing swine field virus isolates that contain the swM gene to those that contain the pdmM gene (Table 4.1).

The Matrix Gene Origin Affects Tissue Tropism, Replication Kinetics, and Pandemic Potential of H1N1 and H1N2 Swine Field Isolates across Multiple Cell Substrates

MDCKs are the most widely used cell line for influenza virus isolation and propagation. SwIAVs, regardless of whether they contained the swM or pdmM gene, were able to efficiently replicate in MDCK cells (Figure 4.1). However, at various time-points of infection, viruses that contained the pdmM gene replicated more efficiently than those that contained the swM gene (Figure 4.1, Supplemental Table 4.1).

Calu-3 cells, a human lung cancer cell line, which are commonly used in cancer research, drug development, and toxicity studies, have been adopted by the CDC as a tool to determine whether certain swine variants have pandemic potential. Although Calu-3 cells grow rapidly, can be passaged multiple times, can become polarized, and secrete mucus, they poorly represent the physiological properties of the respiratory tract. NHBEs better recapitulate the human respiratory tract by forming a pseudostratified columnar epithelium which has the ability to form tight junctions and fully differentiate indicated by the presence of cilia and mucus. SwIAVs, regardless of whether they contained the swM or pdmM gene, were able to efficiently gain entry into NHBE cells, indicated by positive NP staining (Figure 4.2), and were able to efficiently replicate in Calu-3 and NHBE cells (Figure 4.2 and Figure 4.3). However, at various time-points of infection, viruses that contained the pdmM gene

replicated more efficiently in Calu-3 than those that contained the swM gene (Figure 4.2, Figure 4.3, Supplemental Table 4.2, and Supplemental Table 4.3). The ability of some swIAVs that contain the pdmM gene to replicate more efficiently in human respiratory cell substrates increases their pandemic potential and therefore could be a concern for public health.

SwIAVs, regardless of whether they contained the swM or pdmM gene, were able to efficiently gain entry into PNE cells, indicated by positive NP staining (Figure 4.4.2). SwIAVs, regardless of whether they contained the swM or pdmM gene were able to efficiently replicate in PNE cells (Figure 4.4). However, at various time-points of infection, viruses which contained the pdmM gene replicated more efficiently than those that contained the swM gene (Figure 4.4, Supplemental Table 4.4).

SwIAVs, regardless of whether they contained the swM or pdmM gene, were able to efficiently gain entry into PTE and PBE cells, indicated by positive NP staining (Figure 4.5.2 and Figure 4.6.2). SwIAVs, regardless of whether they contained the swM or pdmM gene were able to efficiently replicate in PTE and PBE cells (Figure 4.5 and Figure 4.6). However, at various time-points of infection, viruses which contained the swM gene replicated more efficiently than those that contained the pdmM gene (Figure 4.5, Figure 4.6, Supplemental Table 4.5, and Supplemental Table 4.6).

Effects of Temperature on Replication Kinetics of H1N1 and H1N2 Swine Isolates in PNE Cells

SwIAVs, which contained the swM gene, replicated more efficiently in PNE cells at 37º Celsius than at 32º Celsius (Figure 4.7). Interestingly, swIAVs which contained the pdmM gene, replicated more efficiently in PNE cells at 32º Celsius than at 37º Celsius (Figure 4.7).

DISCUSSION

H1N1 viruses have been circulating in North American pigs for over a century but the pdmM matrix has become dominant in the past decade since its first introduction in 2009 (12, 14, 16). The pdmM gene has been implicated as a determinate of respiratory transmission in hosts, increasing the number of potential "variant" infections (17, 20-22). The data presented above shows that regardless of matrix origin, swine isolates are able to replicate in human respiratory cells, but those that contain the pdmM gene replicate more efficiently. As expected, all the swine isolates regardless of matrix origin were able to replicate in the swine respiratory cells. However, in the PTE, and PBE cells, viruses that contained the swM gene replicated more efficiently.

One question remains: how do swine viruses effectively transmit to humans. A clue to a possible answer may lie within the results of the kinetics at different temperatures. Within the PNE cells, viruses which contained the pdmM gene replicated more efficiently at both 37º and 32º C. It is known that temperature plays an important role in ability of viruses, specifically influenza, to replicate within the nasal epithelium and transmit. It is also been reported that differences in temperature modulates the innate immune response within the nasal epithelium (23-25). The temperature within the human nasal tract fluctuates between 30º C and 34º C, depending on inhalation or expiration (26, 27). Therefore, temperature may play a role in the ability of these swine viruses to transmit. Future studies aim to evaluate differences in innate immune responses to swine influenza due to temperature in both primary human nasal epithelial cells (HNECs) and PNEs.

Although the matrix gene has been implicated in the ability of swine influenza to transmit to humans, other genes may play a role. For example, the viral polymerase, which is comprised of the PB1, PB2, and PA gene segments has been shown to be a major host

determinant. Specifically in a single residue of the PB2 gene E627K which is correlated with increased viral replication and aerosol transmission in animals (28-35). Literature has also shown that other residues A271 and D701N are able to compensate for the absence of the E627K amino acid change. Therefore, for the following viruses the A271 amino acid change may have contributed to their increase in viral replication: CA/07/09, Sw/NC/702/15, Sw/MO/664/13, Sw/IN/351/11, and Sw/IL/300/11. Another gene that is known to play a key role in evading the host's Type-1 interferon response is the NP gene segment. The influenza viral NP gene segment has been shown to play a key role in MX sensitivity (36-40). Specifically, Y52, I100, P283, and Y313 have been shown to be essential to reduced MX sensitivity in cell culture and *in vivo* (41-43). None of the viruses used within this study contained these amino acid changes.

Lastly, the HA gene segment is essential for viral attachment as well as entry. The HA surface protein of influenza binds to sialic acid residues on host cells. As stated before, the upper respiratory tract of swine predominately contains cells expressing α2,6-SA, while cells expressing α2,3-SA dominate in the lower airways (3-5). It has been shown that H1 viruses that contain L190 and L225 have a higher affinity for α2,6-sialic acid (44, 45). None of the viruses used within this study possessed this amino acid change. A recent study also surveyed swine field isolates from farms in the United States. Using glycan arrays, it was determined that swine field isolates A/sw/NC/154072/2015 and A/sw/NC/152701/2015 preferentially bind to α2,6-silaic acid. We hypothesize that the viruses Sw/NC/076/15 and Sw/NC/702/15 since they were viruses isolated from the same farm, but different animals preferentially bind to α2,6-silaic acid. Therefore, we attribute partially the increase in viral replication, tissue tropism, and increase pandemic potential of the swine isolates used within this study to the presence of the pdmM gene. Future studies within our lab will explore the

relationship of the matrix gene with other gene segments using reverse genetics in both *in vitro* and *in vivo* models. Future directions within the lab will also look at experimental infections in both mice and ferrets to determine susceptibility, transmissibility, and immune response.

Table 4.1.1: Genetic Similarity of CA/07/09 (pdmM), to various swine isolates: Sw/IL/A01395201/2013 (pdmM), Sw/IL/201/13, Sw/NC/152702/2015 N2 (pdmM), Sw/NC/702/15 N2, Sw/NC/154076/2015 (pdmM), Sw/NC/076/15, Sw/MO/A01444664/2013 N2 (swM), Sw/MO/664/13 N2, Sw/IN/A00968351/2011 (swM), Sw/IN/351/11, and Sw/IL/A00857300/2011 (swM), Sw/IL/7300/11.

Influenza Strain	PB2	PB1	PA	HA	NP	NA	M	NS
CA/07/09 (pdmM)	100%	100%	100%	100%	100%	100%	100%	100%
Sw/IL/201/13 (pdmM)	98.2%	98.7%	98.3%	96.7%	98.1%	97.2%	98.7%	99.0%
Sw/NC/702/15 N2 (pdmM)	93.8%	93.8%	93.8%	76.6%	98.0%	50.3%	98.8%	93.6%
Sw/NC/076/15 (pdmM)	94.1%	93.7%	94.1%	91.7%	98.6%	80.5%	98.5%	93.9%
Sw/MO/664/13 N2 (swM)	94.4%	94.3%	93.8%	76.3%	94.8%	50.1%	87.7%	94.8%
Sw/IN/351/11 (swM)	94.1%	94.1%	93.9%	93.3%	95.1%	79.9%	88.1%	94.7%
Sw/IL/300/11 (swM)	94.4%	94.0%	94.1%	92.9%	95.0%	80.3%	87.5%	94.9%

Table 4.1.2: Genetic Similarity of Sw/IL/A01395201/2013 (pdmM), Sw/IL/201/13, to various swine isolates: Sw/NC/152702/2015 N2 (pdmM), Sw/NC/702/15 N2, Sw/NC/154076/2015 (pdmM), Sw/NC/076/15, Sw/MO/A01444664/2013 N2 (swM), Sw/MO/664/13 N2, Sw/IN/A00968351/2011 (swM), Sw/IN/351/11, and Sw/IL/A00857300/2011 (swM), Sw/IL/7300/11.

Influenza Strain	PB2	PB1	PA	HA	NP	NA	M	NS
Sw/IL/201/13 (pdmM)	100%	100%	100%	100%	100%	100%	100%	100%
Sw/NC/702/15 N2 (pdmM)	92.4%	93.0%	92.6%	76.1%	96.9%	0%	97.8%	92.8%
Sw/NC/076/15 (pdmM)	92.8%	92.9%	93.0%	90%	97.4%	79.3%	97.4%	93.3%
Sw/MO/664/13 N2 (swM)	93.0%	93.5%	92.8%	76.3%	93.5%	50.0%	88.0%	94.0%
Sw/IN/351/11 (swM)	92.7%	93.2%	92.8%	91.6%	93.9%	78.6%	88.2%	93.9%
Sw/IL/300/11 (swM)	93.0%	93.1%	93.0%	91.1%	93.6%	79.2%	87.8%	94.2%

Table 4.1.3: Genetic Similarity of Sw/NC/152702/2015 N2 (pdmM), Sw/NC/702/15 N2, to various swine isolates: Sw/NC/154076/2015 (pdmM), Sw/NC/076/15, Sw/MO/A01444664/2013 N2 (swM), Sw/MO/664/13 N2, Sw/IN/A00968351/2011 (swM), Sw/IN/351/11, and Sw/IL/A00857300/2011 (swM), Sw/IL/7300/11.

Influenza Strain	PB2	PB1	PA	HA	NP	NA	M	NS
Sw/NC/702/15 N2 (pdmM)	100%	100%	100%	100%	100%	100%	100%	100%
Sw/NC/076/15 (pdmM)	98.5%	99.7%	99.3%	77.3%	97.3%	49.7%	98.6%	95.3%
Sw/MO/664/13 N2 (swM)	98.1%	96.8%	98.2%	90.0%	93.2%	90.0%	88.0%	96.2%
Sw/IN/351/11 (swM)	97.6%	98.9%	98.7%	76.9%	93.5%	54.5%	88.4%	96.1%
Sw/IL/300/11 (swM)	98.3%	97.4%	98.5%	76.8%	93.5%	51.2%	88.1%	96.3%

Table 4.1.4: Genetic Similarity of Sw/NC/154076/2015 (pdmM), Sw/NC/076/15, to various swine isolates: Sw/MO/A01444664/2013 N2 (swM), Sw/MO/664/13 N2, Sw/IN/A00968351/2011 (swM), Sw/IN/351/11, and Sw/IL/A00857300/2011 (swM), Sw/IL/7300/11.

Influenza Strain	PB2	PB1	PA	HA	NP	NA	M	NS
Sw/NC/076/15 (pdmM)	100%	100%	100%	100%	100%	100%	100%	100%
Sw/MO/664/13 N2 (swM)	98.3%	96.7%	98.4%	76.7%	94.1%	50.8%	87.6%	98.1%
Sw/IN/351/11 (swM)	97.8%	98.9%	99.0%	97.2%	94.4%	96.7%	87.9%	98.1%
Sw/IL/300/11 (swM)	98.3%	97.3%	98.7%	96.6%	94.2%	96.8%	87.7%	98.3%

Table 4.1.5: Genetic Similarity of Sw/MO/A01444664/2013 N2 (swM), Sw/MO/664/13 N2, to various swine isolates: Sw/IN/A00968351/2011 (swM), Sw/IN/351/11, and Sw/IL/A00857300/2011 (swM), Sw/IL/7300/11.

Influenza Strain	PB2	PB1	PA	HA	NP	NA	M	NS
Sw/MO/664/13 N2 (swM)	100%	100%	100%	100%	100%	100%	100%	100%
Sw/IN/351/11 (swM)	98.6%	97.4%	99.0%	76.2%	99.1%	50.9%	98.1%	99.1%
Sw/IL/300/11 (swM)	99.1%	97.6%	99.1%	76.2%	99.0%	50.9%	96.6%	99.3%

Table 4.1.6: Genetic Similarity of Sw/IN/A00968351/2011 (swM), Sw/IN/351/11, to Sw/IL/A00857300/2011 (swM), Sw/IL/7300/11.

Influenza Strain	PB2	PB1	PA	HA	NP	NA	M	NS
Sw/IN/351/11 (swM)	100%	100%	100%	100%	100%	100%	100%	100%
Sw/IL/300/11 (swM)	98.7%	98.1%	99.1%	98.6%	99.1%	98.3%	96.6%	99.3%

Figure 4.1: Replication Kinetics of H1N1 and H1N2 Swine Isolates in MDCK Cells.

MDCK cells were grown in 12-well tissue culture plates. Cells were infected with CA/07/09 (pdmM), Sw/IL/A01395201/2013 (pdmM), Sw/NC/154076/2015 (pdmM), Sw/NC/152702/2015 N2 (pdmM), Sw/MO/A01444664/2013 N2 (swM), Sw/IN/A00968351/2011 (swM) and Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Data represents one independent experiment with n=6 wells at each time-point. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection.

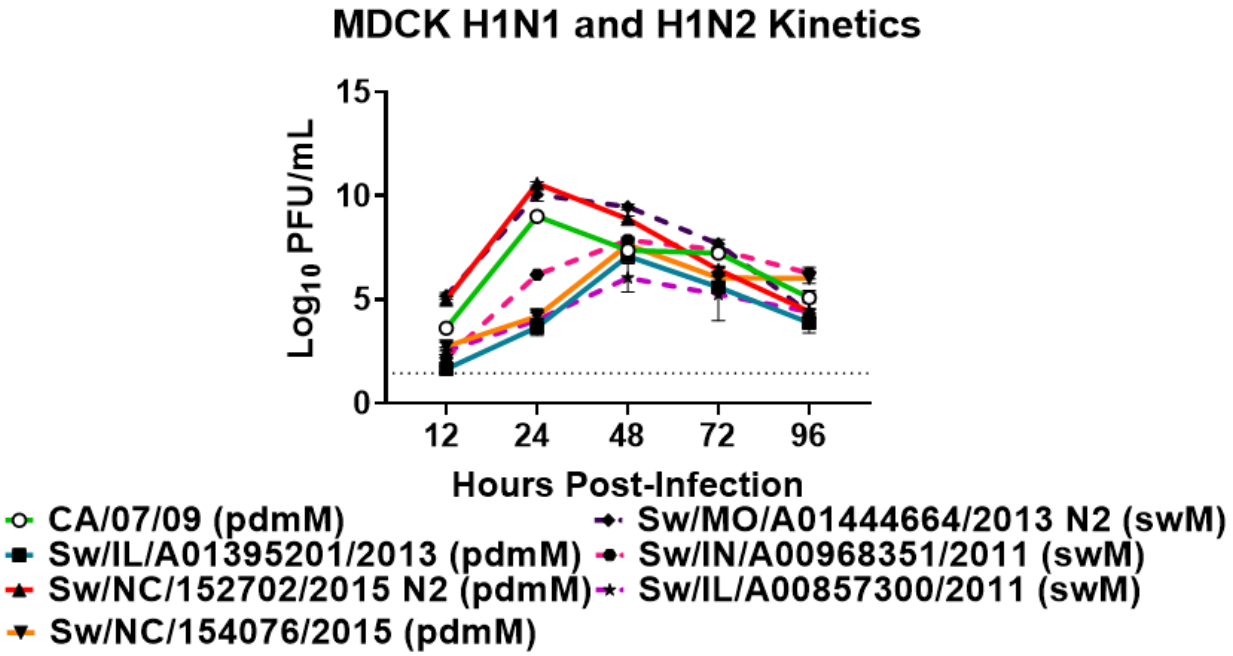


Figure 4.2: Replication Kinetics of H1N1 and H1N2 Swine Isolates in Calu-3 Cells.

MDCK cells were grown in 12-well tissue culture plates. Cells were infected with CA/07/09 (pdmM), Sw/IL/A01395201/2013 (pdmM), Sw/NC/154076/2015 (pdmM), Sw/NC/152702/2015 N2 (pdmM), Sw/MO/A01444664/2013 N2 (swM), Sw/IN/A00968351/2011 (swM) and Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Data represents one independent experiment with n=6 wells at each time-point. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection.

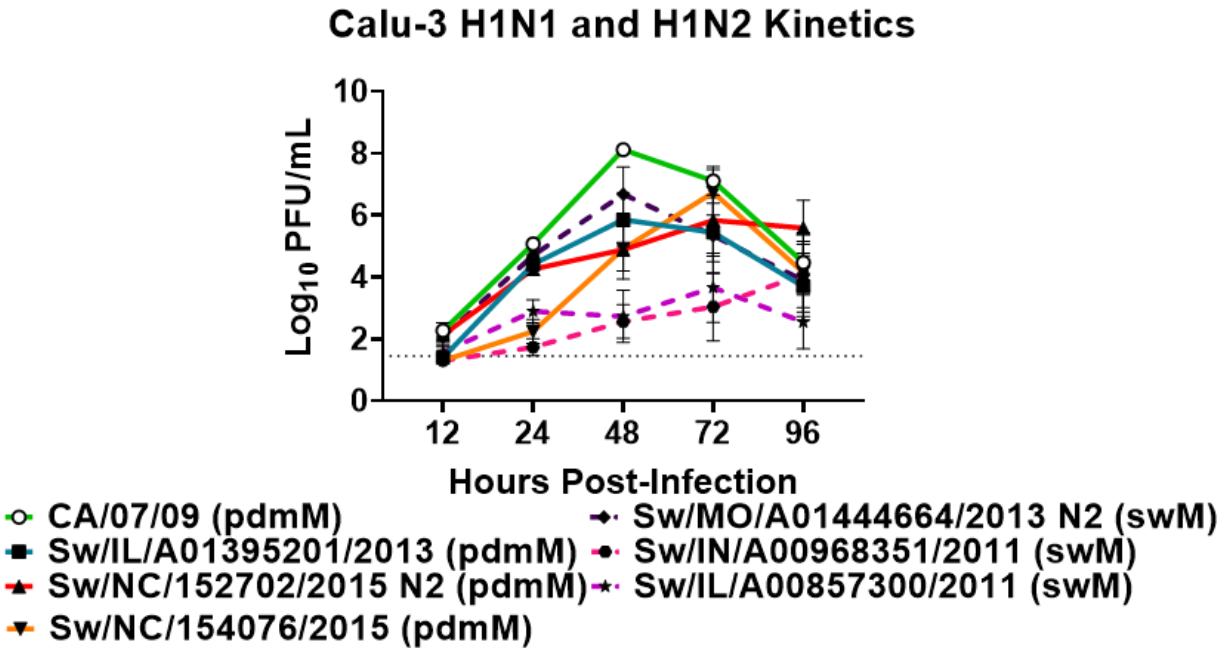


Figure 4.3.1: Replication Kinetics of H1N1 and H1N2 Swine Isolates in NHBE Cells. NHBE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with CA/07/09 (pdmM), Sw/IL/A01395201/2013 (pdmM), Sw/NC/154076/2015 (pdmM), Sw/NC/152702/2015 N2 (pdmM), Sw/MO/A01444664/2013 N2 (swM), Sw/IN/A00968351/2011 (swM) and Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Data represents one independent experiment with n=6 wells at 12 and 24 hours, n=5 wells at 48 hours, n=4 wells at 72 hours, and n=3 wells at 96 hours. At 24, 48, and 72 hours a well from each virus was taken for immunohistochemistry. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection.

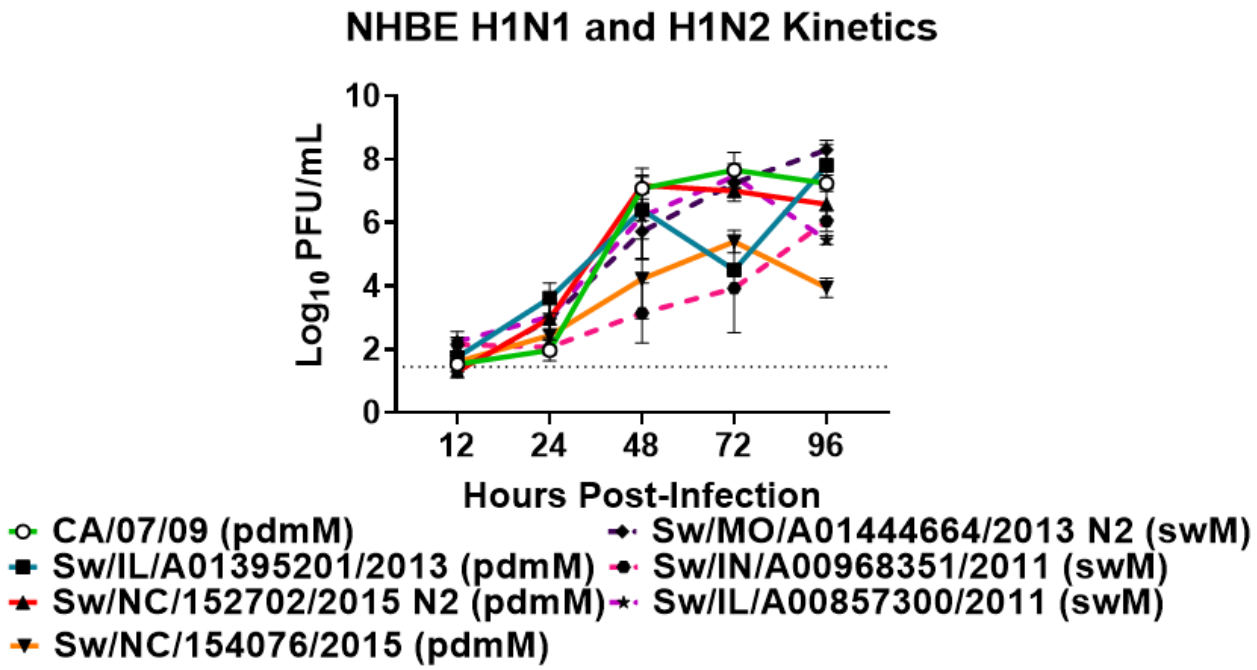


Figure 4.3.2: Immunohistochemistry of NHBE Cells. Influenza nucleoprotein to detect virus inside NHBE cells in air-liquid interface (ALI)=red, beta and acetylated tubulin to detect the presence of cilia on NHBE cells in ALI=green, dapi, nuclear stain=blue, and Phallodin to detect the presence of tight junctions, F-actin=purple, white bar indicates a measurement of 25 microns **A.** CA/07/09 (pdmM) **B**. Sw/IL/201/13 (pdmM) C. Sw/NC/076 (pdmM), **D.** Sw/NC/702/15 N2 (pdmM) **E.** Sw/MO/664/13 N2 (swM) **F.** Sw/IN/351/11 (swM). **G.** Sw/IL/300/11 (swM).

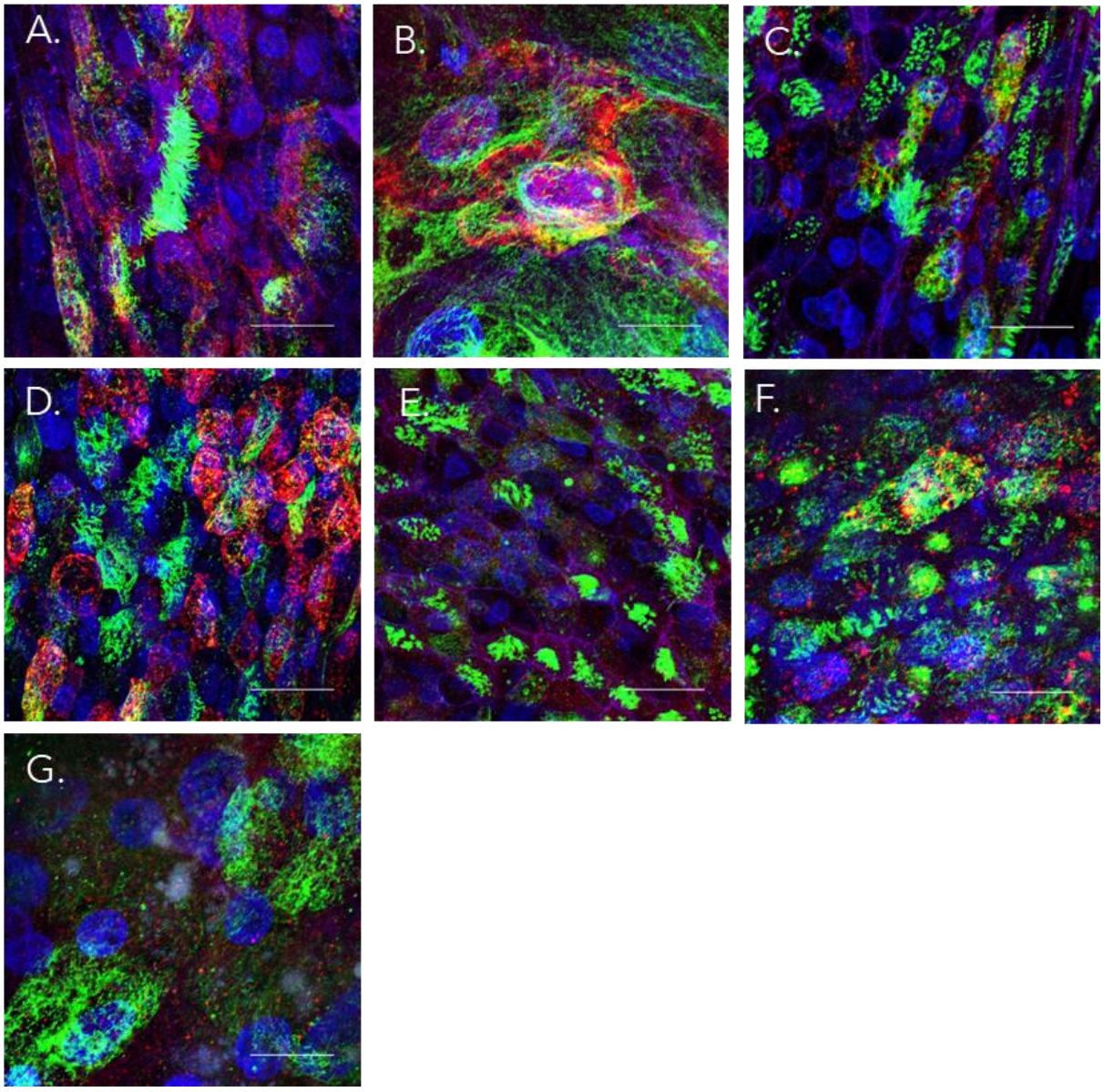


Figure 4.4.1: Replication Kinetics of H1N1 and H1N2 Swine Isolates in PNE Cells. PNE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with CA/07/09 (pdmM), Sw/IL/A01395201/2013 (pdmM), Sw/NC/154076/2015 (pdmM), Sw/NC/152702/2015 N2 (pdmM), Sw/MO/A01444664/2013 N2 (swM), Sw/IN/A00968351/2011 (swM) and Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Data represents one independent experiment with n=6 wells at 12 and 24 hours, n=5 wells at 48 hours, n=4 wells at 72 hours, and n=3 wells at 96 hours. At 24, 48, and 72 hours a well from each virus was taken for immunohistochemistry. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection.

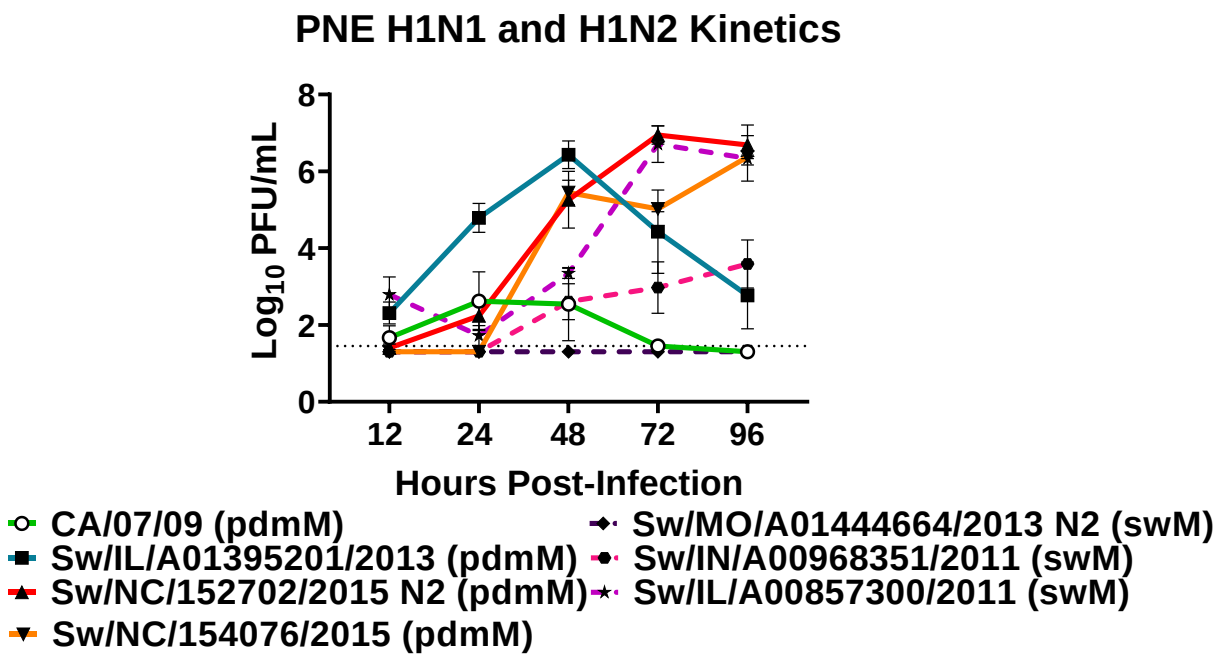


Figure 4.4.2: Immunohistochemistry of PNE Cells. Influenza nuclear protein to stain virus inside PNE cells in air-liquid interface (ALI)=red, beta and acetylated tubulin to detect the presence of cilia on PNE cells in ALI=green, dapi, nuclear stain=blue, and Phallodin to detect the presence of tight junctions, F-actin=purple, white bar indicates a measurement of 25 microns **A.** CA/07/09 (pdmM) **B**. Sw/IL/201/13 (pdmM) C. Sw/NC/076 (pdmM), **D.** Sw/NC/702/15 N2 (pdmM) **E.** Sw/MO/664/13 N2 (swM) **F.** Sw/IN/351/11 (swM). **G.** Sw/IL/300/11 (swM).

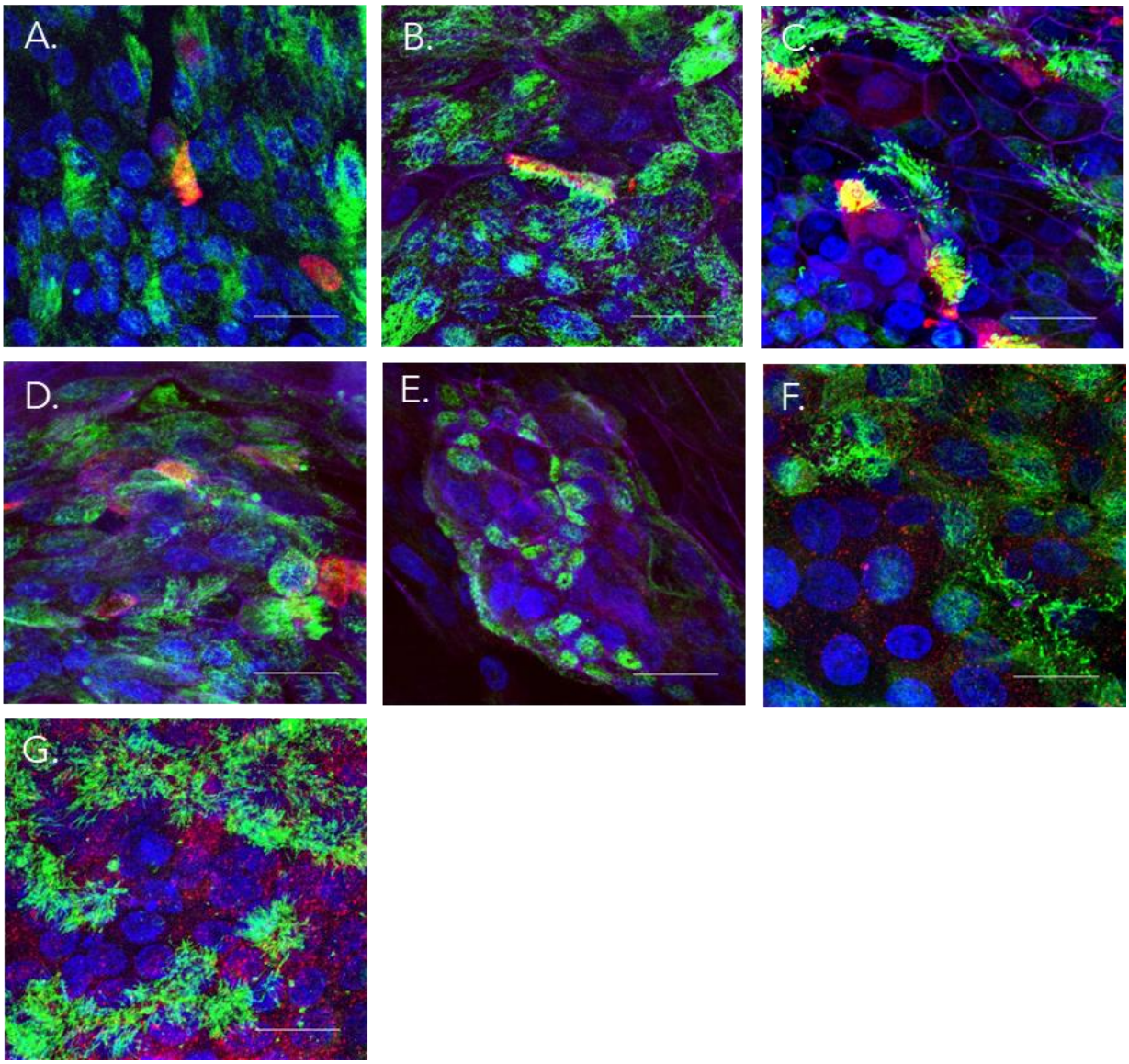


Figure 4.5.1: Replication Kinetics of H1N1 and H1N2 Swine Isolates in PTE Cells. PTE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with CA/07/09 (pdmM), Sw/IL/A01395201/2013 (pdmM), Sw/NC/154076/2015 (pdmM), Sw/NC/152702/2015 N2 (pdmM), Sw/MO/A01444664/2013 N2 (swM), Sw/IN/A00968351/2011 (swM) and Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Data represents one independent experiment with n=6 wells at 12 and 24 hours, n=5 wells at 48 hours, n=4 wells at 72 hours, and n=3 wells at 96 hours. At 24, 48, and 72 hours a well from each virus was taken for immunohistochemistry. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection.

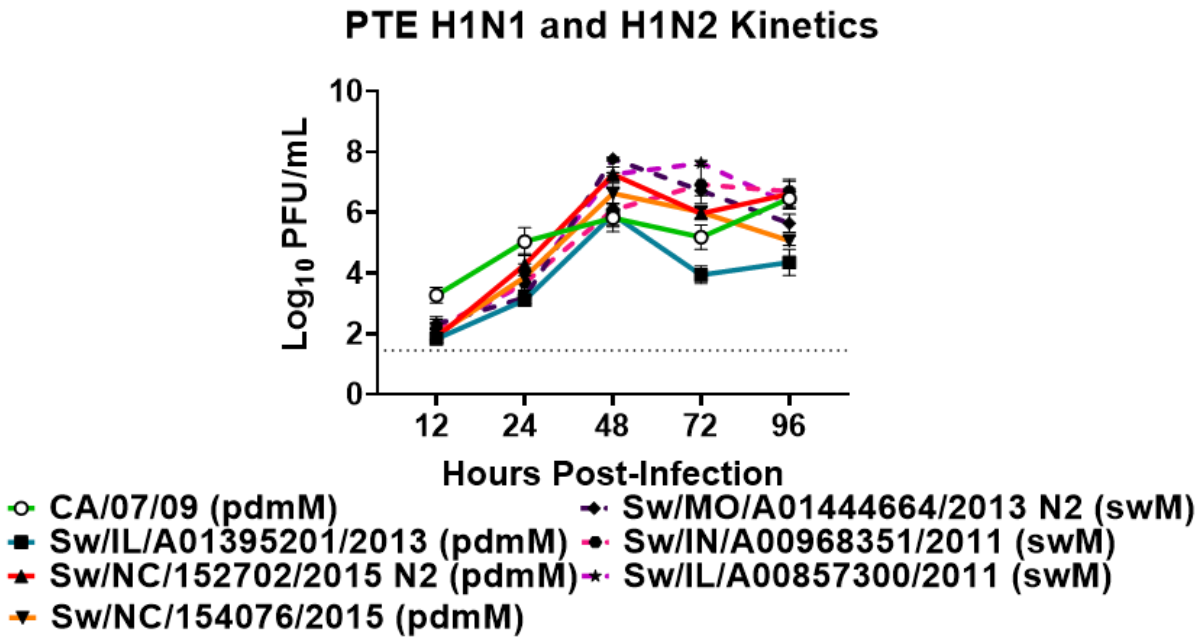


Figure 4.5.2: Immunohistochemistry of PTE Cells. Influenza nuclear protein to stain virus inside PTE cells in air-liquid interface (ALI)=red, beta and acetylated tubulin to detect the presence of cilia on PTE cells in ALI=green, dapi, nuclear stain=blue, and Phallodin to detect the presence of tight junctions, F-actin=purple, white bar indicates a measurement of 25 microns **A.** CA/07/09 (pdmM) **B**. Sw/IL/201/13 (pdmM) C. Sw/NC/076 (pdmM), **D.** Sw/NC/702/15 N2 (pdmM) **E.** Sw/MO/664/13 N2 (swM) **F.** Sw/IN/351/11 (swM). **G.** Sw/IL/300/11 (swM).

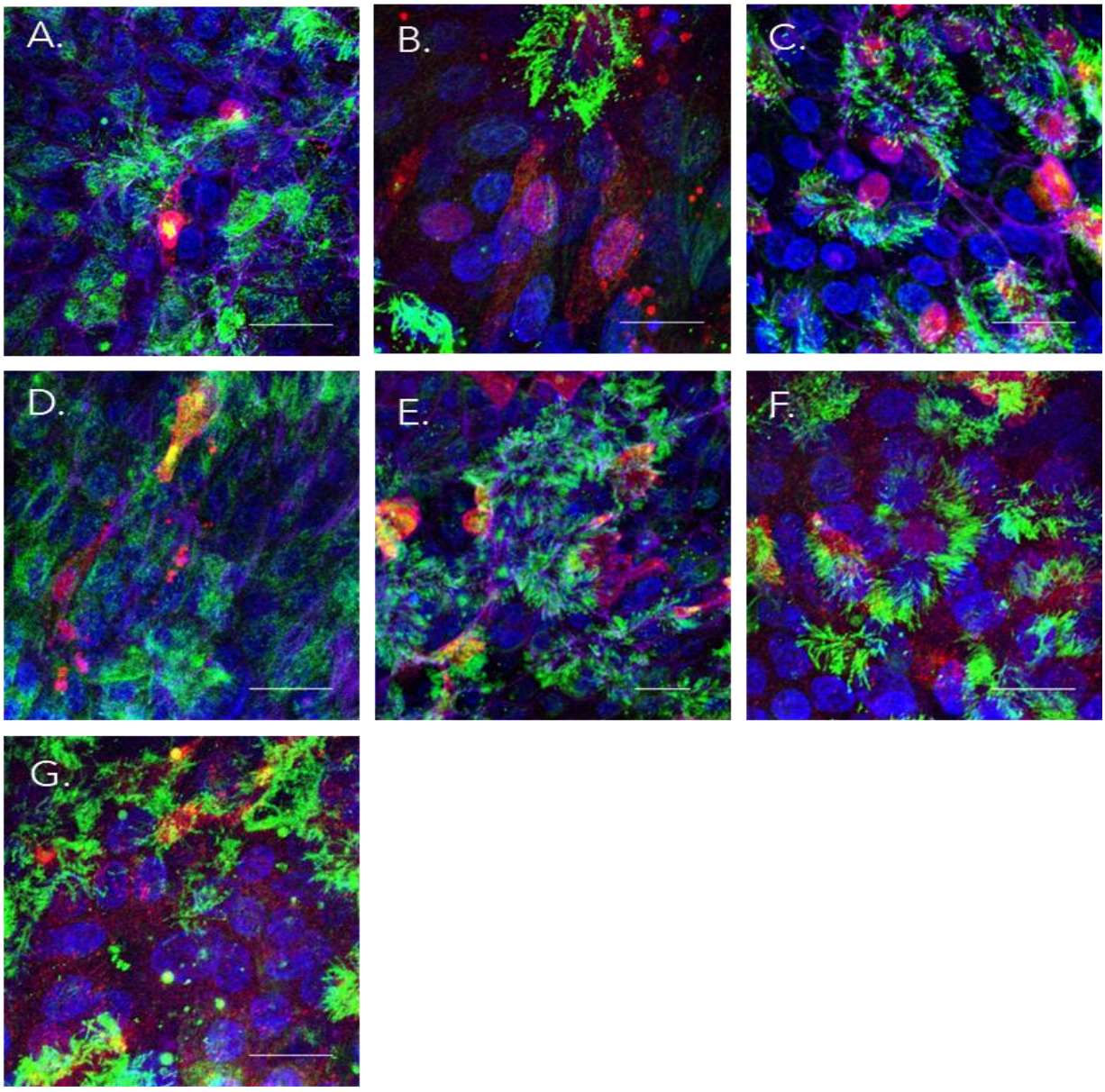


Figure 4.6.1: Replication Kinetics of H1N1 and H1N2 Swine Isolates in PBE Cells. PBE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with CA/07/09 (pdmM), Sw/IL/A01395201/2013 (pdmM), Sw/NC/154076/2015 (pdmM), Sw/NC/152702/2015 N2 (pdmM), Sw/MO/A01444664/2013 N2 (swM), Sw/IN/A00968351/2011 (swM) and Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Data represents one independent experiment with n=6 wells at 12 and 24 hours, n=5 wells at 48 hours, n=4 wells at 72 hours, and n=3 wells at 96 hours. At 24, 48, and 72 hours a well from each virus was taken for immunohistochemistry. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection.

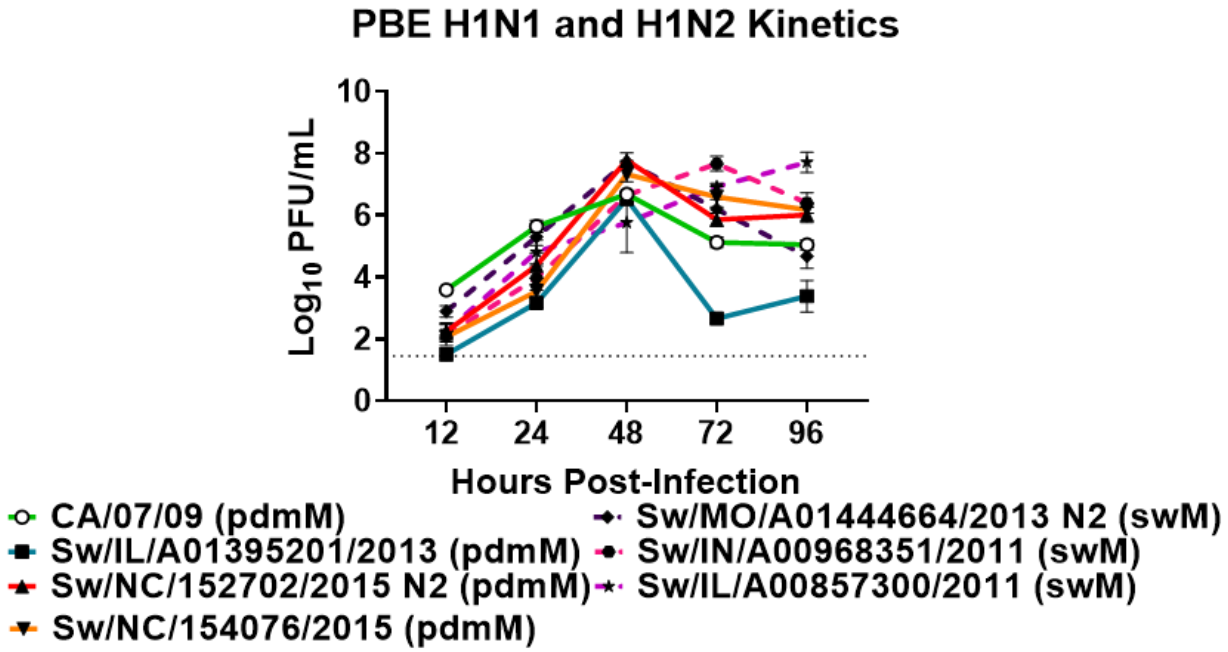


Figure 4.6.2: Immunohistochemistry of PBE Cells. Influenza nuclear protein to stain virus inside PBE cells in air-liquid interface (ALI)=red, beta and acetylated tubulin to detect the presence of cilia on PBE cells in ALI=green, dapi, nuclear stain=blue, and Phallodin to detect the presence of tight junctions, F-actin=purple, white bar indicates a measurement of 25 microns **A.** CA/07/09 (pdmM) **B**. Sw/IL/201/13 (pdmM) C. Sw/NC/076 (pdmM), **D.** Sw/NC/702/15 N2 (pdmM) **E.** Sw/MO/664/13 N2 (swM) **F.** Sw/IN/351/11 (swM). **G.** Sw/IL/300/11 (swM).

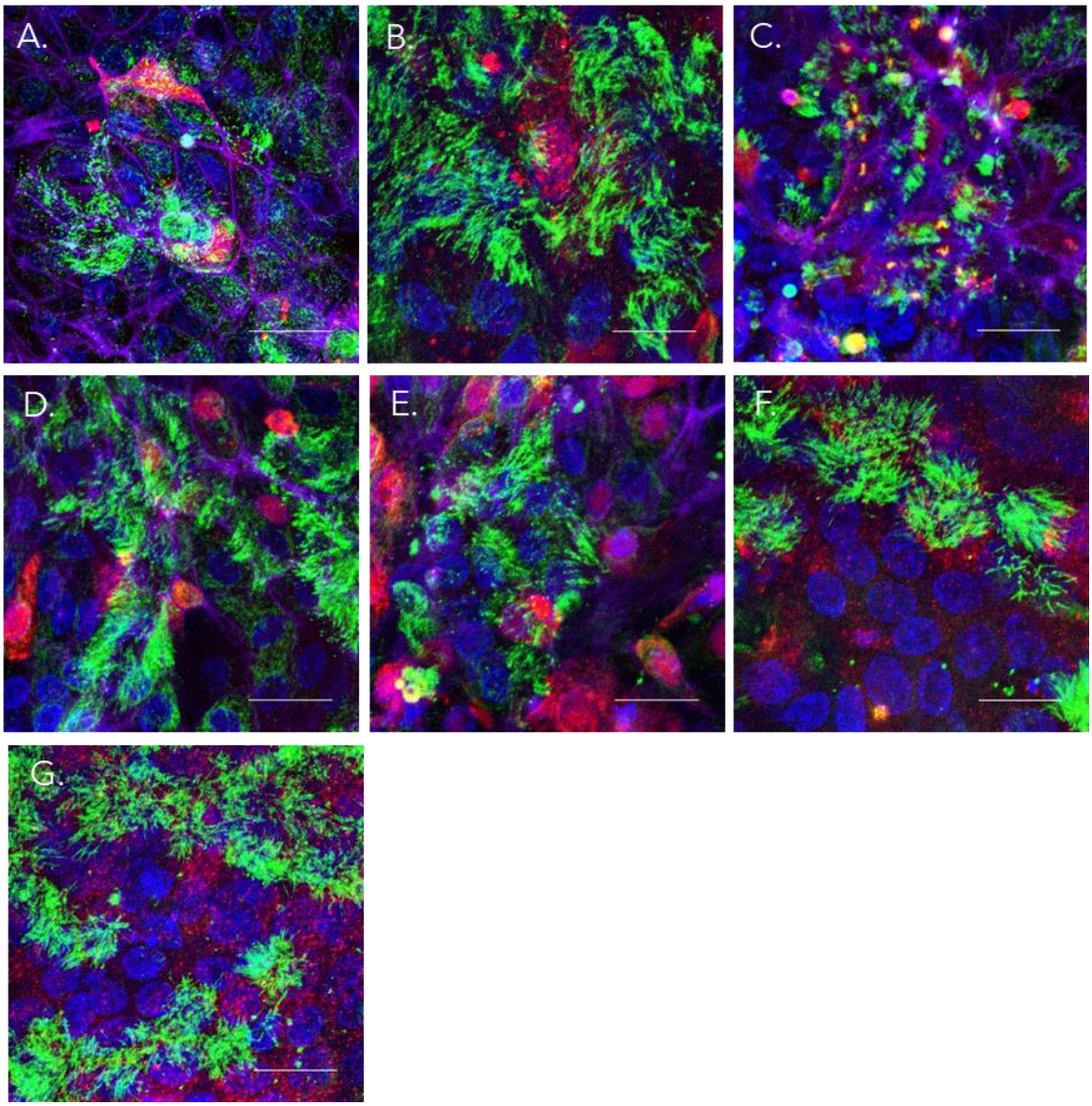
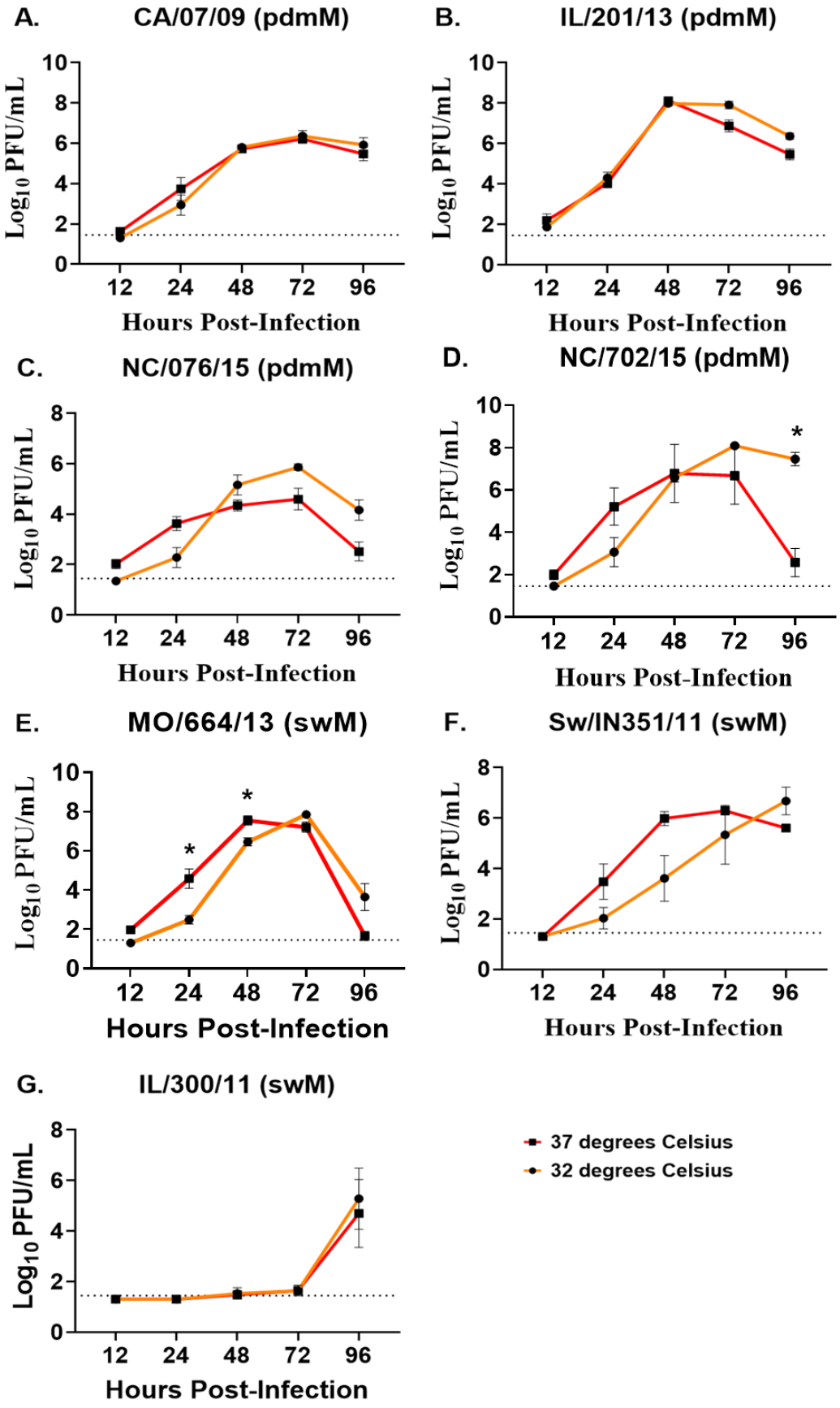


Figure 4.7: Temperature Replication Kinetics of H1N1 and H1N2 Swine Isolates in PNE Cells. PNE cells were grown on trans-wells and allowed to fully differentiate in ALI. Once cilia and mucus secretion were present, cells were infected with **A.** CA/07/09 (pdmM), **B.** Sw/IL/A01395201/2013 (pdmM), **C.** Sw/NC/154076/2015 (pdmM), **D.** Sw/NC/152702/2015 N2 (pdmM), **E.** Sw/MO/A01444664/2013 N2 (swM), **F.** Sw/IN/A00968351/2011 (swM) and **G.** Sw/IL/A00857300/2011 (swM) at 250 PFU/well. Replication kinetics for each virus were done side by side at both 32º Celsius and 37º Celsius. Data represents one independent experiment, in a different donor than above, with n=6 wells at 12 and 24 hours, n=5 wells at 48 hours, n=4 wells at 72 hours, and n=3 wells at 96 hours. At 24, 48, and 72 hours a well from each virus was taken for immunohistochemistry. For each virus the same set of wells was used for all subsequent time-points. Error bars represent +/- SEM. Dashed line represents limit of detection. *P<0.05 by mixed effects analysis with repeated measures.


A.
CA/07/09 (pdmM)
B.
IL/201/13 (pdmM)
C.
NC/076/15 (pdmM)
D.
NC/702/15 (pdmM)
E.
MO/664/13 (swM)
F.
Sw/IN351/11 (swM)
G.
IL/300/11 (swM)
Log10 PFU/mL
Hours Post-Infection
12
24
48
72
96
0
2
4
6
8
10
*
37 degrees Celsius
32 degrees Celsius

Supplemental Table 4.1. MDCK Kinetics 2-Way ANOVA Multiple Comparisons

A. CA/07/09 (pdmM) versus pandemic matrix (pdmM) or swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
CA/07/09 (pdmM) vs. Sw/IL/201/13 (pdmM)	******, <0.0001**	*****, 0.0004**	NS, 0.9618	****, 0.0055**	NS, 0.0628
CA/07/09 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	****, 0.0027**	******, <0.0001**	****, 0.0062**	NS, 0.0614	NS, 0.3125
CA/07/09 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.0964	******, <0.0001**	NS, 0.9254	NS, 0.4720	NS, 0.2055
CA/07/09 (pdmM) vs. Sw/MO/664/13 N2 (swM)	*****, 0.0007**	NS, 0.1487	*****, 0.0007**	NS, 0.6924	NS, 0.3352
CA/07/09 (pdmM) vs. Sw/IN/351/11 (swM)	****, 0.0023**	******, <0.0001**	NS, 0.6677	NS, 0.9822	NS, 0.0951
CA/07/09 (pdmM) vs. Sw/IL/300/11 (swM)	***, 0.0175**	****, 0.0023**	NS, 0.5634	NS, 0.7123	NS, 0.9920

B. Matrix (pdmM) versus swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/IL/201/13 (pdmM) vs. Sw/MO/664/13 N2 (swM)	******, <0.0001**	******, <0.0001**	******, <0.0001**	****, 0.0012**	NS, 0.7883
Sw/IL/201/13 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.2847	***, 0.0127**	NS, 0.1545	****, 0.0037**	*****, 0.0010**
Sw/IL/201/13 (pdmM) vs. Sw/IL/300/11 (swM)	***, 0.0445**	NS, 0.9983	NS, 0.7487	NS, >0.9999	NS, 0.9975
Sw/NC/702/15 N2 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.9756	NS, 0.6986	NS, 0.1194	***, 0.0106**	NS, 0.9996
Sw/NC/702/15 N2 (pdmM) vs. Sw/IN/351/11 (swM)	******, <0.0001**	******, <0.0001**	***, 0.0122**	****, 0.0041**	****, 0.0056**
Sw/NC/702/15 N2 (pdmM) vs. Sw/IL/300/11 (swM)	******, <0.0001**	*****, 0.0005**	NS, 0.0622	NS, 0.9498	NS, >0.9999
Sw/NC/076/15 (pdmM) vs. Sw/MO/664/13 N2 (swM)	******, <0.0001**	******, <0.0001**	******, <0.0001**	NS, 0.2218	****, 0.0067**
Sw/NC/076/15 (pdmM) vs Sw/IN/351/11 (swM)	NS, 0.4796	*****, 0.0006**	NS, 0.9316	NS, 0.3450	NS, 0.9945
Sw/NC/076/15 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9824	NS, 0.9999	NS, 0.3699	NS, 0.9955	NS, 0.7216

C. Swine matrix (swM) versus swine matrix (swM) or pandemic matrix (pdmM) versus pandemic matrix (pdmM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/MO/664/13 N2 (swM) vs. Sw/IN/351/11 (swM)	******, <0.0001**	*****, 0.0006**	*****, 0.0006**	NS, 0.8784	****, 0.0038**
Sw/MO/664/13 N2 (swM) vs. Sw/IL/300/11 (swM)	******, <0.0001**	*****, 0.0002**	***, 0.0291**	NS, 0.5506	NS, >0.9999
Sw/IN/351/11 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.8466	NS, 0.0799	NS, 0.2798	NS, 0.6473	NS, 0.6152
Sw/IL/201/13 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	******, <0.0001**	******, <0.0001**	*****, 0.0006**	NS, 0.1557	NS, 0.4688
Sw/IL/201/13 (pdmM) vs. Sw/NC/076/15 (pdmM)	***, 0.0251**	NS, 0.8848	NS, 0.2928	NS, 0.9841	****, 0.0016**
Sw/NC/702/15 N2 (pdmM) vs. Sw/NC/076/15 (pdmM)	*****, 0.0002**	******, <0.0001**	*****, 0.0003**	NS, 0.9881	****, 0.0081**

Differences in replication kinetics of viruses analyzed using a 2-Way ANOVA with multiple comparisons. Values are expressed as not significant (NS) with corresponding p-value or values are expressed as significant (*) with corresponding p-value. #=statistics not calculated due to mean difference value of zero.

Supplemental Table 4.2. Calu-3 Kinetics 2-Way ANOVA Multiple Comparisons

A. CA/07/09 (pdmM) versus pandemic matrix (pdmM) or swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
CA/07/09 (pdmM) vs. Sw/IL/201/13 (pdmM)	NS, 0.1463	****, 0.0041**	NS, 0.2796	NS, 0.6924	NS, 0.9970
CA/07/09 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, >0.9999	NS, 0.0544	NS, 0.1350	NS, 0.9328	NS, 0.9685
CA/07/09 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.0984	****, 0.0054**	***, 0.0495**	NS, 0.9992	NS, >0.9999
CA/07/09 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.9939	NS, 0.2857	NS, 0.6760	NS, 0.8102	NS, 0.9995
CA/07/09 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.0984	*****, 0.0003**	****, 0.0012**	NS, 0.1000	NS, >0.9999
CA/07/09 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.4488	***, 0.0154**	***, 0.0110**	NS, 0.1941	NS, 0.7183

B. Matrix (pdmM) versus swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/IL/201/13 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.1296	NS, 0.5706	NS, 0.9918	NS, >0.9999	NS, >0.9999
Sw/IL/201/13 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.9338	*****, 0.0009**	NS, 0.1020	NS, 0.6546	NS, >0.9999
Sw/IL/201/13 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9865	NS, 0.0703	NS, 0.2216	NS, 0.8732	NS, 0.9761
Sw/NC/702/15 N2 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, >0.9999	NS, 0.5103	NS, 0.8027	NS, >0.9999	NS, 0.8983
Sw/NC/702/15 N2 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.4469	*****, 0.0005**	NS, 0.4245	NS, 0.6038	NS, 0.9233
Sw/NC/702/15 N2 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.8553	NS, 0.1140	NS, 0.6332	NS, 0.8161	NS, 0.2797
Sw/NC/076/15 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.0724	****, 0.0087**	NS, 0.7129	NS, 0.9466	NS, >0.9999
Sw/NC/076/15 (pdmM) vs Sw/IN/351/11 (swM)	#	NS, 0.9215	NS, 0.2228	NS, 0.1808	NS, >0.9999
Sw/NC/076/15 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.8501	NS, 0.8786	NS, 0.4804	NS, 0.3406	NS, 0.9318

C. Swine matrix (swM) versus swine matrix (swM) or pandemic matrix (pdmM) versus pandemic matrix (pdmM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/MO/664/13 N2 (swM) vs. Sw/IN/351/11 (swM)	NS, 0.0724	*****, 0.0003**	***, 0.0391**	NS, 0.7909	NS, >0.9999
Sw/MO/664/13 N2 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.6295	***, 0.0325**	NS, 0.0888	NS, 0.9394	NS, 0.9586
Sw/IN/351/11 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.8501	NS, 0.2637	NS, >0.9999	NS, 0.9995	NS, 0.9097
Sw/IL/201/13 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, 0.5808	NS, 0.9844	NS, 0.9848	NS, >0.9999	NS, 0.8194
Sw/IL/201/13 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.9338	***, 0.0180**	NS, 0.9739	NS, 0.9186	NS, >0.9999
Sw/NC/702/15 N2 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.4496	***, 0.0214**	NS, >0.9999	NS, 0.9922	NS, 0.9608

Differences in replication kinetics of viruses analyzed using a 2-Way ANOVA with multiple comparisons. Values are expressed as not significant (NS) with corresponding p-value or values are expressed as significant (*) with corresponding p-value. #=statistics not calculated due to mean difference value of zero.

Supplemental Table 4.3. NHBE Kinetics Mixed-effect Analysis Multiple Comparisons

A. CA/07/09 (pdmM) versus pandemic matrix (pdmM) or swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
CA/07/09 (pdmM) vs. Sw/IL/201/13 (pdmM)	NS, 0.9496	NS, 0.1668	NS, 0.9527	NS, 0.0537	NS, 0.9714
CA/07/09 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, 0.7095	NS, 0.7625	NS, >0.9999	NS, 0.9208	NS, 0.8576
CA/07/09 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.9999	NS, 0.9867	NS, 0.4835	NS, 0.1242	****, 0.0095**
CA/07/09 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.7905	NS, 0.7771	NS, 0.8536	NS, 0.9936	NS, 0.2937
CA/07/09 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.3206	NS, >0.9999	NS, 0.0912	NS, 0.3517	NS, 0.2687
CA/07/09 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.4668	NS, 0.4923	NS, 0.9935	NS, 0.9999	***, 0.0439**

B. Matrix (pdmM) versus swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/IL/201/13 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.2509	NS, 0.9707	NS, 0.9843	***, 0.0301**	NS, 0.9841
Sw/IL/201/13 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.7394	NS, 0.1700	NS, 0.1542	NS, 0.9988	NS, 0.4355
Sw/IL/201/13 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.7851	NS, 0.9603	NS, >0.9999	***, 0.0225**	NS, 0.2619
Sw/NC/702/15 N2 (pdmM) vs. Sw/MO/664/13 N2 (swM)	#	NS, >0.9999	NS, 0.6965	NS, 0.9980	NS, 0.2471
Sw/NC/702/15 N2 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.0862	NS, 0.7919	NS, 0.0772	NS, 0.4809	NS, 0.9518
Sw/NC/702/15 N2 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.2013	NS, >0.9999	NS, 0.9812	NS, 0.9553	NS, 0.4814
Sw/NC/076/15 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.7108	NS, 0.9948	NS, 0.9463	NS, 0.1063	****, 0.0038**
Sw/NC/076/15 (pdmM) vs Sw/IN/351/11 (swM)	NS, 0.5607	NS, 0.9940	NS, 0.9897	NS, 0.9215	NS, 0.0645
Sw/NC/076/15 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.6377	NS, 0.9766	NS, 0.4838	NS, 0.0664	NS, 0.1222

C. Swine matrix (swM) versus swine matrix (swM) or pandemic matrix (pdmM) versus pandemic matrix (pdmM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/MO/664/13 N2 (swM) vs. Sw/IN/351/11 (swM)	NS, 0.0862	NS, 0.8065	NS, 0.4838	NS, 0.4275	***, 0.0496**
Sw/MO/664/13 N2 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.2013	NS, >0.9999	NS, 0.9999	NS, 0.9995	***, 0.0213**
Sw/IN/351/11 (swM) vs. Sw/IL/300/11 (swM)	NS, >0.9999	NS, 0.4974	NS, 0.5427	NS, 0.3814	NS, 0.6736
Sw/IL/201/13 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, 0.2509	NS, 0.9745	NS, 0.5815	***, 0.0176**	NS, 0.7549
Sw/IL/201/13 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.9978	NS, 0.6956	NS, 0.6601	NS, 0.3732	NS, 0.0823
Sw/NC/702/15 N2 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.7108	NS, 0.9936	NS, 0.3999	NS, 0.1156	NS, 0.0840

Differences in replication kinetics of viruses analyzed using a Mixed-effect analysis with multiple comparisons. Values are expressed as not significant (NS) with corresponding p-value or values are expressed as significant (*) with corresponding p-value. #=statistics not calculated due to mean difference value of zero.

Supplemental Table 4.4. PNE Kinetics Mixed-effect Analysis Multiple Comparisons

A. CA/07/09 (pdmM) versus pandemic matrix (pdmM) or swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
CA/07/09 (pdmM) vs. Sw/IL/201/13 (pdmM)	NS, 0.7289	NS, 0.2633	NS, 0.0861	NS, 0.3213	NS, 0.6761
CA/07/09 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, 0.9739	NS, 0.9989	NS, 0.3653	******, <0.0001**	***, 0.0364**
CA/07/09 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.8762	NS, 0.6302	NS, 0.2184	*****, 0.0001**	******, <0.0001**
CA/07/09 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.8762	NS, 0.6302	NS, 0.8234	NS, 0.9247	#
CA/07/09 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.8762	NS, 0.6302	NS, >0.9999	NS, 0.4527	NS, 0.2464
CA/07/09 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.4641	NS, 0.9037	NS, 0.9669	****, 0.0051**	NS, 0.0534

B. Matrix (pdmM) versus swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/IL/201/13 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.1179	****, 0.0021**	****, 0.0010**	NS, 0.2915	NS, 0.6761
Sw/IL/201/13 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.1179	****, 0.0021**	****, 0.0061**	NS, 0.8911	NS, 0.9763
Sw/IL/201/13 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9659	****, 0.0012**	****, 0.0041**	NS, 0.5472	NS, 0.1809
Sw/NC/702/15 N2 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.9338	NS, 0.2783	***, 0.0393**	****, 0.0012**	***, 0.0364**
Sw/NC/702/15 N2 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.9338	NS, 0.2783	NS, 0.1604	***, 0.0389**	NS, 0.1217
Sw/NC/702/15 N2 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.1950	NS, 0.8871	NS, 0.3223	NS, 0.9986	NS, 0.9986
Sw/NC/076/15 (pdmM) vs. Sw/MO/664/13 N2 (swM)	#	#	****, 0.0015**	******, <0.0001**	******, <0.0001**
Sw/NC/076/15 (pdmM) vs Sw/IN/351/11 (swM)	#	#	***, 0.0259**	NS, 0.2576	NS, 0.1754
Sw/NC/076/15 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.1559	NS, 0.6812	***, 0.0128**	NS, 0.1841	NS, >0.9999

C. Swine matrix (swM) versus swine matrix (swM) or pandemic matrix (pdmM) versus pandemic matrix (pdmM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/MO/664/13 N2 (swM) vs. Sw/IN/351/11 (swM)	#	#	NS, 0.3112	NS, 0.3798	NS, 0.2464
Sw/MO/664/13 N2 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.1559	NS, 0.6812	***, 0.0168**	****, 0.0081**	NS, 0.0534
Sw/IN/351/11 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.1559	NS, 0.6812	NS, 0.7352	***, 0.0408**	NS, 0.1930
Sw/IL/201/13 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, 0.1708	****, 0.0076**	NS, 0.7753	NS, 0.4378	NS, 0.1397
Sw/IL/201/13 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.1179	****, 0.0021**	NS, 0.4585	NS, 0.9948	NS, 0.1988
Sw/NC/702/15 N2 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.9338	NS, 0.2783	NS, >0.9999	***, 0.0149**	NS, 0.9901

Differences in replication kinetics of viruses analyzed using a Mixed-effect analysis with multiple comparisons. Values are expressed as not significant (NS) with corresponding p-value or values are expressed as significant (*) with corresponding p-value. #=statistics not calculated due to mean difference value of zero.

Supplemental Table 4.5. PTE Kinetics Mixed-effect Analysis Multiple Comparisons

A. CA/07/09 (pdmM) versus pandemic matrix (pdmM) or swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
CA/07/09 (pdmM) vs. Sw/IL/201/13 (pdmM)	***, 0.0127**	NS, 0.0572	NS, >0.9999	NS, 0.3062	NS, 0.1203
CA/07/09 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	***, 0.0152**	NS, 0.8311	***, 0.0473**	NS, 0.6160	NS, 0.9999
CA/07/09 (pdmM) vs. Sw/NC/076/15 (pdmM)	*, 0.0293	NS, 0.5487	NS, 0.5455	NS, 0.5782	NS, 0.1827
CA/07/09 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.1055	NS, 0.0695	***, 0.0146**	NS, 0.1451	NS, 0.6024
CA/07/09 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.2917	NS, 0.3052	NS, 0.9998	NS, 0.3735	NS, 0.9983
CA/07/09 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.0981	NS, 0.4738	NS, 0.0538	***, 0.0426**	NS, 0.9974

B. Matrix (pdmM) versus swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/IL/201/13 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.4022	NS, 0.9999	***, 0.0410**	****, 0.0042**	NS, 0.3664
Sw/IL/201/13 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.9635	NS, 0.7584	NS, >0.9999	NS, 0.0757	NS, 0.1018
Sw/IL/201/13 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9761	NS, 0.9453	NS, 0.1417	****, 0.0035**	NS, 0.1529
Sw/NC/702/15 N2 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.4739	NS, 0.2283	****, 0.0036**	NS, 0.1831	NS, 0.5760
Sw/NC/702/15 N2 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.9816	NS, 0.8829	NS, 0.6654	NS, 0.7630	NS, >0.9999
Sw/NC/702/15 N2 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9914	NS, 0.9467	NS, >0.9999	****, 0.0089**	NS, 0.9761
Sw/NC/076/15 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.7828	NS, 0.7746	NS, 0.1506	NS, 0.2116	NS, 0.6483
Sw/NC/076/15 (pdmM) vs Sw/IN/351/11 (swM)	NS, 0.9965	NS, 0.9998	NS, 0.9858	NS, 0.7864	NS, 0.1869
Sw/NC/076/15 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9998	NS, 0.9998	NS, 0.7255	****, 0.0094**	***, 0.0284**

C. Swine matrix (swM) versus swine matrix (swM) or pandemic matrix (pdmM) versus pandemic matrix (pdmM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/MO/664/13 N2 (swM) vs. Sw/IN/351/11 (swM)	NS, 0.9998	NS, 0.8601	NS, 0.3803	NS, 0.9998	NS, 0.4789
Sw/MO/664/13 N2 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.9856	NS, 0.9746	NS, 0.5165	***, 0.0424**	NS, 0.5605
Sw/IN/351/11 (swM) vs. Sw/IL/300/11 (swM)	NS, >0.9999	NS, >0.9999	NS, 0.7032	NS, 0.8925	NS, 0.9282
Sw/IL/201/13 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, >0.9999	NS, 0.1752	NS, 0.1211	***, 0.0152**	NS, 0.1227
Sw/IL/201/13 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.9990	NS, 0.6827	NS, 0.7556	***, 0.0141**	NS, 0.7130
Sw/NC/702/15 N2 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, >0.9999	NS, 0.9884	NS, 0.5618	NS, >0.9999	NS, 0.2335

Differences in replication kinetics of viruses analyzed using a Mixed-effect analysis with multiple comparisons. Values are expressed as not significant (NS) with corresponding p-value or values are expressed as significant (*) with corresponding p-value. #=statistics not calculated due to mean difference value of zero.

Supplemental Table 4.6. PBE Kinetics Mixed-effect Analysis Multiple Comparisons

A. CA/07/09 (pdmM) versus pandemic matrix (pdmM) or swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
CA/07/09 (pdmM) vs. Sw/IL/201/13 (pdmM)	***, 0.0004	****, <0.0001	NS, 0.6928	***, 0.0005	NS, 0.2849
CA/07/09 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	*, 0.0313	NS, 0.2042	NS, 0.0518	NS, 0.2473	NS, 0.1925
CA/07/09 (pdmM) vs. Sw/NC/076/15 (pdmM)	**, 0.0313	***, 0.0006	NS, 0.3114	NS, 0.0901	*, 0.0397
CA/07/09 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.0921	NS, 0.9456	***, 0.0001	NS, 0.1967	NS, 0.9524
CA/07/09 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.0515	NS, 0.0743	NS, >0.9999	**, 0.0020	NS, 0.1774
CA/07/09 (pdmM) vs. Sw/IL/300/11 (swM)	*, 0.0178	NS, 0.5136	NS, 0.9429	**, 0.0026	*, 0.0312

B. Matrix (pdmM) versus swine matrix (swM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/IL/201/13 (pdmM) vs. Sw/MO/664/13 N2 (swM)	**, 0.0058	**, 0.0023	***, 0.0001	**, 0.0031	NS, 0.5198
Sw/IL/201/13 (pdmM) vs. Sw/IN/351/11 (swM)	NS, 0.6951	NS, 0.5454	NS, 0.9840	***, 0.0001	NS, 0.0655
Sw/IL/201/13 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.3129	NS, 0.0523	NS, 0.9746	****, <0.0001	*, 0.0218
Sw/NC/702/15 N2 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.4926	NS, 0.5510	NS, >0.9999	NS, 0.9530	NS, 0.2736
Sw/NC/702/15 N2 (pdmM) vs. Sw/IN/351/11 (swM)	NS, >0.9999	NS, 0.9879	*, 0.0430	*, 0.0123	NS, 0.9407
Sw/NC/702/15 N2 (pdmM) vs. Sw/IL/300/11 (swM)	NS, >0.9999	NS, 0.9826	NS, 0.5005	NS, 0.0557	NS, 0.0983
Sw/NC/076/15 (pdmM) vs. Sw/MO/664/13 N2 (swM)	NS, 0.0850	**, 0.0092	NS, 0.6609	NS, 0.9647	NS, 0.2189
Sw/NC/076/15 (pdmM) vs Sw/IN/351/11 (swM)	NS, >0.9999	NS, 0.9500	NS, 0.3294	NS, 0.2676	NS, 0.9862
Sw/NC/076/15 (pdmM) vs. Sw/IL/300/11 (swM)	NS, 0.9958	NS, 0.1694	NS, 0.7091	NS, 0.9427	NS, 0.1419

C. Swine matrix (swM) versus swine matrix (swM) or pandemic matrix (pdmM) versus pandemic matrix (pdmM)					
Multiple Comparison Groups	**Time-Points**				
	12	**24**	**48**	**72**	**96**
Sw/MO/664/13 N2 (swM) vs. Sw/IN/351/11 (swM)	NS, 0.4692	NS, 0.2065	*, 0.0115	NS, 0.0823	NS, 0.1720
Sw/MO/664/13 N2 (swM) vs. Sw/IL/300/11 (swM)	NS, 0.4132	NS, 0.9335	NS, 0.5064	NS, 0.4473	*, 0.0297
Sw/IN/351/11 (swM) vs. Sw/IL/300/11 (swM)	NS, >0.9999	NS, 0.7217	NS, 0.9539	NS, 0.2548	NS, 0.2619
Sw/IL/201/13 (pdmM) vs. Sw/NC/702/15 N2 (pdmM)	NS, 0.3845	NS, 0.2198	*, 0.0301	***, 0.0004	NS, 0.1029
Sw/IL/201/13 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.3987	NS, 0.8044	NS, 0.1729	**, 0.0030	NS, 0.1119
Sw/NC/702/15 N2 (pdmM) vs. Sw/NC/076/15 (pdmM)	NS, 0.9972	NS, 0.5883	NS, 0.8039	NS, 0.5515	NS, 0.9893

Differences in replication kinetics of viruses analyzed using a Mixed-effect analysis with multiple comparisons. Values are expressed as not significant (NS) with corresponding p-value or values are expressed as significant (*) with corresponding p-value. #=statistics not calculated due to mean difference value of zero

REFERENCES

1. CDC. 2020. Disease Burden of Influenza. https://wwwcdcgov/flu/about/burden/indexhtml#:~:text=While%20the%20impact%20of%20flu,61%2C000%20deaths%20annually%20since%202010.

2. CDC. 2019. 2009 H1N1 Pandemic (H1N1 pdm09 virus). https://wwwcdcgov/flu/pandemic-resources/2009-h1n1-pandemichtml.

3. Punyadarsaniya D, Liang C-H, Winter C, Petersen H, Rautenschlein S, Hennig-Pauka I, Schwegmann-Wessels C, Wu C-Y, Wong C-H, Herrler G. 2011. Infection of differentiated porcine airway epithelial cells by influenza virus: differential susceptibility to infection by porcine and avian viruses. PloS one 6:e28429-e28429.

4. Trebbien R, Larsen LE, Viuff BM. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virology Journal 8:434.

5. Van Poucke SG, Nicholls JM, Nauwynck HJ, Van Reeth K. 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7:38.

6. Vincent KVRaAL. 2019. Influenza Viruses, p 576-593, Diseases of Swine doi:10.1002/9781119350927.ch36.

7. Rajao DS, Vincent AL, Perez DR. 2019. Adaptation of Human Influenza Viruses to Swine. Frontiers in veterinary science 5:347-347.

8. Van Reeth K, Vincent AL. Diseases of Swine 11th Edition doi:https://doi.org/10.1002/9781119350927.ch36. John Wiley & Sons, Inc.

9. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang K-C. 2010.

Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Veterinary Research 6:4.

10. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. 2006. Avian flu: influenza virus receptors in the human airway. Nature 440:435-6.

11. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JSM. 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.

12. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. 2000. Evolution of Swine H3N2 Influenza Viruses in the United States. Journal of Virology 74:8243.

13. Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Reeth KV, Brown IH, Swenson SL, Simon G, Saito T, Berhane Y, Ciacci-Zanella J, Pereda A, Davis CT, Donis RO, Webby RJ, Vincent AL, Lowen AC. 2016. A Phylogeny-Based Global Nomenclature System and Automated Annotation Tool for H1 Hemagglutinin Genes from Swine Influenza A Viruses. mSphere 1:e00275-16.

14. Brockwell-Staats C, Webster RG, Webby RJ. 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza and other respiratory viruses 3:207-213.

15. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73:8851-6.

16. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton J-C, Lowe J, Gramer M,

Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerging infectious diseases 17:1624-1629.

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

18. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125.

19. Mena I, Nelson MI, Quezada-Monroy F, Dutta J, Cortes-Fernández R, Lara-Puente JH, Castro-Peralta F, Cunha LF, Trovão NS, Lozano-Dubernard B. 2016. Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. Elife 5:e16777.

20. Campbell PJ, Danzy S, Kyriakis CS, Deymier MJ, Lowen AC, Steel J. 2014. The M Segment of the 2009 Pandemic Influenza Virus Confers Increased Neuraminidase Activity, Filamentous Morphology, and Efficient Contact Transmissibility to A/Puerto Rico/8/1934-Based Reassortant Viruses. Journal of Virology 88:3802.

21. Chou Y-y, Albrecht RA, Pica N, Lowen AC, Richt JA, García-Sastre A, Palese P, Hai R. 2011. The M Segment of the 2009 New Pandemic H1N1 Influenza Virus Is Critical for Its High Transmission Efficiency in the Guinea Pig Model. Journal of Virology 85:11235.

22. Prevention CfDCa. 2020. Reported Infections with Variant Influenza Viruses in the United States.

23. Wohlgemuth N, Ye Y, Fenstermacher KJ, Liu H, Lane AP, Pekosz A. 2017. The

M2 protein of live, attenuated influenza vaccine encodes a mutation that reduces replication in human nasal epithelial cells. Vaccine 35:6691-6699.

24. Forero A, Fenstermacher K, Wohlgemuth N, Nishida A, Carter V, Smith EA, Peng X, Hayes M, Francis D, Treanor J, Morrison J, Klein SL, Lane A, Katze MG, Pekosz A. 2017. Evaluation of the innate immune responses to influenza and live-attenuated influenza vaccine infection in primary differentiated human nasal epithelial cells. Vaccine 35:6112-6121.

25. Pharo EA, Williams SM, Boyd V, Sundaramoorthy V, Durr PA, Baker ML. 2020. Host-Pathogen Responses to Pandemic Influenza H1N1pdm09 in a Human Respiratory Airway Model. Viruses 12.

26. Lindemann J, Leiacker R, Rettinger G, Keck T. 2002. Nasal mucosal temperature during respiration. Clin Otolaryngol Allied Sci 27:135-9.

27. Keck T, Leiacker R, Riechelmann H, Rettinger G. 2000. Temperature profile in the nasal cavity. Laryngoscope 110:651-4.

28. Neumann G, Kawaoka Y. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerging infectious diseases 12:881.

29. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67:1761-4.

30. Massin P, Werf Svd, Naffakh N. 2001. Residue 627 of PB2 Is a Determinant of Cold Sensitivity in RNA Replication of Avian Influenza Viruses. Journal of Virology 75:5398-5404.

31. Chen R, Holmes EC. 2006. Avian Influenza Virus Exhibits Rapid Evolutionary Dynamics. Molecular Biology and Evolution 23:2336-2341.

32. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. 2007. Growth of H5N1 Influenza A Viruses in the Upper Respiratory Tracts of Mice. PLOS Pathogens 3:e133.

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

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

35. Manzoor R, Sakoda Y, Nomura N, Tsuda Y, Ozaki H, Okamatsu M, Kida H. 2009. PB2 protein of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) determines its replication potential in pigs. J Virol 83:1572-8.

36. La Gruta NL, Kedzierska K, Stambas J, Doherty PC. 2007. A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol 85:85-92.

37. Randall RE, Goodbourn S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. Journal of General Virology 89:1-47.

38. Killip MJ, Fodor E, Randall RE. 2015. Influenza virus activation of the interferon system. Virus Res 209:11-22.

39. Xiao H, Killip MJ, Staeheli P, Randall RE, Jackson D. 2013. The human interferon-induced MxA protein inhibits early stages of influenza A virus infection by retaining the

incoming viral genome in the cytoplasm. J Virol 87:13053-8.

40. Ciancanelli MJ, Abel L, Zhang SY, Casanova JL. 2016. Host genetics of severe influenza: from mouse Mx1 to human IRF7. Curr Opin Immunol 38:109-20.

41. Riegger D, Hai R, Dornfeld D, Mänz B, Leyva-Grado V, Sánchez-Aparicio MT, Albrecht RA, Palese P, Haller O, Schwemmle M, García-Sastre A, Kochs G, Schmolke M, Lyles DS. 2015. The Nucleoprotein of Newly Emerged H7N9 Influenza A Virus Harbors a Unique Motif Conferring Resistance to Antiviral Human MxA. Journal of Virology 89:2241-2252.

42. Mänz B, Dornfeld D, Götz V, Zell R, Zimmermann P, Haller O, Kochs G, Schwemmle M. 2013. Pandemic Influenza A Viruses Escape from Restriction by Human MxA through Adaptive Mutations in the Nucleoprotein. PLOS Pathogens 9:e1003279.

43. Zimmermann P, Mänz B, Haller O, Schwemmle M, Kochs G. 2011. The Viral Nucleoprotein Determines Mx Sensitivity of Influenza A Viruses. Journal of Virology 85:8133-8140.

44. Bateman Allen C, Busch Marc G, Karasin Alexander I, Bovin N, Olsen Christopher W. 2008. Amino Acid 226 in the Hemagglutinin of H4N6 Influenza Virus Determines Binding Affinity for α2,6-Linked Sialic Acid and Infectivity Levels in Primary Swine and Human Respiratory Epithelial Cells. Journal of Virology 82:8204-8209.

45. Obadan AO, Santos J, Ferreri L, Thompson AJ, Carnaccini S, Geiger G, Gonzalez Reiche AS, Rajão DS, Paulson JC, Perez DR. 2019. Flexibility In Vitro of Amino Acid 226 in the Receptor-Binding Site of an H9 Subtype Influenza A Virus and Its Effect In Vivo on Virus Replication, Tropism, and Transmission. J Virol 93.

CHAPTER 5

DISCUSSION

Swine and avian species are susceptible to multiple viral and bacterial pathogens. These viral and bacterial pathogens are commonly referred to as the Porcine Respiratory Disease Complex (PRDC) and the Avian Respiratory Disease Complex (ARDC). Diseases of the PRDC include but are not limited to, swine influenza virus (swIAV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, and *Bordetella* (1-6)**.** Diseases of the ARDC include, but are not limited to avian influenza virus (avIAV), Infectious Bronchitis Virus (IBV), Infectious Laryngotracheitis Virus (ILTV), Avian pneumovirus, Newcastle Disease Virus (NDV), *Mycoplasma gallisepticum*, *Escheria Coli*, and *Staphylococcus* (7-12). These viral and bacterial pathogens of swine and avian are known to infect the respiratory epithelium and are an important health concern for swine and poultry producers, since this causes tremendous economic loss to the industry.

In our first study we established primary PNE, PTE, and PBE cells that recapitulate the physical and functional properties of the respiratory tract, develop tight junctions and have TEERs (Figure 2.2, 2.3, and 2.4). Primary PNE, PTE, and PBE cells also have the ability to fully differentiate, indicated by the presence of cilia and mucus (Figures 2.3 and 2.4). Adding primary PNE, PTE, and PBE cells to surveillance studies can help assess the pandemic potential of emerging influenza viruses for humans as well as help understand the dynamics of polymicrobial infections in swine. Swine primary PNE, PTE, and PBE cells developed through the methodology of this dissertation, revealed the presence of α2,3-SA and a2,6-SA indicated by positive lectin staining with MAL2 or SNA respectively (Figure

23 and 24). Pigs, susceptible to avian and human IAVs, are proposed as a "mixing vessel" linked to the generation of novel IAVs that may infect humans based on the distribution of sialic acid receptors within their respiratory tract. The upper respiratory tract of swine predominately contains cells expressing α2,6-SA, while cells expressing α2,3-SA dominate in the lower airways (13-15). The swine airway epithelium also has less sialylated glycans that humans and swine have a greater proportion of α2,6-SA than the human respiratory tract (16). In human bronchial and tracheal cells, it has been reported that α2,3-SA are expressed in ciliated cells, while α2,6 SA are primarily expressed in non-ciliated cells (17-19).

In our second study, our lab proved that primary PNE, PTE, and PBE cells are a feasible model for risk assessment of swine and avian influenza viruses. The data presented in chapter 3 of this dissertation shows that H1N1 and H3N2 viruses of swine all replicated efficiently in porcine cell substrates as expected. Interestingly, these H1N1 and H3N2 viruses were also able to replicate in human cell substrates, indicating their possibility for pandemic potential (Figure 3.1 and Figure 3.2). In the United States alone there has been 9 cases of H1N1, 25 cases of H1N2, and 430 cases of H3N2 variant swine virus infections since 2009/2010 (20, 21).

Furthermore, H7N2 viruses of avian, NY/94 and NJ/02 were able to replicate in at least one porcine substrate. However, all of the avian viruses were able to replicate in human substrates, indicating their possibility for pandemic potential (Figure 3.3). In addition to the pandemics of avian origin mentioned above, there have been multiple reports of human infections with H7 low path avian influenza subtypes, such as seasonal waves of low path human H7N9 in China (22-32). There also has been reports of human infections with the H7N2 subtype, notably the A/NY/16 used in this study. Highly pathogenic H7

viruses have been known to emerge from ancestral low pathogenic avian influenza (33-35).

In the third study conducted as a component of this dissertation, our lab showed that the matrix gene origin of swine influenza H1N1 and H1N2 isolates affects the viral tropism, replication kinetics, and pandemic potential in primary airway models. Utilizing the primary porcine cell and human cell substrates developed within the first and second aim of this dissertation, viruses which contained the swM gene or pdmM gene were utilized to create a panel of H1N1 and H1N2 swine viruses for *in vitro* studies. H1N1 and H1N2 viruses have been circulating in pigs for over a century and the pdmM matrix has dominated (36-38). The pdmM gene has been implicated as a determinate of respiratory transmission in hosts, increasing the number of potential "variant" infections (20, 39-41). The data presented in the fourth chapter of this dissertation shows that regardless of matrix origin, swine isolates are able to replicate within the human respiratory tract, but those that contain the pdmM gene replicate more efficiently. All the swine isolates regardless of matrix origin were able to replicate with the swine respiratory tract, which is not surprising. However, in the PTE, and PBE cells viruses which contained the swM gene replicated more efficiently. This begs the question then how do swine viruses effectively transmit to humans (Chapter 4 Figures).

A clue to a possible answer may lie within the results of the PNE. Within the PNE cells, viruses which contained the pdmM gene replicated more efficiently at both 37º and 32º Celsius (Chapter 4 Figures). It is known that temperature plays an important role in ability of viruses, specifically influenza, to replicate within the nasal epithelium and transmit. It is also been reported that differences in temperature modulates the innate immune response within the nasal epithelium (42-44). The temperature within the human nasal tract fluctuates between 30º Celsius and 34º Celsius, depending on inhalation or

expiration (45, 46).

Although the matrix gene has been implicated in the ability of swine influenza to transmit to humans, other genes may play a role. For example, the viral polymerase, which is comprised of the PB1, PB2, and PA gene segment has been shown to be a major host determinant. Specifically in a single residue of the PB2 gene E627K which is correlated with increased viral replication and transmission in animals (47-54). Literature has also shown that other residues A271 and D701N are able to compensate for the absence of the E627K amino acid change. Therefore, for the following viruses the A271 amino acid change may have contributed to their increase in viral replication: CA/07/09, Sw/NC/702/15, Sw/MO/664/13, Sw/IN/351/11, and Sw/IL/300/11. Another gene that is known to play a key role in evading the host's Type-1 interferon response is the NP gene segment. The influenza viral NP gene segment has been shown to play a key role in MX sensitivity (55-59). Specifically, Y52, I100, P283, and Y313 have been shown to be essential to reduced MX sensitivity in cell culture and *in vivo*(60-62). None of the viruses used within this study contained these amino acid changes.

Lastly, the HA gene segment is essential for viral attachment as well as entry. The HA surface protein of influenza binds to sialic acid residues on host cells. As stated before, the upper respiratory tract of swine predominately contains cells expressing a2,6-SA, while cells expressing a2,3-SA dominate in the lower airways (13-15). It has been shown that viruses that contain L226 have a higher affinity for a2,6-sialic acid (63, 64). None of the viruses used within this study possessed this amino acid change. A recent study also surveyed swine field isolates from farms in the United States. Using glycan arrays, it was determined that swine field isolates A/sw/NC/154072/2015 and A/sw/NC/152701/2015 preferentially bind to a2,6-silaic acid. It is hypothesized that the viruses Sw/NC/076/15 and

Sw/NC/702/15 since they were viruses isolated from the same farm, but different animals preferentially bind to a2,6-silaic acid (65). Therefore, we contribute the increase in viral replication, tissue tropism, and increase pandemic potential of the swine isolates used within this study to the presence of the pdmM gene.

Utilizing these porcine and human cell substrates as a tool for pandemic assessment along with increased surveillance will help detect possible variants that may pose a threat to human health. These cell substrates can also be utilized as a tool for the isolation of difficult viruses and to learn more about reassortment events and virus evolution. Furthermore, these cell substrates can be utilized to learn more about innate responses of the airway epithelium to influenza. Future studies aim to evaluate differences in innate immune responses to swine influenza due to temperature in both primary human nasal epithelial cells (HNECs) and PNEs. Future studies within our lab will also explore the relationship of the matrix gene with other gene segments using reverse genetics in both *in vitro* and *in vivo* models. Future directions within the lab will also look at experimental infections in both mice and ferrets to determine susceptibility, transmissibility, and immune response. Theses *in vivo* studies will allow us to explore the role of other innate and adaptive immune cell subtypes induced upon influenza viral infection and will allow us more freedom to target specific pathways using purposely bred knockout mice.

REFERENCES

1. Qin S, Ruan W, Yue H, Tang C, Zhou K, Zhang B. 2018. Viral communities associated with porcine respiratory disease complex in intensive commercial farms in Sichuan province, China. Scientific Reports 8:13341.

2. Van Reeth K, Vincent AL. Diseases of Swine 11th Edition doi:https://doi.org/10.1002/9781119350927.ch36. John Wiley & Sons, Inc.

3. Opriessnig T, Giménez-Lirola L, Halbur P. 2011. Polymicrobial respiratory disease in pigs. Animal Health Research Reviews 12:133.

4. Fablet C, Marois-Créhan C, Simon G, Grasland B, Jestin A, Kobisch M, Madec F, Rose N. 2012. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. Veterinary microbiology 157:152-163.

5. Schmidt C, Cibulski S, Andrade C, Teixeira T, Varela A, Scheffer C, Franco A, de Almeida L, Roehe P. 2016. Swine influenza virus and association with the porcine respiratory disease complex in pig farms in southern Brazil. Zoonoses and public health 63:234-240.

6. Galán-Relaño A, Luque I, Gómez-Gascón L, Morales-Partera A, Tarradas C, Carrasco L, Gómez-Laguna J. 2015. Seroprevalence against selected pathogens involved in porcine respiratory disease complex in free-range fattening pigs in Spain. Veterinary Record:vetrec-2015-103432.

7. Thomas JK, Noppenberger J. 2007. Avian influenza: a review. Am J Health Syst Pharm 64:149-65.

8. Stipkovits L, Egyed L, Palfi V, Beres A, Pitlik E, Somogyi M, Szathmary S, Denes B. 2012. Effect of low-pathogenicity influenza virus H3N8 infection on Mycoplasma gallisepticum infection of chickens. Avian Pathol 41:51-7.

9. Haghighat-Jahromi M, Asasi K, Nili H, Dadras H, Shooshtari AH. 2008. Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Arch Virol 153:651-5.

10. França M, Howerth EW, Carter D, Byas A, Poulson R, Afonso CL, Stallknecht DE. 2014. Co-infection of mallards with low-virulence Newcastle disease virus and low-pathogenic avian influenza virus. Avian Pathol 43:96-104.

11. Cavanagh D. 2007. Coronavirus avian infectious bronchitis virus. Vet Res 38:281-97.

12. Scheiblauer H, Reinacher M, Tashiro M, Rott R. 1992. Interactions between bacteria and influenza A virus in the development of influenza pneumonia. J Infect Dis 166:783-91.

13. Punyadarsaniya D, Liang C-H, Winter C, Petersen H, Rautenschlein S, Hennig-Pauka I, Schwegmann-Wessels C, Wu C-Y, Wong C-H, Herrler G. 2011. Infection of differentiated porcine airway epithelial cells by influenza virus: differential susceptibility to infection by porcine and avian viruses. PloS one 6:e28429-e28429.

14. Trebbien R, Larsen LE, Viuff BM. 2011. Distribution of sialic acid receptors and influenza A virus of avian and swine origin in experimentally infected pigs. Virology Journal 8:434.

15. Van Poucke SG, Nicholls JM, Nauwynck HJ, Van Reeth K. 2010. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. Virol J 7:38.

16. Chan RW, Karamanska R, Van Poucke S, Van Reeth K, Chan IW, Chan MC, Dell A, Peiris JS, Haslam SM, Guan Y, Nicholls JM. 2013. Infection of swine ex vivo tissues with avian viruses including H7N9 and correlation with glycomic analysis. Influenza Other

Respir Viruses 7:1269-82.

17. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang K-C. 2010. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Veterinary Research 6:4.

18. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. 2006. Avian flu: influenza virus receptors in the human airway. Nature 440:435-6.

19. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JSM. 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.

20. Prevention CfDCa. 2020. Reported Infections with Variant Influenza Viruses in the United States.

21. Control CfD, Prevention. 2017. Reported Infections with Variant Influenza Viruses in the United States.

22. Swayne DE. 2009. Avian influenza. John Wiley & Sons.

23. Capua I, Marangon S. 2000. The avian influenza epidemic in Italy, 1999—2000: a review. Avian Pathology 29:289-294.

24. Capua I, Alexander DJ. 2007. Avian influenza infections in birds–a moving target. Influenza and other respiratory viruses 1:11-18.

25. Capua I, Alexander DJ. 2004. Avian influenza: recent developments. Avian Pathology 33:393-404.

26. Belser JA, Pulit-Penaloza JA, Sun X, Brock N, Pappas C, Creager HM, Zeng H, Tumpey TM, Maines TR. 2017. A novel A (H7N2) influenza virus isolated from a veterinarian caring for cats in a New York City animal shelter causes mild disease and transmits poorly in the ferret model. Journal of virology 91:e00672-17.

27. Naguib MM, Verhagen JH, Mostafa A, Wille M, Li R, Graaf A, Järhult JD, Ellström P, Zohari S, Lundkvist Å, Olsen B. 2019. Global patterns of avian influenza A (H7): virus evolution and zoonotic threats. FEMS Microbiology Reviews 43:608-621.

28. Mostafa A, Abdelwhab EM, Mettenleiter TC, Pleschka S. 2018. Zoonotic potential of influenza A viruses: a comprehensive overview. Viruses 10:497.

29. Bonfanti L, Monne I, Tamba M, Santucci U, Massi P, Patregnani T, Piccolomini LL, Natalini S, Ferri G, Cattoli G. 2014. Highly pathogenic H7N7 avian influenza in Italy. Veterinary Record.

30. Joseph U, Su YC, Vijaykrishna D, Smith GJ. 2017. The ecology and adaptive evolution of influenza A interspecies transmission. Influenza and other respiratory viruses 11:74-84.

31. Abdelwhab E, Veits J, Mettenleiter T. 2014. Prevalence and control of H7 avian influenza viruses in birds and humans. Epidemiology & Infection 142:896-920.

32. Abdelwhab E-SM, Veits J, Tauscher K, Ziller M, Teifke JP, Stech J, Mettenleiter TC. 2016. A unique multibasic proteolytic cleavage site and three mutations in the HA2 domain confer high virulence of H7N1 avian influenza virus in chickens. Journal of virology 90:400-411.

33. Anonymous. 2007. Avian influenza A/(H7N2) outbreak in the United Kingdom. Euro Surveill 12:E070531.2.

34. Marinova-Petkova A, Laplante J, Jang Y, Lynch B, Zanders N, Rodriguez M, Jones J, Thor S, Hodges E, De La Cruz JA, Belser J, Yang H, Carney P, Shu B, Berman L, Stark T, Barnes J, Havers F, Yang P, Trock SC, Fry A, Gubareva L, Bresee JS, Stevens J, Daskalakis D, Liu D, Lee CT, Torchetti MK, Newbury S, Cigel F, Toohey-Kurth K, St George K, Wentworth DE, Lindstrom S, Davis CT. 2017. Avian Influenza A(H7N2) Virus

in Human Exposed to Sick Cats, New York, USA, 2016. Emerging infectious diseases 23:2046-2049.

35. Terebuh P, Adija A, Edwards L, Rowe T, Jenkins S, Kleene J, Fukuda K, Katz JM, Bridges CB. 2018. Human infection with avian influenza A(H7N2) virus-Virginia, 2002. Influenza Other Respir Viruses 12:529-532.

36. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. 2000. Evolution of Swine H3N2 Influenza Viruses in the United States. Journal of Virology 74:8243.

37. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton J-C, Lowe J, Gramer M, Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerging infectious diseases 17:1624-1629.

38. Brockwell-Staats C, Webster RG, Webby RJ. 2009. Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza and other respiratory viruses 3:207-213.

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

40. Campbell PJ, Danzy S, Kyriakis CS, Deymier MJ, Lowen AC, Steel J. 2014. The M Segment of the 2009 Pandemic Influenza Virus Confers Increased Neuraminidase Activity, Filamentous Morphology, and Efficient Contact Transmissibility to A/Puerto Rico/8/1934-Based Reassortant Viruses. Journal of Virology 88:3802.

41. Chou Y-y, Albrecht RA, Pica N, Lowen AC, Richt JA, García-Sastre A, Palese P,

Hai R. 2011. The M Segment of the 2009 New Pandemic H1N1 Influenza Virus Is Critical for Its High Transmission Efficiency in the Guinea Pig Model. Journal of Virology 85:11235.

42. Wohlgemuth N, Ye Y, Fenstermacher KJ, Liu H, Lane AP, Pekosz A. 2017. The M2 protein of live, attenuated influenza vaccine encodes a mutation that reduces replication in human nasal epithelial cells. Vaccine 35:6691-6699.

43. Forero A, Fenstermacher K, Wohlgemuth N, Nishida A, Carter V, Smith EA, Peng X, Hayes M, Francis D, Treanor J, Morrison J, Klein SL, Lane A, Katze MG, Pekosz A. 2017. Evaluation of the innate immune responses to influenza and live-attenuated influenza vaccine infection in primary differentiated human nasal epithelial cells. Vaccine 35:6112-6121.

44. Pharo EA, Williams SM, Boyd V, Sundaramoorthy V, Durr PA, Baker ML. 2020. Host-Pathogen Responses to Pandemic Influenza H1N1pdm09 in a Human Respiratory Airway Model. Viruses 12.

45. Lindemann J, Leiacker R, Rettinger G, Keck T. 2002. Nasal mucosal temperature during respiration. Clin Otolaryngol Allied Sci 27:135-9.

46. Keck T, Leiacker R, Riechelmann H, Rettinger G. 2000. Temperature profile in the nasal cavity. Laryngoscope 110:651-4.

47. Neumann G, Kawaoka Y. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerging infectious diseases 12:881.

48. Subbarao EK, London W, Murphy BR. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67:1761-4.

49. Massin P, Werf Svd, Naffakh N. 2001. Residue 627 of PB2 Is a Determinant of Cold Sensitivity in RNA Replication of Avian Influenza Viruses. Journal of Virology

75:5398-5404.

50. Chen R, Holmes EC. 2006. Avian Influenza Virus Exhibits Rapid Evolutionary Dynamics. Molecular Biology and Evolution 23:2336-2341.

51. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. 2007. Growth of H5N1 Influenza A Viruses in the Upper Respiratory Tracts of Mice. PLOS Pathogens 3:e133.

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

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

54. Manzoor R, Sakoda Y, Nomura N, Tsuda Y, Ozaki H, Okamatsu M, Kida H. 2009. PB2 protein of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) determines its replication potential in pigs. J Virol 83:1572-8.

55. La Gruta NL, Kedzierska K, Stambas J, Doherty PC. 2007. A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol 85:85-92.

56. Randall RE, Goodbourn S. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. Journal of General Virology 89:1-47.

57. Killip MJ, Fodor E, Randall RE. 2015. Influenza virus activation of the interferon system. Virus Res 209:11-22.

58. Xiao H, Killip MJ, Staeheli P, Randall RE, Jackson D. 2013. The human interferon-induced MxA protein inhibits early stages of influenza A virus infection by retaining the incoming viral genome in the cytoplasm. J Virol 87:13053-8.

59. Ciancanelli MJ, Abel L, Zhang SY, Casanova JL. 2016. Host genetics of severe influenza: from mouse Mx1 to human IRF7. Curr Opin Immunol 38:109-20.

60. Riegger D, Hai R, Dornfeld D, Mänz B, Leyva-Grado V, Sánchez-Aparicio MT, Albrecht RA, Palese P, Haller O, Schwemmle M, García-Sastre A, Kochs G, Schmolke M, Lyles DS. 2015. The Nucleoprotein of Newly Emerged H7N9 Influenza A Virus Harbors a Unique Motif Conferring Resistance to Antiviral Human MxA. Journal of Virology 89:2241-2252.

61. Mänz B, Dornfeld D, Götz V, Zell R, Zimmermann P, Haller O, Kochs G, Schwemmle M. 2013. Pandemic Influenza A Viruses Escape from Restriction by Human MxA through Adaptive Mutations in the Nucleoprotein. PLOS Pathogens 9:e1003279.

62. Zimmermann P, Mänz B, Haller O, Schwemmle M, Kochs G. 2011. The Viral Nucleoprotein Determines Mx Sensitivity of Influenza A Viruses. Journal of Virology 85:8133-8140.

63. Bateman Allen C, Busch Marc G, Karasin Alexander I, Bovin N, Olsen Christopher W. 2008. Amino Acid 226 in the Hemagglutinin of H4N6 Influenza Virus Determines Binding Affinity for α2,6-Linked Sialic Acid and Infectivity Levels in Primary Swine and Human Respiratory Epithelial Cells. Journal of Virology 82:8204-8209.

64. Obadan AO, Santos J, Ferreri L, Thompson AJ, Carnaccini S, Geiger G, Gonzalez Reiche AS, Rajão DS, Paulson JC, Perez DR. 2019. Flexibility In Vitro of Amino Acid 226 in the Receptor-Binding Site of an H9 Subtype Influenza A Virus and Its Effect In Vivo on Virus Replication, Tropism, and Transmission. J Virol 93.

65. Bakre AA, Jones LP, Kyriakis CS, Hanson JM, Bobbitt DE, Bennett HK, Todd KV, Orr-Burks N, Murray J, Zhang M, Steinhauer DA, Byrd-Leotis L, Cummings RD, Fent J, Coffey T, Tripp RA. 2020. Molecular epidemiology and glycomics of swine influenza viruses circulating in commercial swine farms in the southeastern and midwest United States. Vet Microbiol 251:108914.